mately 50% immature myeloid cells, including myeloblasts and promyelocytes (Figure 2A and B). Flow cytometric analysis revealed that the percentage of $CD34^+$ cells within HAX1-iPS cell-derived blood cells was significantly higher than in normal iPS cell-derived blood cells (Figure 2C), which also showed that the percentage of phenotypically immature myeloid cells was higher in HAX1-iPS cell-derived blood cells than in normal iPS cell-derived blood cells.

Immunocytochemical analysis for lactoferrin and gelatinase, which are constitutive proteins of neutrophil specific granules observed in mature neutrophils, showed that the proportion of these granule-positive cells was significantly lower in HAX1-iPS cell-derived blood cells than in normal iPS cell-derived blood cells (Figure 2D). NE is a protease stored in primary granules of neutrophilic granulocytes that are formed at the promyelocytic phase of granulocyte differentiation. *ELANE* mRNA expression in myeloid progenitors and the protein level of NE in plasma are markedly reduced in SCN patients with mutations in *ELANE* or *HAX1*.[20] Consistent with this, the proportion of NE-positive cells was significantly lower in blood cells derived from HAX1-iPS cells than in those derived from normal iPS cells (Figure 2E). Thus, the level of functionally mature neutrophils decreased during *in vitro* granulopoietic differentiation of HAX1-iPS cells.

Next, we analyzed the colony-forming potential of HAX1-iPS cell-derived myeloprogenitor cells. Significantly fewer colonies, which were classified as granulocyte-macrophage (GM) or granulocyte (G) colony-forming units (CFU), were derived from HAX1-iPS cells than from control iPS cells. Furthermore, the colonies derived from HAX1-iPS cells were predominantly CFU-GM (Figure 2F). Thus, maturation arrest occurred at the clonogenic progenitor stage during *in vitro* neutrophil differentiation of HAX1-iPS cells.

SCN is characterized by severe neutropenia with very low absolute neutrophil counts in peripheral blood, and many SCN patients respond to G-CSF treatment.[1,2] In colony-forming assays using bone marrow cells of SCN patients, primitive myeloid progenitor cells have reduced responsiveness to hematopoietic cytokines including G-CSF.[21,22] Therefore, we next examined the response of HAX1-iPS cell-derived blood cells to G-CSF using a colony-forming assay. Although the number of colonies derived from HAX1-iPS cells slightly increased following the addition of G-CSF, it remained significantly lower than the number of colonies derived from control iPS cells in the absence of G-CSF (Figure 2F). These results indicate that the responsiveness of HAX1-iPS-derived blood cells to G-CSF was insufficient to restore the neutrophil count to a normal level and are consistent with the fact that the absolute neutrophil counts of SCN patients remain low following G-CSF therapy.[19,21]

Neutrophils derived from HAX1-iPS cells are predisposed to undergo apoptosis due to their reduced $\Delta\psi_m$

Previous studies have shown HAX1 to localize to mitochondria[6] and to mediate anti-apoptotic activity.[7] Interestingly, this apoptotic predisposition of neutrophils due to their reduced $\Delta\psi_m$ was observed in HAX1-deficient patients,[5] prompting us to examine apoptosis in HAX1-iPS cell-derived blood cells. Consistent with these reports, HAX1-iPS cell-derived blood cells showed a significantly higher percentage of Annexin V-positive cells than in control cells (Figure 3A). In addition, a mitochondrial membrane potential assay revealed that the percentage of cells with a low $\Delta\psi_m$ was significantly higher in HAX1-iPS cell-derived blood cells than in blood cells derived from control iPS cells (Figure 3B). By contrast, the percentage of cells with a low $\Delta\psi_m$ was similar in undifferentiated HAX1-iPS cells and undifferentiated control iPS cells (*Online Supplementary Figure S3*).

Thus, increased apoptosis due to reduced $\Delta\psi_m$ causes defective granulopoiesis during neutrophil differentiation from HAX1-iPS cells, similar to the process observed in SCN patients with *HAX1* gene deficiency.

Lentiviral transduction of HAX1 cDNA improves maturation arrest and apoptotic predisposition of HAX1-iPS cells

Because most *HAX1* gene mutations in SCN patients are nonsense mutations resulting in a premature stop codon and protein truncation,[23] loss of the HAX1 protein is believed to cause severe neutropenia. To uncover the pathophysiological hallmarks of this disease, we performed lentiviral transduction of *HAX1* cDNA into HAX1-iPS cells.

We constructed lentiviral vectors that expressed *HAX1* cDNA and EGFP as a marker gene (pCSII-EF-IEGFP; EGFP

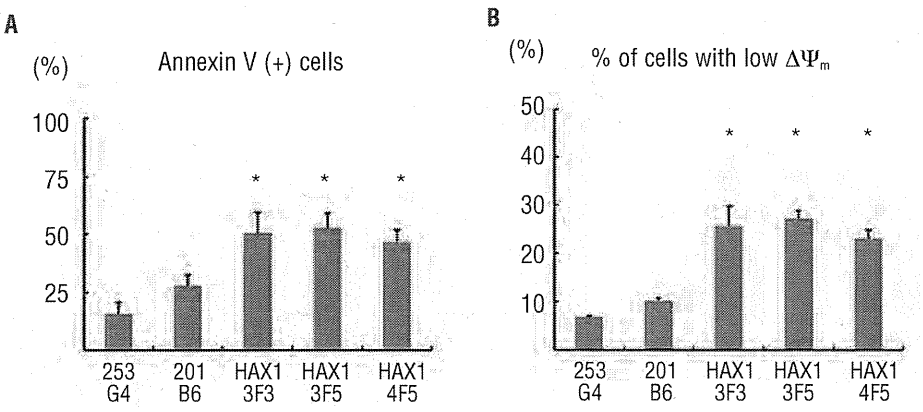

Figure 3. Neutrophils derived from HAX1-iPS cells are predisposed to undergo apoptosis due to their reduced $\Delta\psi m$. Annexin V assay (A) and mitochondrial membrane potential assay (B) of iPS cell-derived cells on Day 26 of neutrophil differentiation using flow cytometry. Cells gated on human $CD45^+$ were analyzed (n = 3; bars represent SDs; *$P<0.05$ to control iPS cells).

only, pCSII-EF-HAX1-IEGFP; HAX1 cDNA and EGFP) (Figure 4A). Efficient transduction of HAX1-iPS cells with these lentiviral vectors (HAX1 3F5+GFP; HAX1 3F5 transduced with pCSII-EF-IEGFP, HAX1 3F5+HAX1; HAX1 3F5 transduced with pCSII-EF-HAX1-IEGFP) was confirmed by a significant increase in HAX1 protein by Western blotting analysis (Figure 4B).

We then differentiated these lentiviral-transduced iPS cells into neutrophils, and examined whether defective granulopoiesis and apoptotic predisposition could be reversed. Morphologically, cells derived from HAX1 3F5+HAX1 showed a higher proportion of mature neutrophils than cells derived from HAX1 3F5+GFP and HAX1 3F5 (Figure 5A and B). Flow cytometric analysis revealed that the proportion of $CD34^+$ cells was significantly lower in the cells derived from HAX1 3F5+HAX1 than HAX1 3F5+GFP and HAX1 3F5 (Figure 5C). Immunocytochemical analysis for lactoferrin and gelatinase showed that the proportion of these granule-positive cells in generated blood cells was significantly higher in HAX 3F5+HAX1 than in HAX13F5+GFP and HAX1 3F5 (Figure 5D). These results indicated that *HAX1* cDNA increased the number of mature neutrophils in the neutrophil differentiation culture from HAX1-iPS cells *in vitro*. In addition, the percentage of NE-positive cells was significantly higher in cells derived from HAX1 3F5+HAX1 than in cells derived from HAX1 3F5+GFP and HAX1 3F5 (Figure 5E). Furthermore, the number of colonies derived from HAX1 3F5+HAX1 was comparable to the number derived from control cells (Figure 5F).

