Genetic correction of *HAX1* in induced pluripotent stem cells from a patient with severe congenital neutropenia improves defective granulopoiesis

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ABSTRACT

HAX1 was identified as the gene responsible for the autosomal recessive type of severe congenital neutropenia. However, the connection between mutations in the *HAX1* gene and defective granulopoiesis in this disease has remained unclear, mainly due to the lack of a useful experimental model for this disease. In this study, we generated induced pluripotent stem cell lines from a patient presenting for severe congenital neutropenia with *HAX1* gene deficiency, and analyzed their *in vitro* neutrophil differentiation potential by using a novel serum- and feeder-free directed differentiation culture system. Cytostaining and flow cytometric analyses of myeloid cells differentiated from patient-derived induced pluripotent stem cells showed arrest at the myeloid progenitor stage and apoptotic predisposition, both of which replicated abnormal granulopoiesis. Moreover, lentiviral transduction of the *HAX1* cDNA into patient-derived induced pluripotent stem cells reversed disease-related abnormal granulopoiesis. This *in vitro* neutrophil differentiation system, which uses patient-derived induced pluripotent stem cells for disease investigation, may serve as a novel experimental model and a platform for high-throughput screening of drugs for various congenital neutrophil disorders in the future.

Introduction

Severe congenital neutropenia (SCN) is a rare myelopoietic disorder resulting in recurrent life-threatening infections due to a lack of mature neutrophils,[1] and individuals with SCN present for myeloid hypoplasia with an arrest of myelopoiesis at the promyelocyte/myelocyte stage.[1,2] SCN is actually a multigene syndrome that can be caused by inherited mutations in several genes. For instance, approximately 60% of SCN patients are known to carry autosomal dominant mutations in the *ELANE* gene, which encodes neutrophil elastase (NE).[3] An autosomal recessive type of SCN was first described by Kostmann in 1956,[4] and defined as Kostmann disease. Although the gene responsible for this classical type of SCN remained unknown for more than 50 years, Klein *et al.* identified mutations in *HAX1* to be responsible for this type of SCN in 2007.[5] HAX1 localizes predominantly to mitochondria, where it controls inner mitochondrial membrane potential ($\Delta\psi_m$) and apoptosis.[6,7] Although an increase in apoptosis in mature neutrophils was presumed to cause neutropenia in HAX1 gene deficiency,[5] the connection between *HAX1* gene mutations and defective granulopoiesis in SCN has remained unclear.

To control infections, SCN patients are generally treated with granulocyte colony-stimulating factor (G-CSF); however, long-term G-CSF therapy associates with an increased risk of myelodysplastic syndrome and acute myeloid leukemia (MDS/AML).[8,9] Although hematopoietic stem cell transplantations are available as the only curative therapy for this disease, they can result in various complications and mortality.[4]

Many murine models of human congenital and acquired diseases are invaluable for disease investigation as well as for novel drug discoveries. However, their use in a research setting can be limited if they fail to mimic strictly the phenotype of the human disease in question. For instance, the *Hax1* knock-out mouse is characterized by lymphocyte loss and neuronal apoptosis, but not neutropenia.[10] Thus, it is not a suitable experimental model for SCN. Induced pluripotent stem (iPS) cells are reprogrammed somatic cells with embryonic stem (ES) cell-like characteristics produced by the introduction of specific transcription factors,[11,16] and they may substitute murine models of human disease. It is believed that iPS cell technology, which generates disease-specific pluripotent stem cells in combination with directed cell differentiation, will contribute enormously to patient-oriented research, including disease pathophysiology, drug screening, cell transplantation, and gene therapy.

In vitro neutrophil differentiation systems, which can reproduce the differentiation of myeloid progenitor cells to mature neutrophils, are needed to understand the pathogenesis of SCN better. Recently, we established a neutrophil differentia-

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tion system from human iPS cells[17] as well as a serum- and feeder-free monolayer hematopoietic culture system from human ES and iPS cells.[18] In this study, we generate iPS cell lines from an SCN patient with *HAX1* gene deficiency and differentiate them into neutrophils *in vitro*. Furthermore, we corrected for the *HAX1* gene deficiency in HAX1-iPS cells by lentiviral transduction with *HAX1* cDNA and analyzed the neutrophil differentiation potential of these cells. Thus, this *in vitro* neutrophil differentiation system from patient-derived iPS cells may be a useful model for future studies in SCN patients with *HAX1* gene deficiency.

