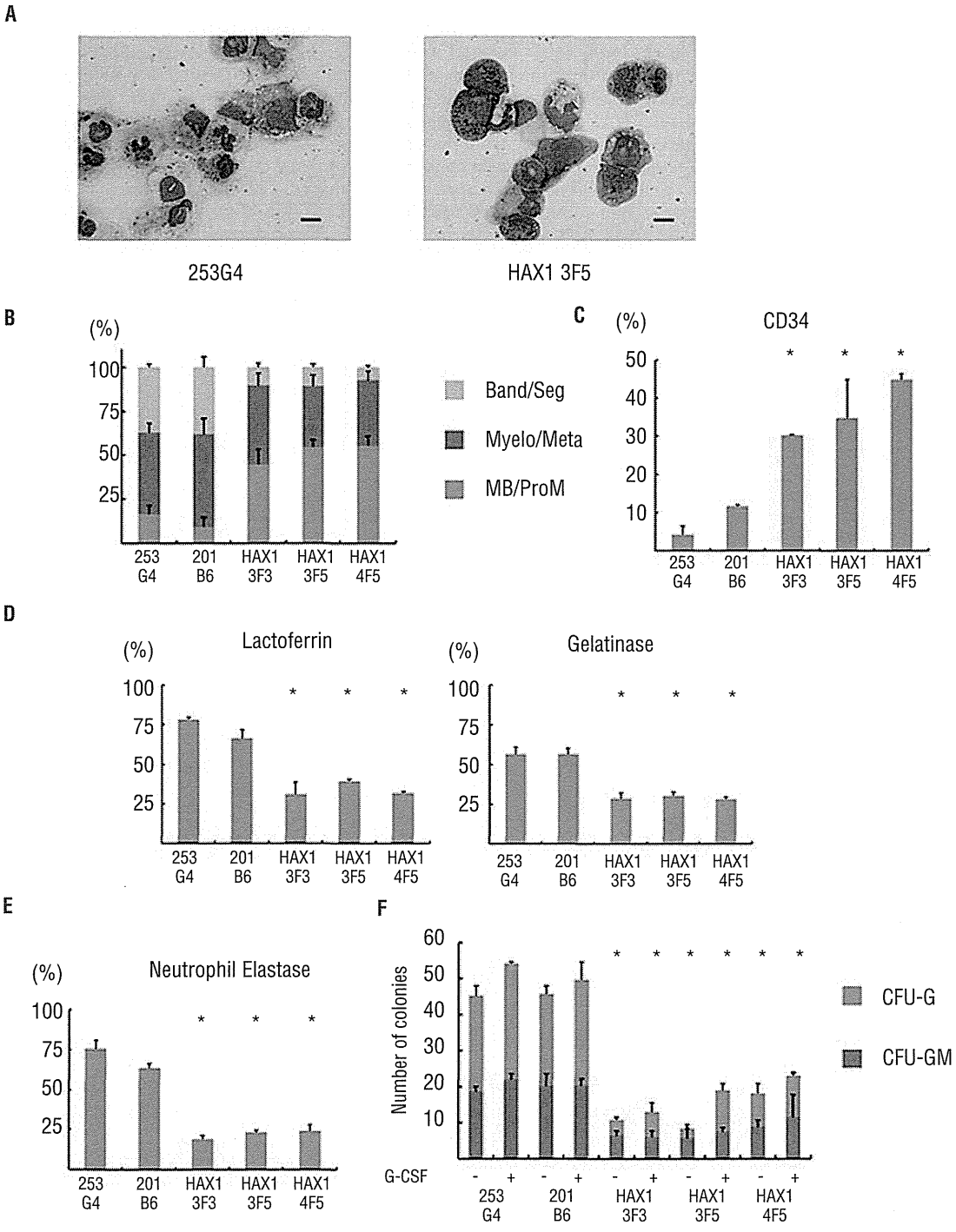

Figure 2. Maturation arrest at the progenitor level in neutrophil differentiation from HAX1-iPS cells. (A) May-Giemsa staining of CD45$^+$ cells derived from normal (253G4) and HAX1-iPS (HAX1 3F5) cells. Scale bars: 10 μm. (B) Morphological classification of CD45$^+$ cells derived from iPS cells. Cells 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