HAX1 3F5+HAX1-derived blood cells showed a significantly lower percentage of Annexin V-positive cells (Figure 6A) and a significantly lower percentage of cells with a low $\Delta\psi_m$ (Figure 6B) than HAX13F5+GFP and HAX1 3F5-derived blood cells. These results indicated that only *HAX1* cDNA transduction improved defective granulopoiesis and apoptotic predisposition due to low $\Delta\psi_m$ in the neutrophil differentiation culture from HAX1-iPS cells *in vitro*.

Discussion

Animal models and *in vitro* cultures consisting of cells derived from patients are often used to investigate disease pathophysiology and to develop novel therapies. Unfortunately, *Hax1* knock-out mice fail to reproduce abnormal granulopoiesis as observed in SCN patients.[10] Moreover, bone marrow cells are not an ideal experimental tool because it is difficult to obtain sufficient blood cells due to the invasiveness of the aspiration procedure. Moreover, the pathophysiological mechanisms occurring during early granulopoiesis are difficult to address in primary patient samples.

Our established culture system efficiently induced directed hematopoietic differentiation, which consisted of myeloid cells at different stages of development, from various control and patient-derived HAX1-iPS cell lines. Furthermore, this *in vitro* neutrophil differentiation system produced sufficient myeloid cells, which enabled us to perform various types of assays. In addition, flow cytom-

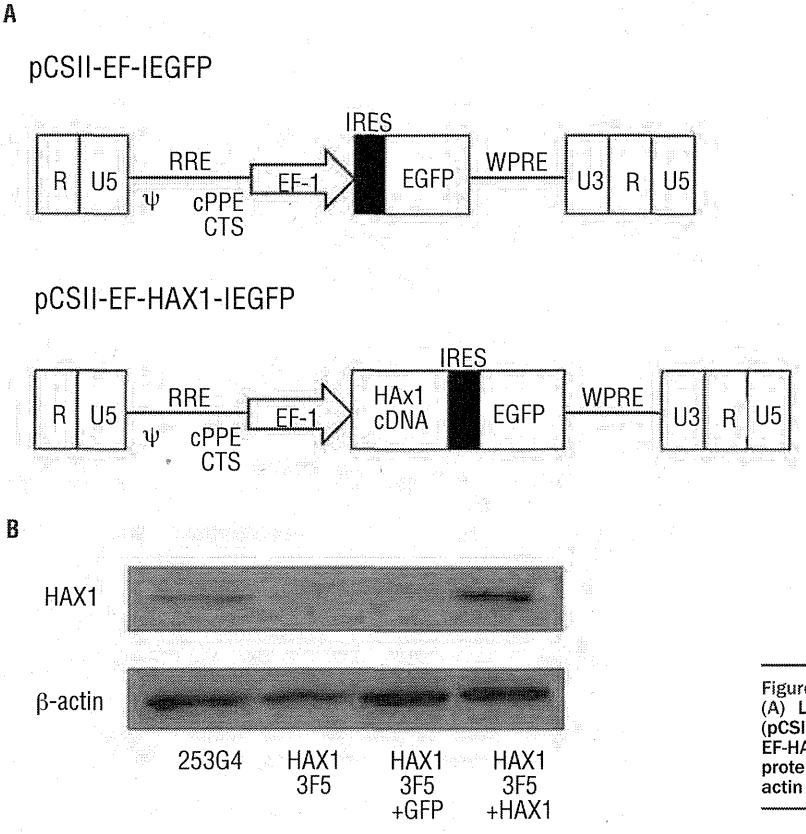

Figure 4. Lentiviral transduction of HAX1-iPS cells. (A) Lentiviral vector constructs with only EGFP (pCSII-EF-IEGFP), and *HAX1* cDNA and EGFP (pCSII-EF-HAX1-IEGFP). (B) Western blot analysis for HAX1 protein in lentivirally-transduced HAX1-iPS cells. β-actin was used as a loading control.