Methods

Human iPS cell generation

Skin biopsy specimens were obtained from an 11-year old male SCN patient with *HAX1* gene deficiency.[19] This study was approved by the Ethics Committee of Kyoto University, and informed consent was obtained from the patient's guardians in accordance with the Declaration of Helsinki. Fibroblasts were expanded in DMEM (Nacalai Tesque, Inc., Kyoto, Japan) containing 10% FBS (vol/vol, Invitrogen, Carlsbad, CA, USA) and 0.5% penicillin and streptomycin (wt/vol, Invitrogen). Generation of iPS cells was performed as described previously.[12] In brief, we introduced *OCT3/4, SOX2, KLF4*, and *cMYC* using ecotropic retroviral transduction into patient's fibroblasts expressing mouse *Slc7a1*. Six days after transduction, cells were harvested and re-plated onto mitotically inactive SNL feeder cells. On the following day, DMEM was replaced with primate ES cell medium (ReproCELL, Kanagawa, Japan) supplemented with basic fibroblast growth factor (5 ng/mL, R&D Systems, Minneapolis, MN, USA). Three weeks later, individual colonies were isolated and expanded.

Maintenance of cells

Control ES (KhES-1) and control iPS (253G4 and 201B6) cells were kindly provided by Drs. Norio Nakatsuji and Shinya Yamanaka (Kyoto University, Kyoto, Japan), respectively. These human ES and iPS cell lines were maintained on mitomycin-C (Kyowa Hakko Kirin, Tokyo, Japan)-treated SNL feeder cells as described previously[17] and subcultured onto new SNL feeder cells every seven days.

Flow cytometric analysis

Cells were stained with antibodies as reported previously.[17] Samples were analyzed using an LSR flow cytometer and Cell Quest software (Becton-Dickinson).

Neutrophil differentiation of iPS cells

In a previous study, we established a serum and feeder-free monolayer hematopoietic culture system from human ES and iPS cells.[18] In this study, we modified this culture system to direct neutrophil differentiation. iPS cell colonies were cultured on growth factor-reduced Matrigel (Becton-Dickinson)-coated cell culture dishes in Stemline II hematopoietic stem cell expansion medium (Sigma-Aldrich, St. Louis, MO, USA) containing the insulin-transferrin-selenium (ITS) supplement (Invitrogen) and cytokines. iPS cells were treated with cytokines as follows: bone morphogenetic protein (BMP) 4 (20 ng/mL, R&D Systems) was added for four days and then replaced with vascular endothelial growth factor (VEGF) 165 (40 ng/mL, R&D Systems) on Day 4. On Day 6, VEGF 165 was replaced with a combination of stem cell factor (SCF, 50 ng/mL, R&D Systems), interleukin (IL)-3 (50 ng/mL, R&D Systems), thrombopoietin (TPO, 5 ng/mL, kindly provided by Kyowa Hakko Kirin), and G-CSF (50 ng/mL, also kindly provided by Kyowa Hakko Kirin). Thereafter, medium was replaced every five days.

Dead cell removal and CD45$^+$ leukocyte separation

Floating cells were collected, followed by the removal of dead cells and cellular debris with the Dead Cell Removal kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD45$^+$ cells were then separated using human CD45 microbeads (Miltenyi Biotec). Cell separation procedures were performed using the autoMACS Pro Separator (Miltenyi Biotec).

Statistical analysis

Statistical analysis was carried out using Student's t-test. $P<0.05$ was considered statistically significant.