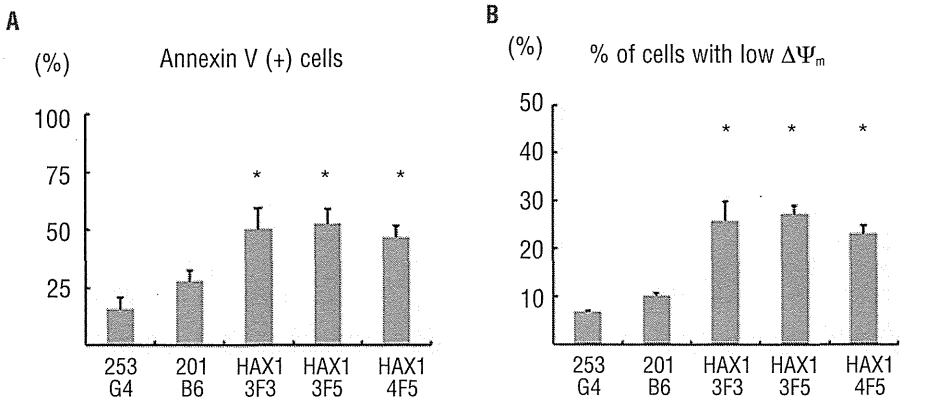

Figure 3. Neutrophils derived from HAX1-iPS cells are predisposed to undergo apoptosis due to their reduced $\Delta\psi$m. Annexin V assay (A) and mitochondrial membrane potential assay (B) of iPS cell-derived cells on Day 26 of neutrophil differentiation using flow cytometry. Cells gated on human $CD45^+$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

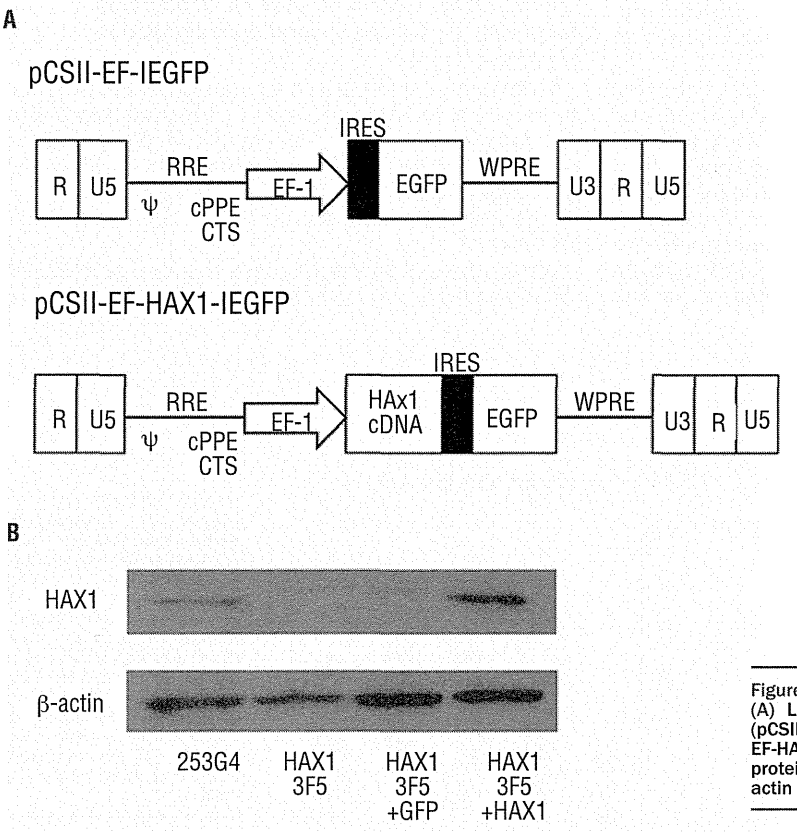

Figure 4. Lentiviral transduction of HAX1-iPS cells. (A) Lentiviral vector constructs with only EGFP (pCSII-EF-IEGFP), and *HAX1* cDNA and EGFP (pCSII-EF-HAX1-IEGFP). (B) Western blot analysis for HAX1 protein in lentivirally-transduced HAX1-iPS cells. β-actin was used as a loading control.