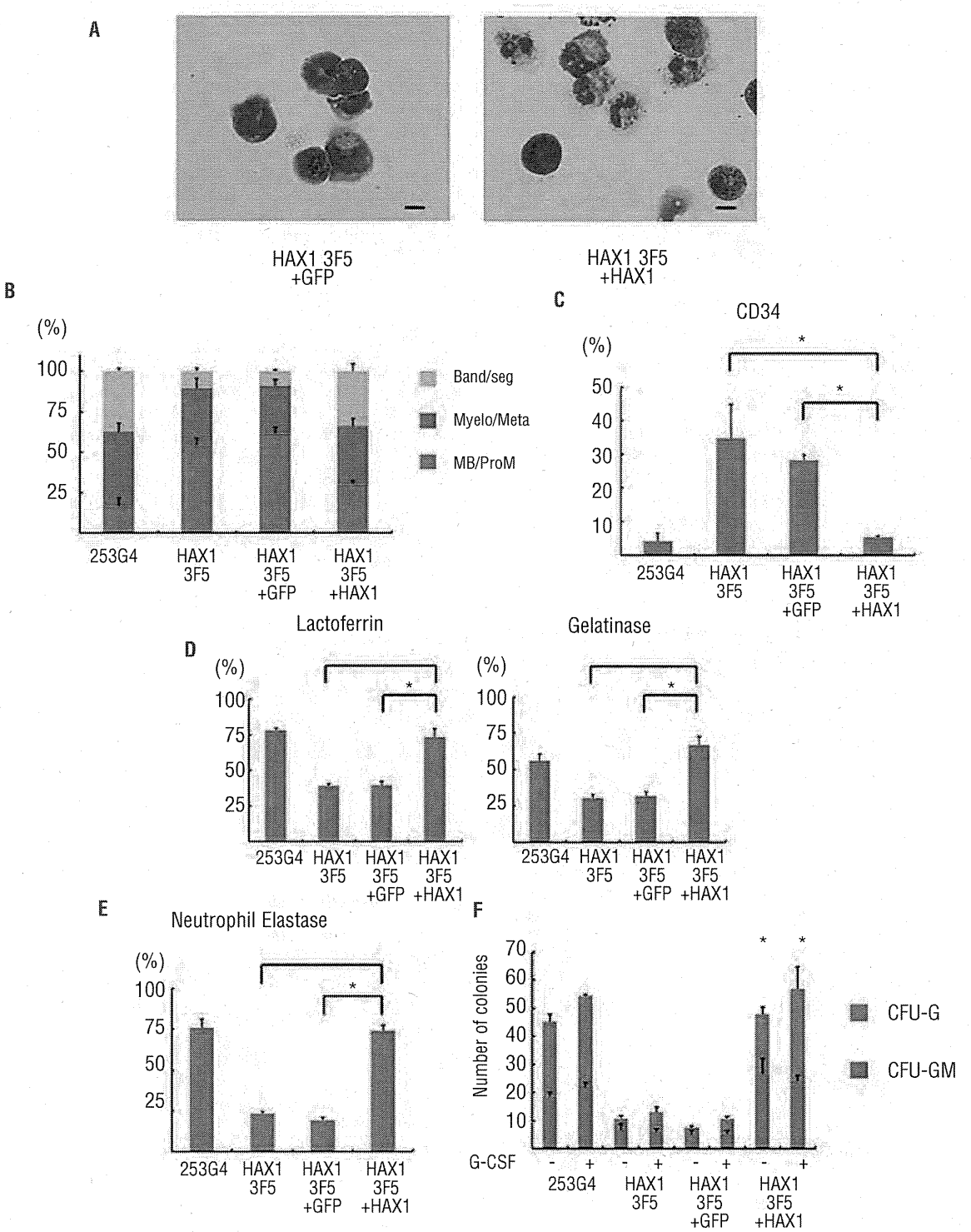

Figure 5. Lentiviral transduction of HAX1 cDNA improves maturation arrest of HAX1-iPS cells. (A) May-Giemsa staining of CD45$^+$ cells derived from HAX1 3F5+GFP and HAX1 3F5+HAX1 cells. Scale bars: 10 μm. (B) Morphological classification of CD45$^+$ cells derived from lentivirally-transduced iPS cells. (n = 3; bars represent SDs). (C) Flow cytometric analysis of CD45$^+$ cells derived from lentivirally-transduced iPS cells. Cells gated on GFP$^+$ human CD45$^+$ DAPI$^-$ were analyzed (n = 3; bars represent SDs; *P<0.05). (D) Immunocytochemical analysis of CD45$^+$ cells derived from lentivirally-transduced iPS cells (n = 3; bars represent SDs; *P<0.05). (E) NE staining of CD45$^+$ cells derived from lentivirally-transduced iPS cells (n = 3; bars represent SDs; *P<0.05). (F) Colony-forming assay of lentivirally-transduced cells derived from iPS cells. The number of colonies derived from 1×10^4 cells is indicated (n = 3; bars represent SD; *P<0.05 compared to HAX1 3F5 and HAX1 3F5+GFP). (A-E) Live CD45$^+$ cells derived from lentivirally-transduced iPS cells on Day 26 of neutrophil differentiation were analyzed. Dead cells and CD45$^-$ cells were depleted using an autoMACS Pro separator (see *Methods*).

etry, a colony-forming assay, and cytostaining of HAX1-iPS cell-derived blood cells quantitatively demonstrated maturation arrest at the progenitor level and apoptotic predisposition due to low $\Delta\psi_m$ resulting in defective granulopoiesis, which were typically observed in SCN patients with *HAX1* gene deficiency. Thus, our culture system may serve as a novel experimental model and a platform for high-throughput screening of drugs for neutropenia in SCN with *HAX1* gene deficiency.

A colony-forming assay showed that the response to G-CSF administration correlated well with the responsiveness of SCN patients to G-CSF therapy. Defective granulopoiesis was recently reported in SCN-iPS cells with a mutation in *ELANE*.[24] Our data showing defective granulopoiesis and reduced response to G-CSF administration are generally consistent with this report. The slight differences in CFU-G/GM colony-forming potential between this previous study and the current study might be due to differences in the causative gene (*HAX1* or *ELANE*) or the culture system used for neutrophil differentiation, and/or to variation in the differentiation capabilities of the clones.

In our serum and feeder-free monolayer culture system, human ES and iPS cells differentiate into hematopoietic and endothelial cells via common KDR'CD34' hemoangiogenic progenitors, which exist during early embryogenesis.[18] Therefore, emergence of abnormal granulopoiesis in this system suggests that disease onset might occur at early hematopoietic stage (yolk sac or fetal liver), which would have never been addressed with patient samples.

We also showed that *HAX1* cDNA transduction could reverse disease-related phenotypes such as abnormal granulopoiesis and apoptotic predisposition. Although little is known about the pathophysiology of SCN with *HAX1* gene deficiency, these results clearly indicated that a loss in HAX1 protein might be the primary cause of neutropenia. These results also indicated the possibility of using patient-derived iPS cells for gene therapy; however, there are technical difficulties that would preclude these cells from being used in a clinical setting. Lentiviral vectors that randomly integrate transgenes can affect the expression of related genes, including cancer-related genes.[25-28] To overcome these problems, we are required to select clones in which transgenes are integrated 'safe harbor' sites and highly expressed without perturbation of neighboring gene expression,[29] or to take the zinc finger nuclease-mediated gene targeting approach[30-32] specifically to a predesigned safe harbor site such as the *AAVS1* locus,[33] which has previously been shown to permit stable expression of transgenes with minimal effects on nearby genes.

The pluripotency of patient-derived iPS cells enables investigation of the pathophysiology of various organ abnormalities and/or dysfunctions. Many types of inherited bone marrow failure syndrome were characterized by multisystem developmental defects that affected the heart, kidney, skeletomuscular system, and central nervous system. Among these, neurological symptoms were frequently seen in SCN patients with *HAX1* gene deficiency,[19,23,34] suggesting that a loss in HAX1 may also affect neural development. Indeed, our patient also presented for epilepsy and severe delays in motor, cognitive, and intellectual development.[19] In patient-derived cells, $\Delta\psi_m$ was not reduced in undifferentiated iPS cells but was reduced in differentiated neutrophils. No marked abnormalities in teratoma formation by HAX1-iPS cells were observed. These results are partially consistent with the fact that SCN patients with a *HAX1* gene deficiency have only neutropenia and neurological symptoms, despite *HAX1* being a ubiquitously expressed gene.[6] Because some of these neurological symptoms cannot be reproduced in the currently available mouse model,[10] additional studies will be necessary to address the effects of *HAX1* on neural development by directed culture models of patient-derived iPS cells.

In conclusion, patient-derived iPS cell-derived myeloid cells were similar in disease presentation to SCN patients with *HAX1* gene deficiency, which could be reversed by gene correction in a novel *in vitro* neutrophil differentiation system. This culture system will serve as a new tool to facilitate disease modeling and drug screening for congenital neutrophil disorders.

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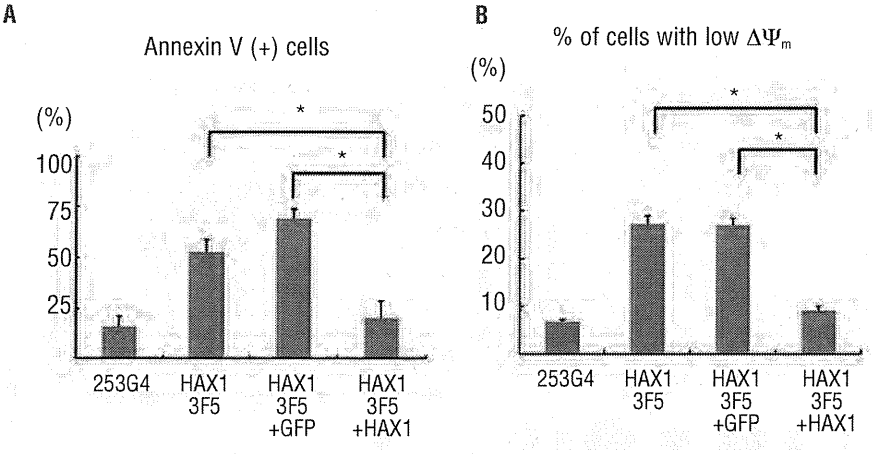

Figure 6. Lentiviral transduction of *HAX1* cDNA prevents HAX1-iPS cells being predisposed to undergo apoptosis. Annexin V assay (A) and mitochondrial membrane potential assay (B) of lentivirally-transduced iPS cell-derived cells on Day 26 of neutrophil differentiation. Cells gated on GFP$^+$ human CD45$^+$ were analyzed (n = 3; bars represent SDs; *P<0.05).

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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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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The authors declare no conflict of interest.