Results

Generation of iPS cell lines from an SCN patient with HAX1 gene deficiency

To generate patient-derived iPS cell lines, dermal fibroblasts were obtained from a male SCN patient with a homozygous 256C-to-T transition resulting in an R86X mutation in the *HAX1* gene.[19] These fibroblasts were reprogrammed to iPS cells after transduction with retroviral vectors encoding *OCT3/4, SOX2, KLF4* and *cMYC*,[12] and a total of 11 iPS cell clones were obtained. From these, we randomly selected three clones for propagation and subsequent analyses. One of these clones (HAX1 4F5) was generated with four factors (*OCT3/4, SOX2, KLF4*, and *cMYC*); the remaining clones (HAX1 3F3 and 3F5) were generated with three factors (*OCT3/4, SOX2*, and *KLF4*).[12]

All of these patient-derived iPS cell clones showed a characteristic human ES cell-like morphology (Figure 1A), and they propagated for serial passages in human ES cell maintenance culture medium. Quantitative PCR analysis showed the expression of *NANOG*, a pluripotent marker gene, to be comparable to that of control ES (KhES-1) and iPS (253G4 and 201B6) cells (Figure 1B). Surface marker analysis indicated that they were also positive for SSEA4, a human ES and iPS cell marker (Figure 1C). DNA sequencing analysis verified an identical mutation in the *HAX1* gene in all established iPS cell clones (Figure 1D). The pluripotency of all iPS cell clones was confirmed by the presence of cell derivatives representing all three germ layers by teratoma formation after subcutaneous injection of undifferentiated iPS cells into immunocompromised NOD/SCID/γc^{null} mice (Figure 1E).

To validate the authenticity of iPS cells further, we investigated the expression of the four genes that were used for iPS cell generation. The expression level of all endogenous genes was comparable to control ES and iPS cells. On the other hand, transgene expression was largely undetectable in patient-derived iPS cell clones (*Online Supplementary Figure S1A*). Chromosomal analysis revealed that all patient-derived iPS cell clones maintained a normal karyotype (*Online Supplementary Figure S1B*). Genetic identity was shown by short tandem repeat analysis (*Online Supplementary Figure S1C*).

Taken collectively, these results indicate that iPS cell clones were comprised of good quality iPS cells derived from the somatic cells of an SCN patient with *HAX1* gene deficiency (HAX1-iPS cells).

Maturation arrest at the progenitor level in neutrophil differentiation from HAX1-iPS cells

The paucity of mature neutrophils in the peripheral blood and a maturation arrest at the promyelocyte/myelocyte stage in the bone marrow are characteristic laboratory findings presented in the SCN patients with *HAX1* gene deficiency. To investigate whether our patient-derived iPS cell model accurately replicated this disease phenotype, we assessed neutrophil differentiation from HAX1-iPS cells by using a serum- and feeder-free monolayer culture system[18] with minor modifications (Online *Supplementary Figure S2*).

In this system, we cultured iPS cell colonies on Matrigel-coated dishes in serum-free medium supplemented with several cytokines and obtained hematopoietic cells as floating cells on approximately Day 26 of differentiation. May-Giemsa staining of floating live CD45$^+$ cells derived from normal iPS cells showed that approximately 40% were mature neutrophils (Figure 2A and B). The remaining cells consisted of immature myeloid cells as well as a small number of macrophages. Cells of other lineages such as erythroid or lymphoid cells were not observed. On the other hand, HAX1-iPS cell-derived blood cells contained only approximately 10% mature neutrophils and approxi-