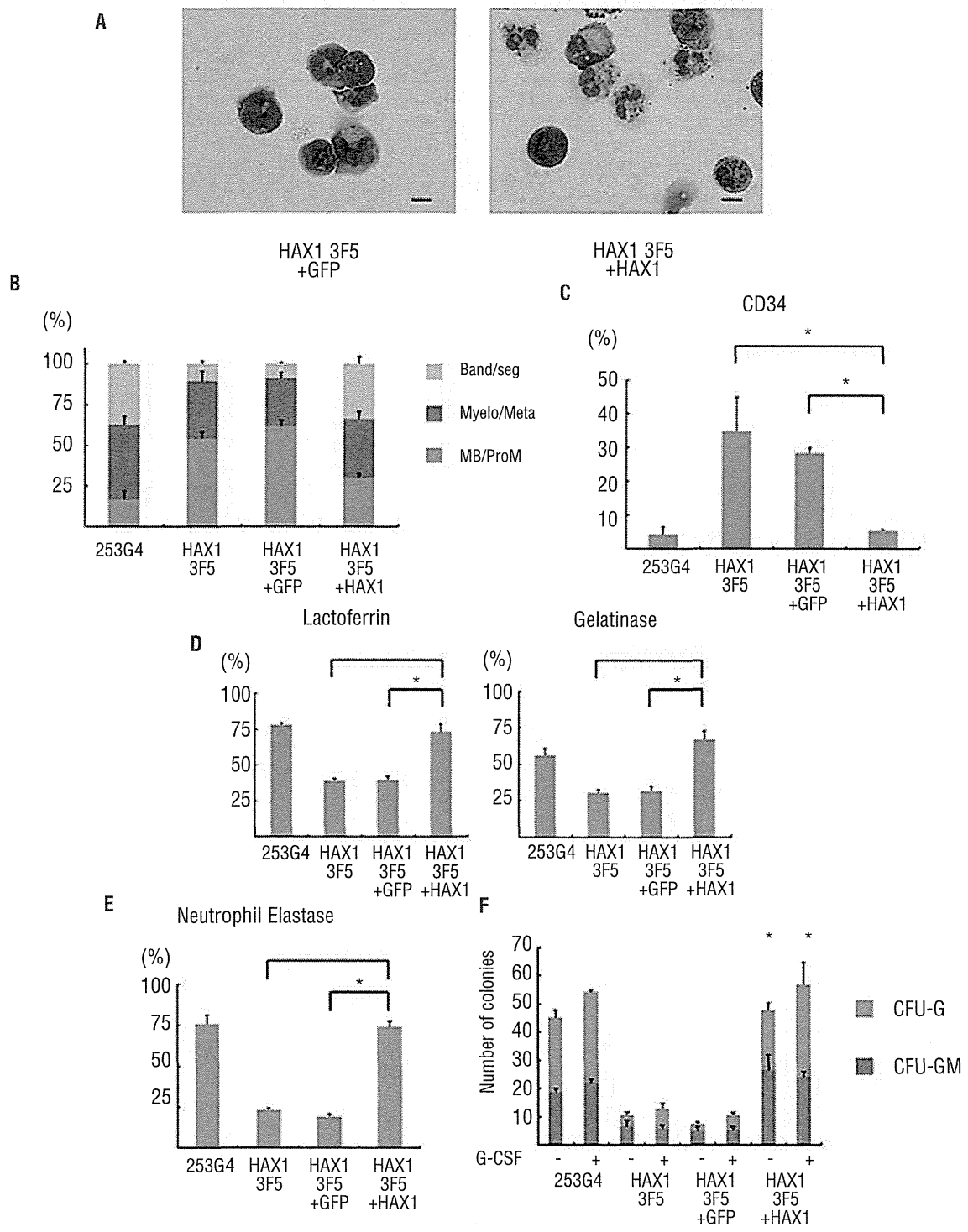

Figure 5. Lentiviral transduction of HAX1 cDNA improves maturation arrest of HAX1-iPS cells. (A) May-Giemsa staining of CD45$^+$ cells derived from HAX1 3F5+GFP and HAX1 3F5+HAX1 cells. Scale bars: 10 μm. (B) Morphological classification of CD45$^+$ cells derived from lentivirally-transduced iPS cells.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

Acknowledgments

The authors would like to thank Dr. Norio Nakatsuji for providing the human ES cell line KhES-1, Dr. Shinya Yamanaka for providing human iPS cell lines 201B6 and 253G4, and Dr. Hiroyuki Miyoshi for providing pCSII-EF-MCS. We are grate-