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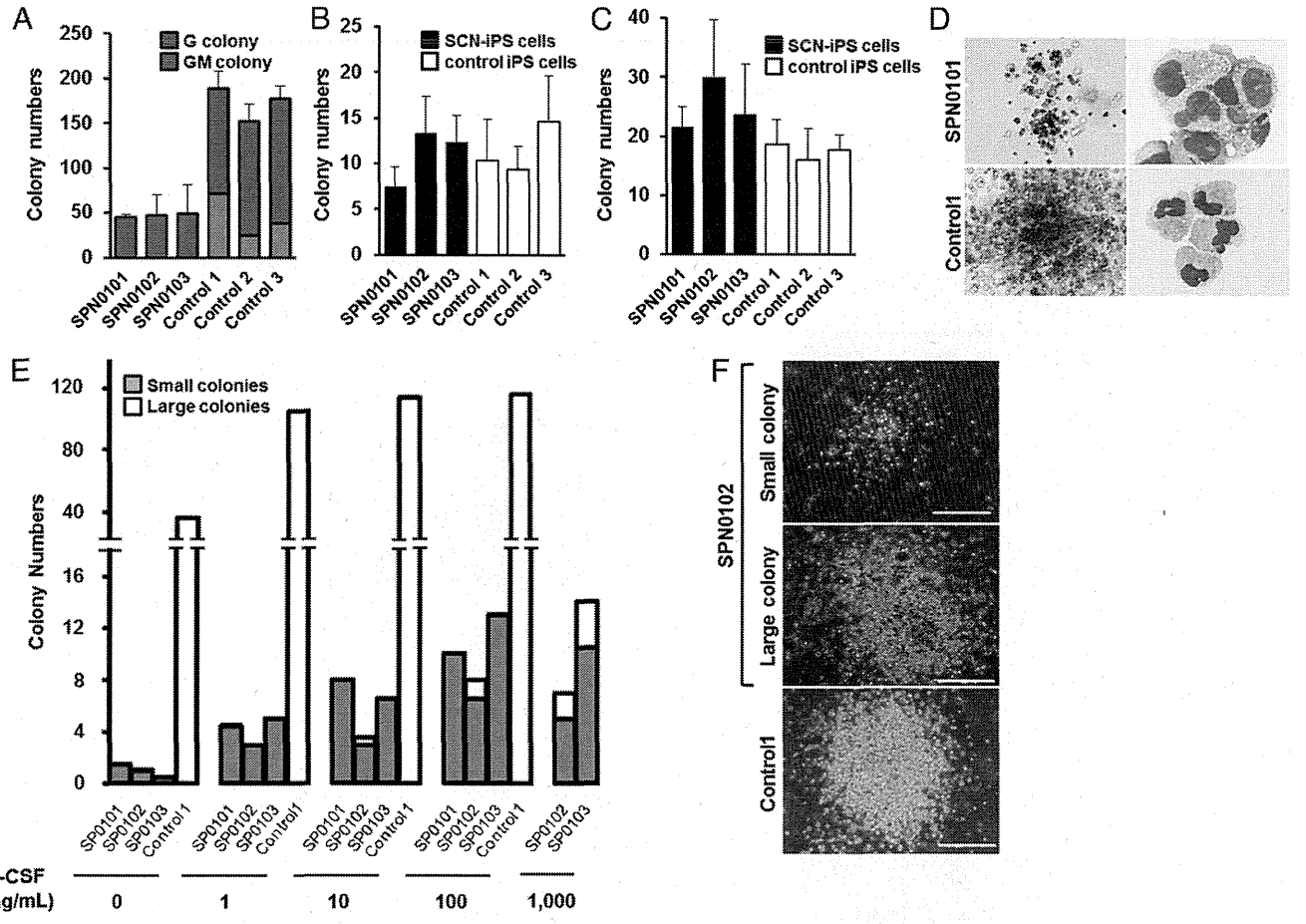

Fig. 1. Impaired neutrophil development from SCN-iPS cells. (*A*–*C*) A hematopoietic colony assay was performed by using 1×10^4 $CD34^+$ cells derived from three SCN-iPS cell clones (SPN0101, SPN0102, and SPN0103) and three control iPS cell clones (controls 1, 2, and 3) in the presence of a cytokine 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-

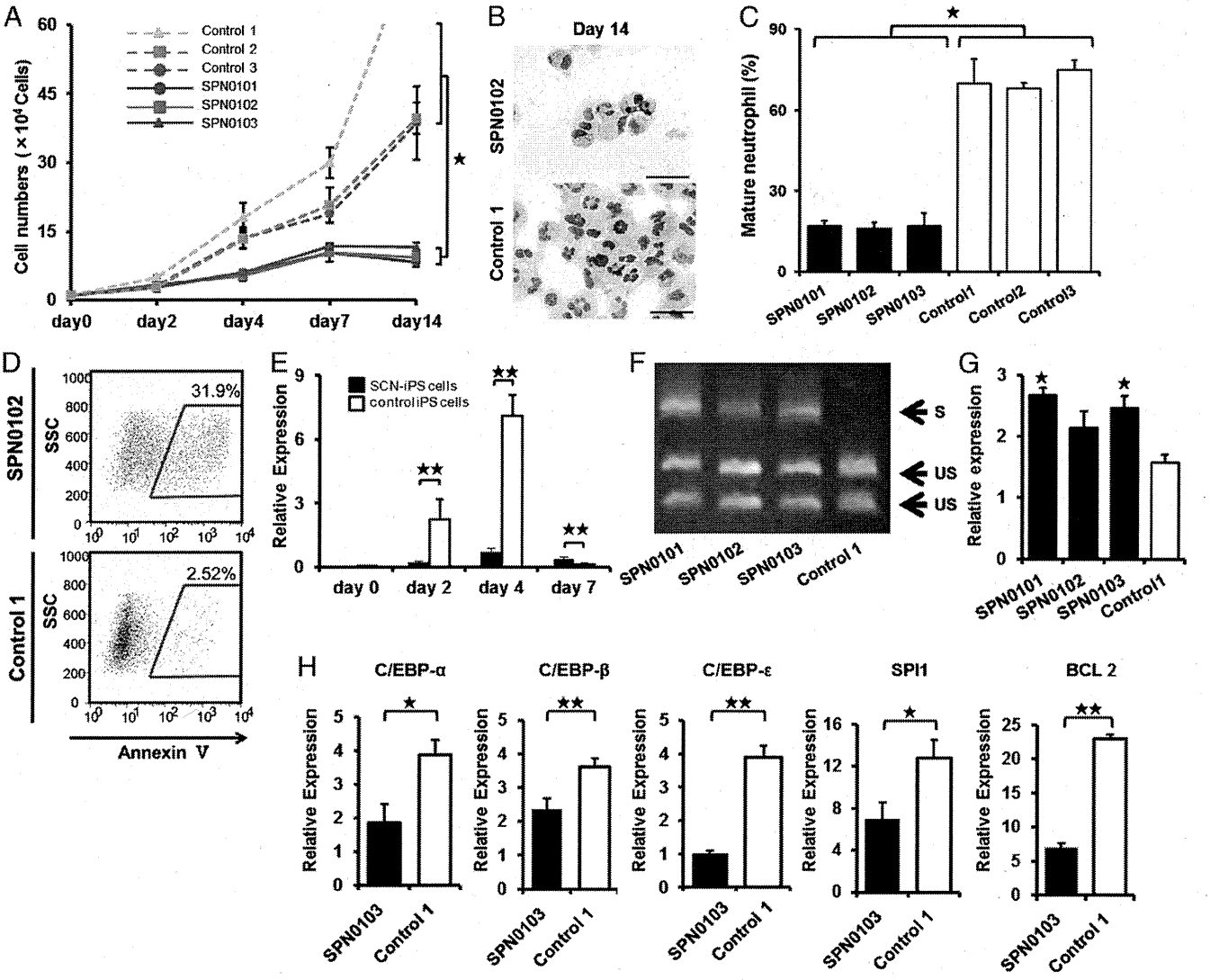

Fig. 2. Analysis of impaired neutrophil development from SCN-iPS cells. (*A*) Total number of nonadherent cells in the suspension culture of 1×10^4 $CD34^+$ cells derived from SCN-iPS and control iPS cells. Data are shown as mean ± SD. $^*P < 0.01$. (*B*) Photographs of nonadherent cells derived from 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