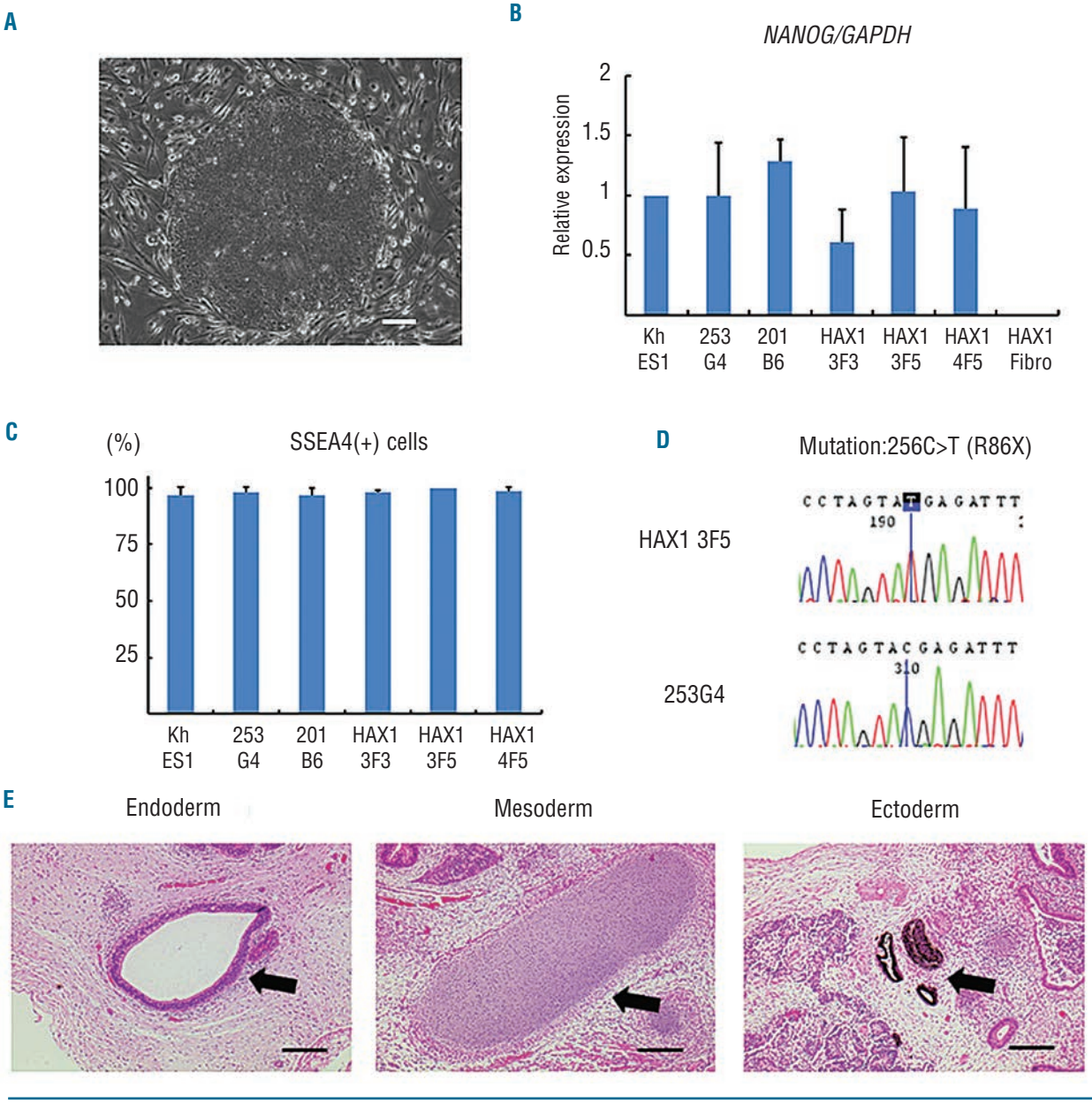

Figure 1. Generation of iPS cell lines from an SCN patient with *HAX1* gene deficiency. (A) Human ES cell-like morphology of HAX1-iPS cells. Scale bar: 200 μm. (B) *NANOG* expression in HAX1-iPS cells, control iPS cells (253G4 and 201B6), and patient-derived fibroblasts (HAX1 Fibro) compared to control ES cells (KhES1). *GAPDH* was used as an internal control (n = 3; bars represent SDs). (C) SSEA-4 expression analysis using flow cytometry. Gated on TRA1-85$^+$DAPI$^-$ cells as viable human iPS (ES) cells (n = 3; bars represent SDs). (D) DNA sequencing analysis of the *HAX1* gene in iPS cells. HAX1-iPS cells showed 256C>T (R86X) mutation that was found in the patient. (E) Teratoma formation from HAX1-iPS cells in the NOD/SCID/γc^{null} (NOG) mouse. Arrows indicate the following; Endoderm: respiratory epithelium; Mesoderm: cartilage; Ectoderm: pigmented epithelium. Scale bars: 200 μm. (A, D-E) Representative data (HAX1 3F5) are shown.

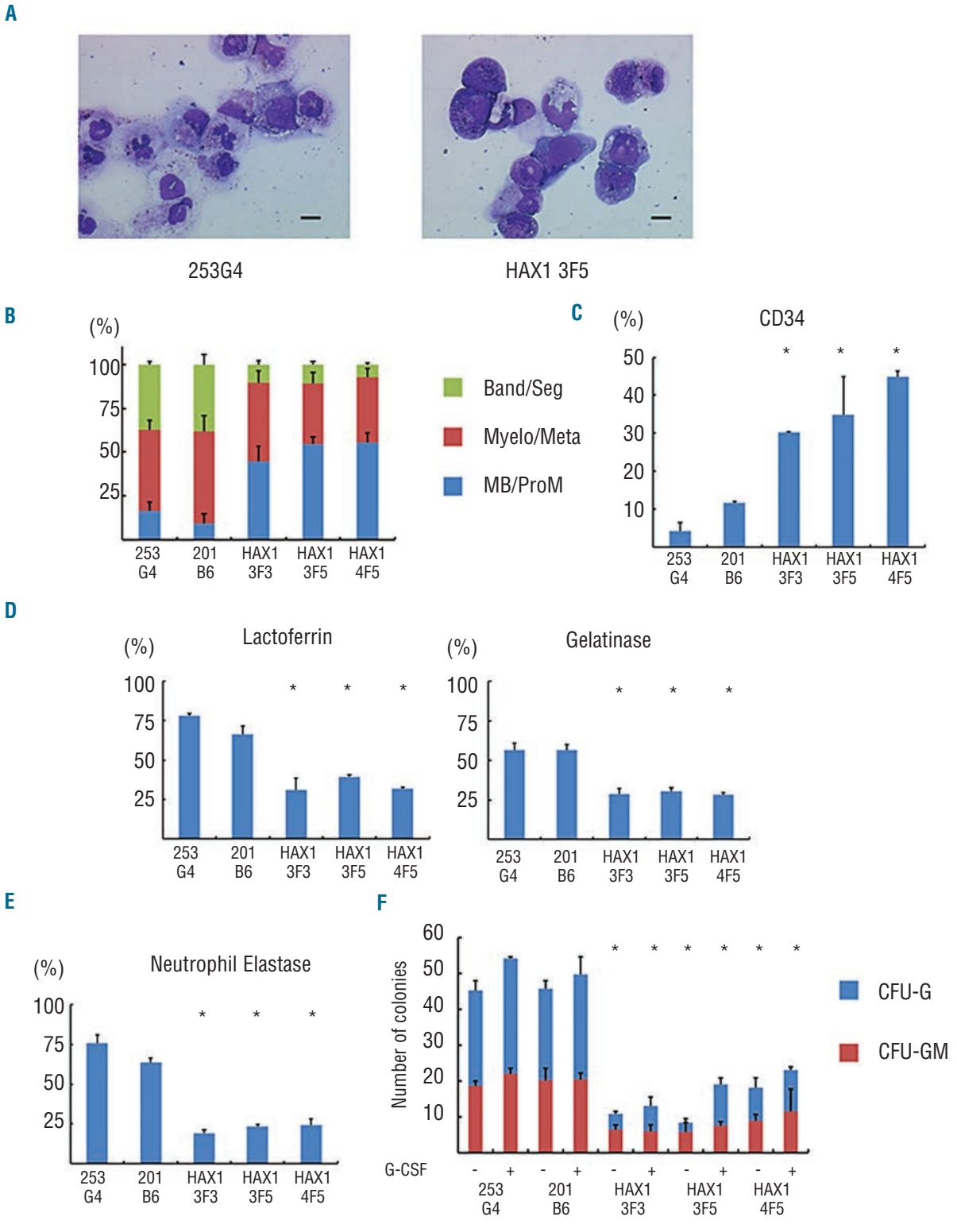

Figure 2. Maturation arrest at the progenitor level in neutrophil differentiation from HAX1-iPS cells. (A) May-Giemsa staining of CD45$^+$ cells derived from normal (253G4) and HAX1-iPS (HAX1 3F5) cells. Scale bars: 10 μm. (B) Morphological classification of CD45$^+$ cells derived from iPS cells. Cells were classified into three groups: myeloblast and promyelocyte (MB/ProM), myelocyte and metamyelocyte (Myelo/Meta), and band and segmented neutrophils (Band/Seg) (n = 3; bars represent SDs). (C) Flow cytometric analysis of CD45$^+$ cells derived from iPS cells. Cells gated on human CD45$^+$ DAPI$^-$ were analyzed (n = 3; bars represent SDs; *P<0.05 compared to control iPS cells). (D) Immunocytochemical analysis of CD45$^+$ cells derived from iPS cells (n = 3; bars represent SDs; *P<0.05 compared to control iPS cells). (E) NE staining of CD45$^+$ cells derived from iPS cells (n = 3; bars represent SDs; *P<0.05 compared to control iPS