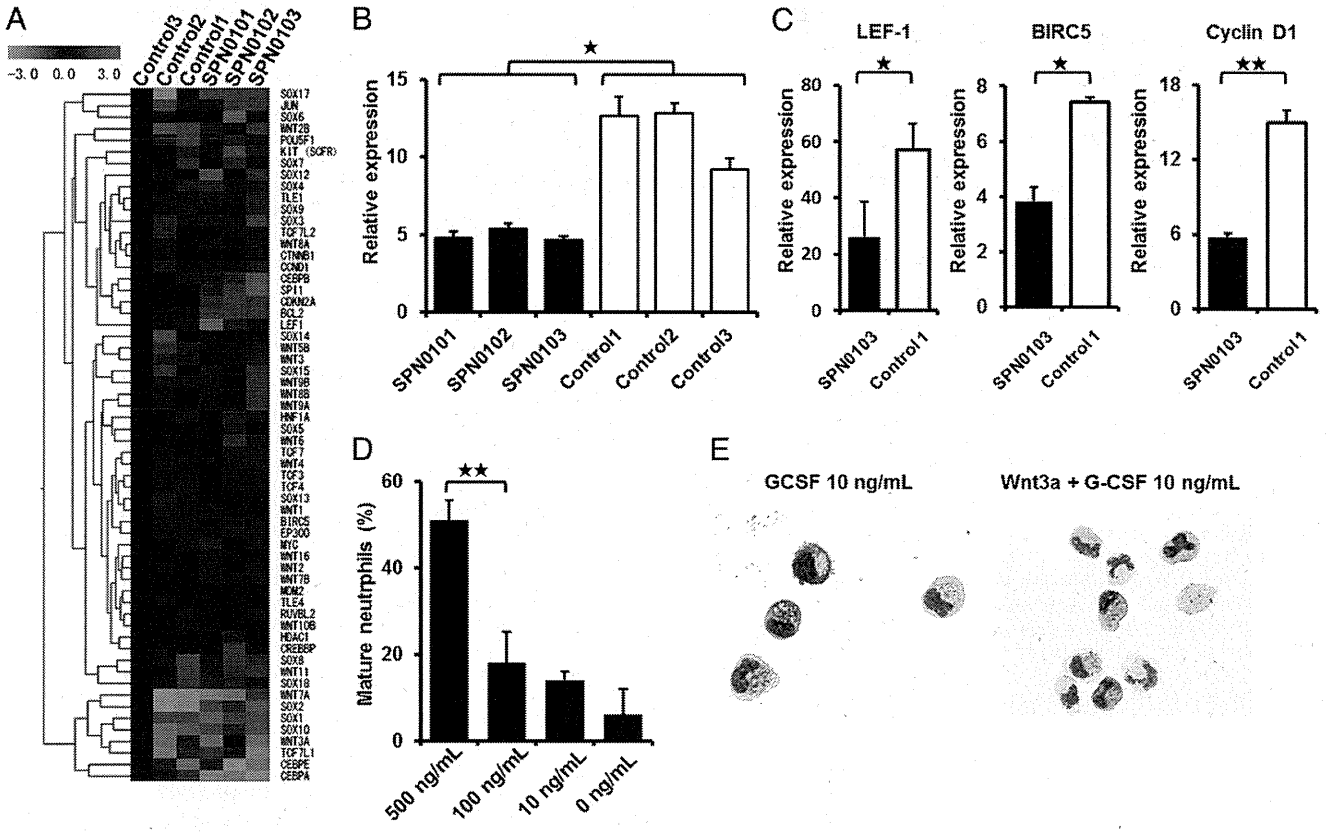

Fig. 3. Effects of Wnt3a on neutrophil development from SCN-iPS cells. (*A*) Heat map showing differential gene expression among SCN-iPS and control iPS cells on day 2. Red, high gene expression; blue, low gene expression compared with gene expression in control 3. (*B*) qRT-PCR analysis of the relative 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).

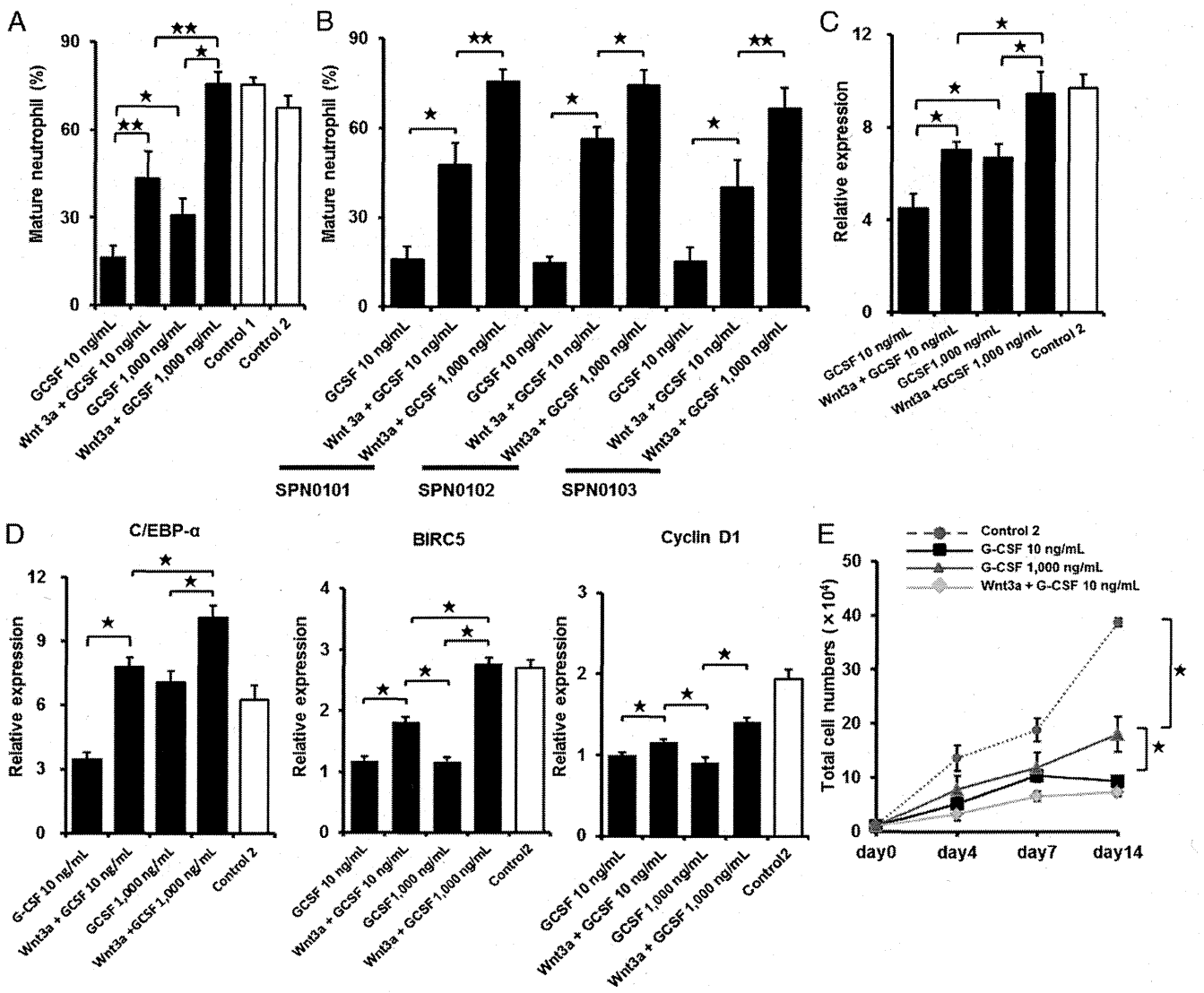

Fig. 4. Effects of Wnt3a in combination with high-dose G-CSF. (*A*) Filled and open bars show the proportion of mature neutrophils among the cells derived from SCN-iPS cells (SPN0101) on day 14 of suspension culture in the presence of neutrophil differentiation medium containing 10 ng/mL G-CSF (GCSF 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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CASE REPORT