cells). (F) Colony-forming assay of cells derived from iPS cells. On Day 16, living adherent cells were collected and cultured in methylcellulose medium (see *Online Supplementary Appendix*). The number of colonies generated from 1×10^4 cells is indicated (n = 3; bars represent SD; *P<0.05 compared to control iPS cells). (A–E) Live CD45$^+$ cells derived from normal and HAX1-iPS cells on Day 26 of neutrophil differentiation were analyzed. Dead cells and CD45$^-$ cells were depleted using an autoMACS Pro separator (see *Methods*).

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

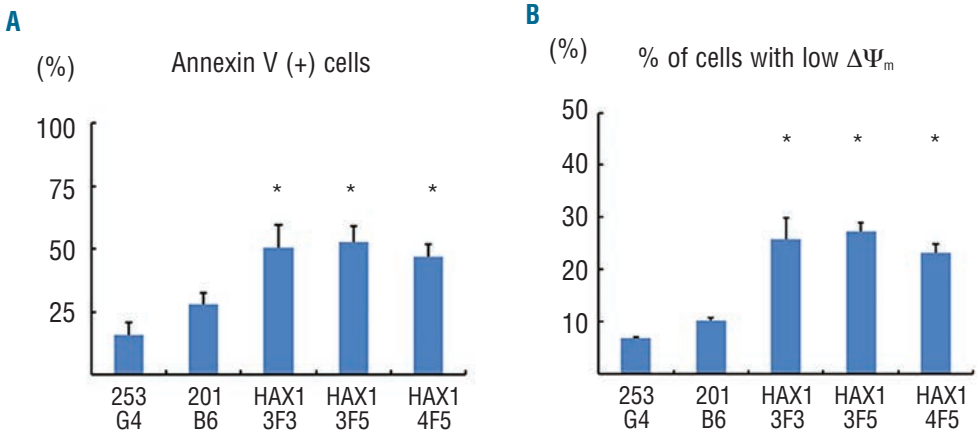

Figure 3. Neutrophils derived from HAX1-iPS cells are predisposed to undergo apoptosis due to their reduced Δψm. Annexin V assay (A) and mitochondrial membrane potential assay (B) of iPS cell-derived cells on Day 26 of neutrophil differentiation using flow cytometry. Cells gated on human $CD45^+$ were 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-

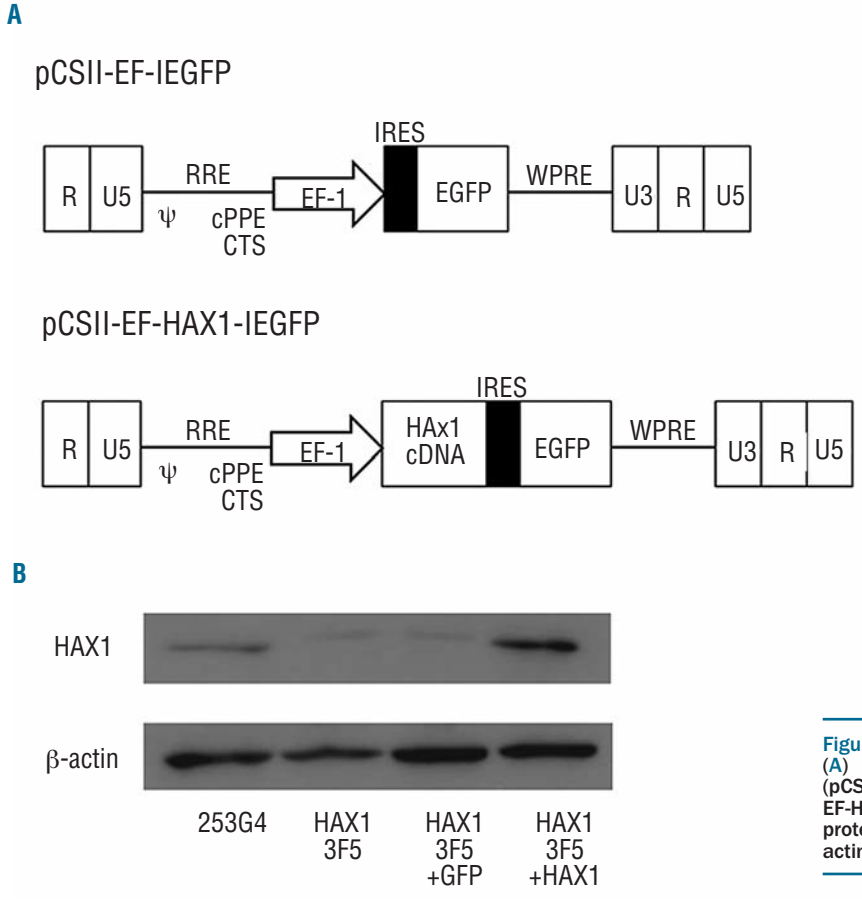

Figure 4. Lentiviral transduction of HAX1-iPS cells. (A) Lentiviral vector constructs with only EGFP (pCSII-EF-IEGFP), and *HAX1* cDNA and EGFP (pCSII-EF-HAX1-IEGFP). (B) Western blot analysis for HAX1 protein in lentivirally-transduced HAX1-iPS cells. β-actin was used as a loading control.