Two cases of partial dominant interferon-γ receptor 1 deficiency that presented with different clinical courses of bacille Calmette–Guérin multiple osteomyelitis

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Abstract We experienced two cases of unrelated Japanese children with bacille Calmette–Guérin (BCG) multiple osteomyelitis with partial interferon (IFN)-γ receptor 1 (IFNGR1) deficiency. Heterozygous small deletions with frame shift (811 del4 and 818 del4) were detected, which were consistent with the diagnosis of partial dominant IFNGR1 deficiency. Case 1: a 2-year-old boy visited us because of limb and neck pain. He had been vaccinated with BCG at 17 months of age. Multiple destructive lesions were observed in the skull, ribs, femur, and vertebral bones. *Mycobacterium bovis* (BCG Tokyo 172 strain by RFLP technique) was detected in the bone specimen. The BCG multiple osteomyelitis was treated successfully without recurrence. Case 2: an 18-month-old girl developed multiple osteomyelitis 9 months after BCG inoculation. Radiologic images showed multiple osteolytic lesions in the skull, ribs, femur, and vertebrae. *M. bovis* (BCG Tokyo 172 strain) was detected in the cultures from a bone biopsy. Her clinical course showed recurrent osteomyelitis and lymphadenitis with no pulmonary involvement. The effects of high-dose antimycobacterial drugs and IFN-γ administration were transient, and complete remission has since been achieved by combination antimycobacterial therapy, including levofloxacin. Partial dominant IFNGR1 deficiency is a rare disorder, but it should be considered when a patient presents with multiple osteomyelitis after BCG vaccination. The cases that are resistant to conventional regimens require additional second-line antituberculous drugs, such as levofloxacin.

Keywords Interferon-γ receptor 1 deficiency · Multiple osteomyelitis · Bacille Calmette–Guérin · Mycobacterial infection · Levofloxacin

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Introduction

Interleukin-12 (IL-12)- and IFN-γ (IFNG)-mediated immunity plays an important role in host defense against intracellular pathogens [1]. Mendelian susceptibility to mycobacterial disease (MSMD) is a rare disorder and sometimes lethal disease that occurs in response to poorly virulent mycobacteria, such as bacille Calmette–Guérin (BCG) and environmental nontuberculous mycobacteria (NTM). In patients with MSMD, different types of mutations in six genes—IFNGR1, IFNGR2, IL12RB1, IL12B, STAT-1, and NEMO—have been revealed [2].

Sasaki et al. [3] previously reported a partial IFNGR1 mutation in three Japanese children with BCG osteomyelitis and in the father of one of the patients. We have followed the two unrelated cases over 10 years since their onset in the same department (Koshigaya Municipal Hospital). Based on our longitudinal experience, we intend to provide important clinical information for the diagnosis and treatment of IFN-γR1 deficiency in Japan.

Case report

Case 1

A Japanese boy became spontaneously positive to a tuberculin purified protein derivative (PPD) skin test at the age of 11 months. There was no family history of tuberculosis. A chest X-ray film showed no abnormal findings. The PPD skin test turned negative after 6 months of prophylactic treatment with isoniazid (INH). He was inoculated with BCG (Tokyo 172 strain) by the multiple puncture technique at the age of 17 months. Nine months later (at 26 months of age), he started to limp and could not move his neck. He visited Koshigaya Municipal Hospital, and multiple osteolytic lesions were observed on his skull, vertebrae (cervical and lumbar), ribs, and femur by X-ray, bone scintigram, and magnetic resonance (MR) imaging. *Mycobacterium* was detected in the bone biopsy. *Mycobacterium bovis* was identified as the BCG Tokyo 172 strain by restriction fragment length polymorphism (RFLP). The BCG osteomyelitis was treated successfully with antimycobacterial therapy with isoniazid (INH), rifampicin (RFP), and streptomycin (SM) for 1.5 years without recurrence. He is now 17 years old and has not had a mycobacterial infection since the treatment.

Case 2 (Fig. 1)

An 18-month-old girl (13 years old at present) developed left axillary lymphadenitis 2 months after BCG inoculation at the age of 8 months. Multiple skin eruptions and abscesses appeared 9 months after the vaccination. At the BCG inoculation site, there were signs of hypertrophic scar and keloid. Granuloma was also observed below the inoculation site. X-ray, skeletal scintigram, and MR imaging showed multiple osteolytic lesions in the skull, ribs, femur, and vertebrae. A bone biopsy specimen of the femur revealed granulomatous inflammation without central necrosis. *M. bovis* (BCG Tokyo 172 strain) was detected in cultures from the bone biopsy by RFLP. She was treated with INH, RFP, and SM, and showed slow improvement. Eighteen months after her initial presentation, she started to develop recurrent osteomyelitis. Additional administration of ethambutol (EB) and IFN-γ was effective but the effect was temporary. She exhibited osteomyelitis soon after discontinuation of EB and RFP. High-dose INH and EB, with the addition of clarithromycin (CAM) and IFN-γ, proved effective. Her osteomyelitis appeared to have subsided. However, later, at the age of 11 years, she experienced a third relapse of the osteomyelitis. Antimycobacterial therapy was started again, but lymphadenitis also developed on her right supraclavicle. The findings from the swollen lymph nodes were nonspecific. Additional administration of high-dose IFN-γ was partially effective against the osteomyelitis and the lymphadenitis. As the cervical lymphadenopathy appeared again, the CAM was changed to levofloxacin (LVFX). A three-drug regimen of INH, RFP, and LVFX for a period of 9 months was successful in achieving remission.

The clinical features of these two unrelated Japanese children with BCG multiple osteomyelitis are summarized in Table 1. Two-color flow cytometric analysis was performed [3] and showed significantly higher levels of IFNGR1 expression on monocytes in both cases. IL-12 and IFN-γ production was normal. Genomic DNA was obtained from peripheral blood mononuclear cells. cDNA sequences were analyzed by polymerase chain reaction. Heterozygous small deletions with frame shift (case 1, 811 del4; case 2,

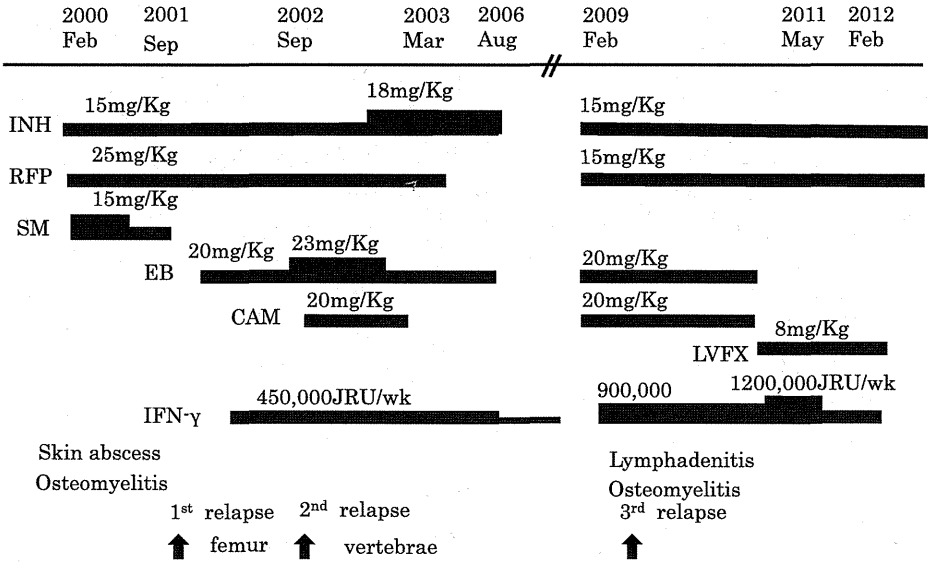

Fig. 1 Recurrent osteomyelitis and lymphadenitis in case 2. *INH* isoniazid, *RFP* rifampicin, *SM* streptomycin, *EB* ethambutol, *CAM* clarithromycin