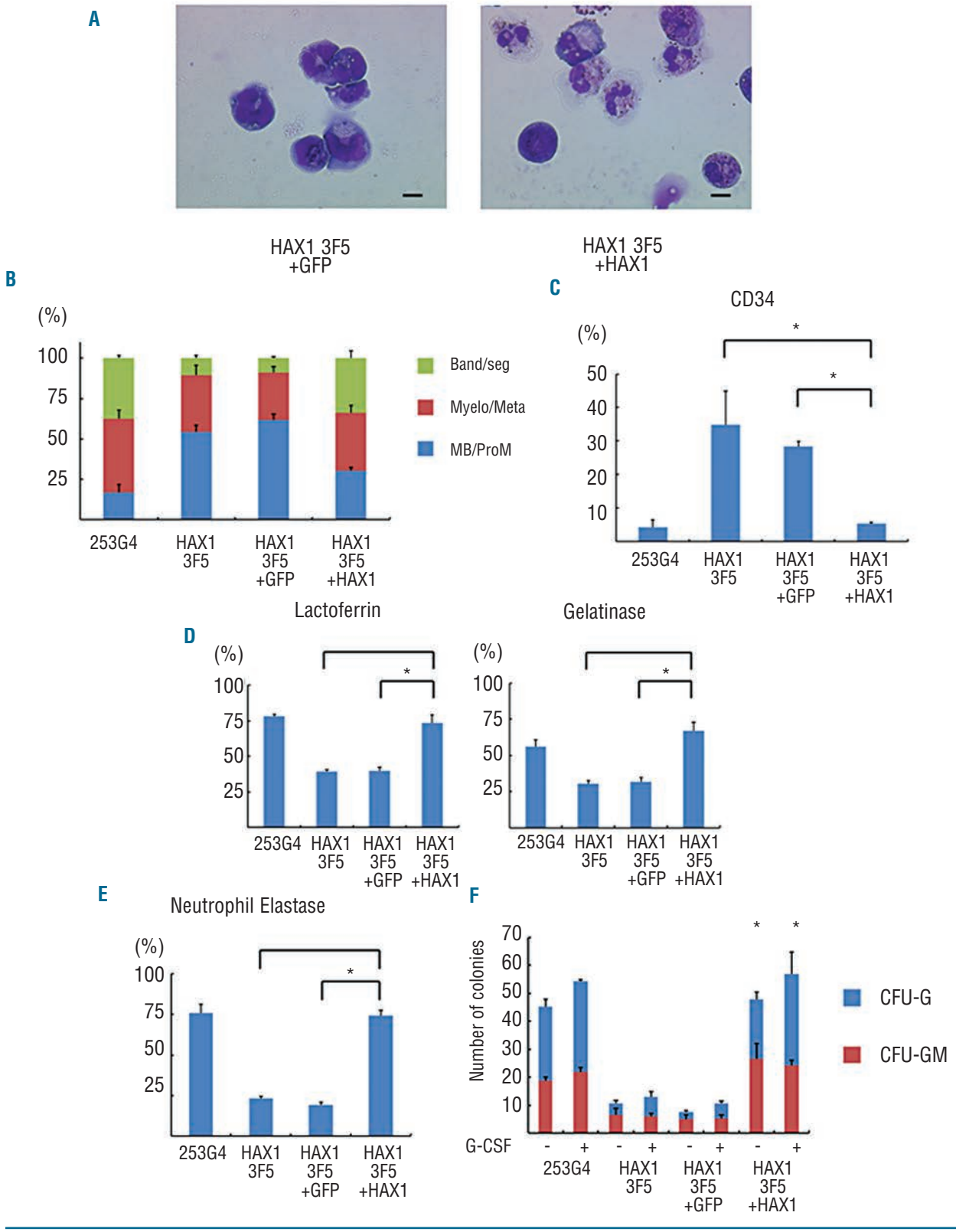

Figure 5. Lentiviral transduction of HAX1 cDNA improves maturation arrest of HAX1-iPS cells. (A) May-Giemsa staining of CD45$^+$ cells derived from HAX1 3F5+GFP and HAX1 3F5+HAX1 cells. Scale bars: 10 μm. (B) Morphological classification of CD45$^+$ cells derived from lentivirally-transduced iPS cells. (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.

Acknowledgments

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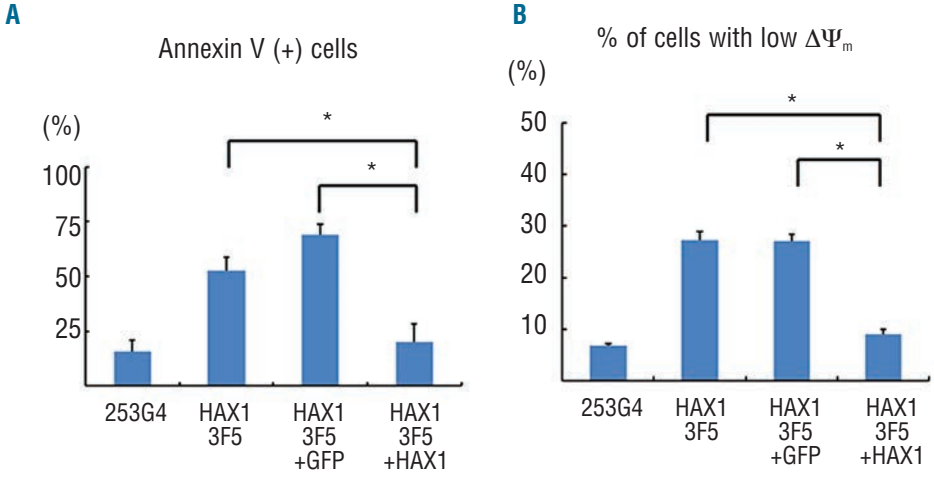

Figure 6. Lentiviral transduction of *HAX1* cDNA prevents HAX1-iPS cells being predisposed to undergo apoptosis. Annexin V assay (A) and mitochondrial membrane potential assay (B) of lentivirally-transduced iPS cell-derived cells on Day 26 of neutrophil differentiation. Cells gated on GFP$^+$ human CD45$^+$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

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Authorship and Disclosures

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