Table 1 Immunological data at the onset of patients with bacille Calmette–Guérin (BCG) osteomyelitis

Case	1 (17 years/M)	2 (13 years/F)
BCG given at	1 year 5 months	8 months
Age at onset	2 years 2 months	1 year 5 months
Type	Multiple	Multiple, recurrent
Histology	Inflammation	Granuloma
Other organs	None	Skin, lymph node
WBCs/μl	5,300	29,600
Lymphocytes/μl	3,657	7,400
IgG, mg/dl	1,370	1,430
IgA, mg/dl	188	104
IgM, mg/dl	602	181
CD3 cells, %	40.7	56.6
CD4:CD8	3	3
CD19 cells, %	10.4	26.4
PHA response	Normal	Normal
Cytokine production IL-12/INF-γ	Normal	Normal

818 del4) were detected, which were consistent with the diagnosis of partial dominant IFNGR1 deficiency (data not shown). Sequence analysis of six coding regions was performed and showed that none of the family members of the patients had any mutations. Furthermore, neither sets of parents were consanguineous. Thus, de novo mutation had occurred in both cases 1 and 2.

Discussion

Bacille Calmette–Guérin vaccines are safe in immunocompetent hosts, and Japanese BCG substrain Tokyo 172 is the safest BCG in the world [4]. Complications of BCG vaccination can be severe and life threatening in infants with immunodeficiency. Systemic adverse reactions to BCG vaccine, including osteomyelitis and disseminated BCG infection, are rare. Toida and Nakata [5] reviewed severe adverse reactions to BCG from 1951 to 2004 in Japan and identified 39 cases (incidence rate, 0.0182 cases per 100,000 vaccinations). Thirteen cases exhibited primary immunodeficiency; 5 of these exhibited chronic granulomatous diseases, 4 exhibited severe combined immunodeficiency, and 4 exhibited IFNGR1 deficiency. Unidentified defects in cellular immunity were observed in 6 cases. The 6 fatal cases had cellular immunodeficiencies. Bone and joint involvement was observed in 27 cases, 15 cases with multiple lesions and 12 cases with single site lesions.

Hoshina et al. [6] analyzed the clinical characteristics and the genetic background of 46 patients with MSMD in Japan from 1999 to 2009, and found that 6 had mutations in the IFN-γR1 gene. All the cases of IFN-γR1 deficiency exhibited multiple osteomyelitis, and disseminated mycobacterial infection recurred in 5 patients. All the patients exhibited the partial dominant type, and 4 of them had 818 del4. Two of the patients were from the same family, and therefore autosomal dominant inheritance was suspected. The 4 others were considered to have occurred spontaneously. In Taiwan, 3 patients from two unrelated families were identified with a hotspot IFNGR1 deletion mutation (818 del4) and exhibited chronic granulomatous disease-like features, presenting as cutaneous granuloma and multiple osteomyelitis infected with NTM [7]. Fewer patients of Asian origin have been reported with partial dominant IFNGR1 deficiency compared with those of Western countries [8]. The clinical phenotype of partial dominant IFNGR1 deficiency is milder than that of complete deficiency. In this type, BCG and NTM are the major pathogens. Complete IFN-γ receptor deficiency is associated with the early onset of severe disease caused by BCG or NTM, whereas the other genetic forms are associated with a milder course of mycobacterial infection [8].

Patients with partial IFGR1 deficiency usually respond well to antibiotic treatment, and for those who do not respond well, additional IFN-γ therapy has been shown to be effective [9]. There is no single standard regimen for the treatment of children with BCG osteomyelitis. *M. bovis* is resistant to pyrazinamides because of the expression of a pyrazinamidase. Case 1 was successfully treated with a long-term combination therapy of INH, RFP, and SM. However, in case 2, conventional therapy was inadequate to fight the infection. Additional administration of EB and relatively low dose IFN-γ was not able to control the intractable osteomyelitis. As NTM infection was also possible, high-dose EB, INH, and CAM were administered. The regimen was effective but temporary. Combination therapy, including LVFX and high-dose INF-γ, was the most successful strategy. Treatment with second-line antituberculous drugs, such as fluoroquinolone, and two first-line drugs (RFP and EB), may be more effective than RFP and EB alone against multidrug-resistant *M. bovis* [10]. LVFX plays an important role as a substitute agent for those patients who are intolerant of first-line antituberculous agents.

IFN-γ receptor deficiency is a rare disorder that should be considered when patients exhibit BCG lymphadenitis and disseminated osteomyelitis. Multifocal mycobacterial osteomyelitis without other organ involvement is only seen in dominant partial IFNGR1 deficiency [6, 8]. This type of immunodeficiency tends to exhibit recurrent mycobacterial infection and resistance to conventional antimycobacterial therapy. LVFX is likely an effective option for cases with the partial dominant type that are resistant.

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Pulmonary fibrosis in dyskeratosis congenita with *TINF2* gene mutation

To the Editor:

Dyskeratosis congenita is a rare inherited disorder of ectodermal dysplasia characterised by the classical mucocutaneous triad of abnormal skin pigmentation, nail dystrophy and leukoplakia [1–3], at least one of which is present in around 80–90% of dyskeratosis congenita cases. Bone marrow failure is another common feature, and a variety of other abnormalities (*e.g.* dental, gastrointestinal, neurological, ophthalmic, pulmonary and skeletal) have been also described [1–3]. The main causes of mortality in dyskeratosis congenita are bone marrow failure, pulmonary disease and malignancy [1]. Three modes of inheritance have been recognised: X-linked recessive, autosomal dominant and autosomal recessive [1, 3]. Eight dyskeratosis congenita genes (*DKC1* (dyskeratosis congenita 1), *TERC* (telomerase RNA component), *TERT* (telomerase reverse transcriptase), *NOP10* (nucleolar protein 10), *NHP2*, *TINF2* (TERF1-interacting nuclear factor 2), *TCAB1* and *RTEL1* (regulation of telomere elongation helicase 1)) have already been identified, and their mutations account for ~60% of all dyskeratosis congenita cases [1]. Among the dyskeratosis congenita genes, mutations in *TERC*, *TERT* and *DKC1* have recently been reported to be associated with familial pulmonary fibrosis and idiopathic pulmonary fibrosis, and pulmonary fibrosis is recognised as one of the features of dyskeratosis congenita. However, the relationship between mutations in the other dyskeratosis congenita genes and pulmonary fibrosis has not yet been clarified. To the best of our knowledge, this is the first case report describing a dyskeratosis congenita patient with pulmonary fibrosis who had a *TINF2* mutation.

A 43-year-old female visited our hospital with cough and progressive dyspnoea. She had never smoked, and had a history of aplastic anaemia, ocular pemphigoid, erythroplasia of Queyrat and infertility. Her father had been diagnosed as having aplastic anaemia and his whole body was pigmented. About 2 years ago, she complained of cough and consulted her personal doctor. Her chest radiographs showed diffuse reticular shadows in the bilateral lung fields. She was referred to a general hospital and was diagnosed with idiopathic interstitial pneumonia. Because her general condition was stable at that time, she was followed up without any specific therapy for 1 year. She was referred to our hospital due to gradual worsening of dyspnoea and admitted for further examinations. Her physical examination was remarkable for skin pigmentation on her whole body, ocular pemphigoid in the left eye and fine crackles in both lung fields. Her fingertip skin was rough but her nails were not dystrophic. Although no leukoplakia was found in the oral mucosa, she had erythroplasia of Queyrat of the vulva. Laboratory data showed elevated lactate dehydrogenase, transaminases, erythrocyte sedimentation rate and sialylated carbohydrate antigen KL-6 with thrombocytopenia. Chest radiographs demonstrated consolidation and reticular shadows in the bilateral lung fields. Furthermore, chest computed tomography revealed consolidation and reticular shadows in both lung fields, as well as bronchiectasis and cystic shadows in the left lung.

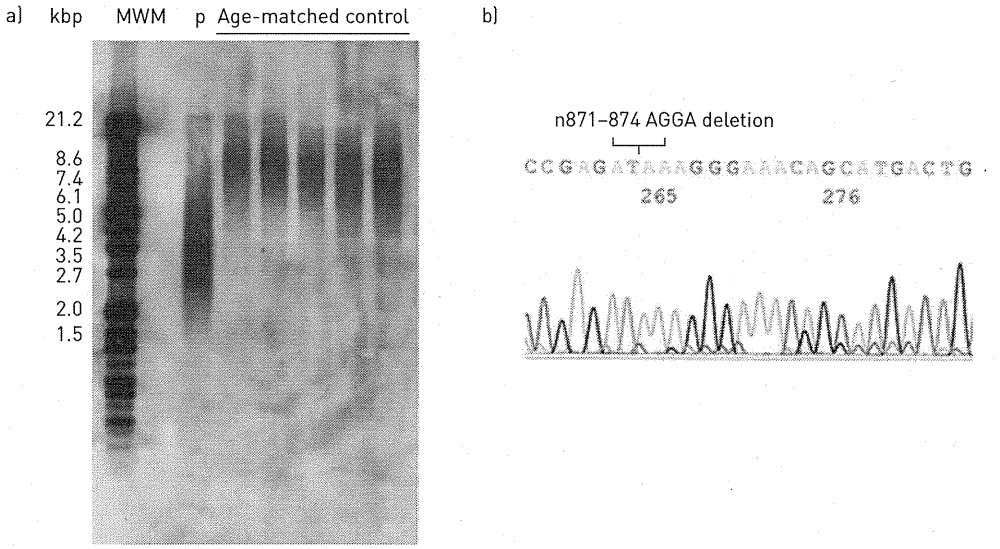

FIGURE 1 a) Southern blot analysis showed shorter telomere length of the patient (P) compared to age-matched healthy controls. MWM: molecular weight marker. b) Gene mutation analysis by direct sequencing showed n871–874 tetranucleotide AGGA deletion in *TINF2* gene.

At this point, we strongly suspected that she had dyskeratosis congenita. To make a definite diagnosis, we first examined the *TERC* and *TERT* genes by direct sequencing. However, no mutations were found in either gene. Southern blot analysis showed short telomere length (fig. 1a), therefore mutations in *TINF2* were next explored. As shown in figure 1b, because direct sequencing showed a n871–874 tetranucleotide AGGA deletion in *TINF2*, she was diagnosed as having dyskeratosis congenita with pulmonary fibrosis associated with *TINF2* mutation. As her respiratory condition progressed, steroid pulse therapy followed by oral prednisolone was conducted. However, no improvement of her symptoms was observed, and bilateral pneumothorax with mediastinal and subcutaneous emphysemas developed. She died of respiratory failure 1 year after starting the treatment.

Dyskeratosis congenita is a rare genetic ectodermal disorder characterised by skin hyperpigmentation, nail dystrophy and leukoplakia of the mucous membranes. Bone marrow failure is a frequent finding and a predisposition to malignancy has been noted. Although pulmonary manifestations of dyskeratosis congenita were believed to be uncommon, DOKAL [1] reported that abnormal pulmonary features may be seen in as many as 10–15% of patients.

Genetically, dyskeratosis congenita is heterogeneous, with three forms having been identified: X-linked recessive, autosomal dominant and autosomal recessive. In the present case, the patient's father had suffered from the same disease; therefore, we suspected that the form of dyskeratosis of this patient was autosomal dominant. The autosomal dominant form of dyskeratosis congenita is caused by heterozygous mutations in the core components of telomerase, *TERC* [4, 5] and *TERT* [6, 7], as well as in the component of the shelterin telomere protection complex, *TINF2* [3]. In this patient, mutation of *TINF2*, but not *TERC* and *TERT*, was confirmed by gene mutation analysis. It has previously been reported that mutations in *DKC1* [8], *TERC* [5] and *TERT* [6] were associated with pulmonary fibrosis in dyskeratosis congenita patients. *DKC1* was not analysed in this patient, because mutation in *DKC1* causes the X-linked form of dyskeratosis congenita. Regarding the relationship between pulmonary fibrosis and *TINF2* mutation in dyskeratosis congenita, WALNE *et al.* [3] have reported that only one patient had pulmonary fibrosis among other clinical features in 33 dyskeratosis congenita patients with *TINF2* mutations. However, they did not describe the patient in detail. To the best of our knowledge, this is the first case report showing pulmonary fibrosis in dyskeratosis congenita with *TINF2* mutation.

TINF2 mutations were reported to be heterozygous mutations in the sixth-found dyskeratosis congenita gene by SAVAGE *et al.* [9] in 2008. *TINF2* encodes TIN2, and is a component of the shelterin telomere-protection complex. The shelterin complex has at least three effects on telomeres: it determines the structure of the telomeric terminus, is implicated in the generation of t-loops and controls the synthesis of telomeric DNA by telomerase [1, 10]. Without the protective activity of shelterin, telomeres are no longer hidden from DNA repair mechanisms and chromosome ends are therefore incorrectly processed by the DNA repair pathways. Approximately 11% of all dyskeratosis congenita has been reported to be accounted for by *TINF2*

mutations and patients with these mutations have significantly shorter telomeres than those with other dyskeratosis congenita subtypes [3]. It has also been reported that most patients with dyskeratosis congenita with *TINF2* mutations have severe disease, and, compared with other dyskeratosis congenita genes, patients with *TINF2* mutations have a high incidence of aplastic anaemia before the age of 10 years [3].

Aberrant repair process by enhanced apoptosis of alveolar epithelial cells plays a critical role in the pathogenesis of pulmonary fibrosis such as idiopathic pulmonary fibrosis, although the precise mechanism is still unclear. The mechanism(s) of pulmonary fibrosis in dyskeratosis congenita has also not yet been clarified. However, because mutations in dyskeratosis congenita genes cause short telomere length with functional deficits in telomere maintenance, telomeres in alveolar epithelial cells may be short. In patients with dyskeratosis congenita, we speculate that aberrant lung repair by enhanced cell death causes pulmonary fibrosis, although the short telomere length in alveolar epithelial cells has not been directly demonstrated.

Herein, we describe the first case report of dyskeratosis congenita with pulmonary fibrosis associated with *TINF2* mutation. This report proved that mutations not only in *TERC*, *TERT* and *DKC1*, but also *TINF2*, cause pulmonary fibrosis in dyskeratosis congenita. However, we do not know why mutations in *TERC*, *TERT* and *DKC1* are frequently found in dyskeratosis congenita patients with pulmonary fibrosis in contrast to the other five genes. In addition, sex hormones, which can increase telomerase activity, are potential therapeutic drugs; however, no standard treatment has been established for pulmonary fibrosis in dyskeratosis congenita patients. Because the clinical characteristics and pathogenesis of pulmonary fibrosis in dyskeratosis congenita is not clear, the accumulation of case-based reports sheds light on the understanding of this devastating disease.

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The first reported case of a dyskeratosis congenita patient with pulmonary fibrosis and *TINF2* mutation http://ow.ly/pheRW

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