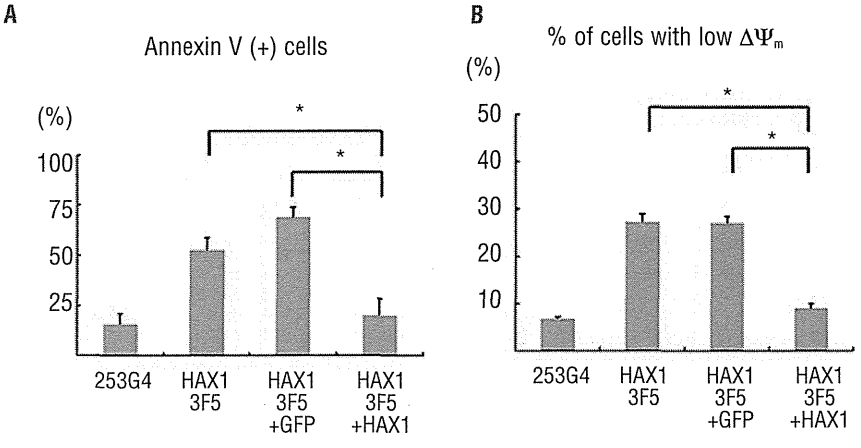

Figure 6. Lentiviral transduction of *HAX1* cDNA prevents HAX1-iPS cells being predisposed to undergo apoptosis. Annexin V assay (A) and mitochondrial membrane potential assay (B) of lentivirally-transduced iPS cell-derived cells on Day 26 of neutrophil differentiation. Cells gated on GFP$^+$ human CD45$^+$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

ful to Kyowa Hakko Kirin for providing TPO and G-CSF. We also thank the Center for Anatomical Studies, Kyoto University Graduate School of Medicine, for immunocytochemical analysis. Funding was provided by grants from the Ministry of Health, Labour and Welfare to KW, TN, and TH, a grant from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to KW, TN, and TH, grants from the Leading Project of MEXT to TN, a grant from Funding Program for World-Leading Innovative Research and Development on Science and Technology (FIRST Program) of Japan Society for the Promotion of Science (JSPS) to TN, grants from the SENSHIN Medical Research Foundation to IK, and grants from the Fujiwara Memorial Foundation to TM. This work was also supported by the Global COE Program "Center for Frontier Medicine" from MEXT, Japan.

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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Robust and Highly-Efficient Differentiation of Functional Monocytic Cells from Human Pluripotent Stem Cells under Serum- and Feeder Cell-Free Conditions

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Abstract

Monocytic lineage cells (monocytes, macrophages and dendritic cells) play important roles in immune responses and are involved in various pathological conditions. The development of monocytic cells from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) is of particular interest because it provides an unlimited cell source for clinical application and basic research on disease pathology. Although the methods for monocytic cell differentiation from ESCs/iPSCs using embryonic body or feeder co-culture systems have already been established, these methods depend on the use of xenogeneic materials and, therefore, have a relatively poor-reproducibility. Here, we established a robust and highly-efficient method to differentiate functional monocytic cells from ESCs/iPSCs under serum- and feeder cell-free conditions. This method produced $1.3\times10^6\pm0.3\times10^6$ floating monocytes from approximately 30 clusters of ESCs/iPSCs 5–6 times per course of differentiation. Such monocytes could be differentiated into functional macrophages and dendritic cells. This method should be useful for regenerative medicine, disease-specific iPSC studies and drug discovery.

Citation: Yanagimachi MD, Niwa A, Tanaka T, Honda-Ozaki F, Nishimoto S, et al. (2013) Robust and Highly-Efficient Differentiation of Functional Monocytic Cells from Human Pluripotent Stem Cells under Serum- and Feeder Cell-Free Conditions. PLoS ONE 8(4): e59243. doi:10.1371/journal.pone.0059243

Editor: Katriina Aalto-Setala, University of Tampere, Finland

Received August 14, 2012; **Accepted** February 13, 2013; **Published** April 3, 2013

Funding: Funding was provided by grants from the Ministry of Health, Labour and Welfare to TN, a grant from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to TN, grants from the Leading Project of MEXT to TN, a grant from Funding Program for World-Leading Innovative Research and Development on Science and Technology (FIRST Program) of Japan Society for the Promotion of Science (JSPS) to TN, grants from JSPS to TN and MKS, grants from the Takeda foundation, Mitsubishi Pharma Research Foundation and Suzuken memorial foundation to MKS and grants from Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to MDY. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Monocytic lineage cells, such as monocytes, macrophages and dendritic cells (DCs), are central to immune responses and play key roles in various pathological conditions. [1–2] Monocytes are the myeloid progeny of hematopoietic stem/progenitor cells [3]; they are a type of mononuclear cell circulating in the bloodstream and act as gatekeepers in innate immunity. While they replenish macrophages and DCs, monocytes themselves respond to various inflammatory stimuli by migrating into inflamed tissues, phagocytosing pathological small particles and producing proinflammatory cytokines and chemokines. Therefore, monocytes not only contribute to host defense against pathogenic microorganisms, but are closely associated with the pathogenesis of chronic sterile inflammation. [4] Macrophages reside in tissues and robustly phagocytose microorganisms and cellular debris. One of the important hallmarks of monocytic lineage cells is their functional plasticity. In response to cytokines and microbial products, macrophages polarize into functionally distinct M1 and M2 cells. [5] Classically activated M1 macrophages are induced by interferon-γ (IFNγ), while alternatively activated M2 macrophages can be induced by IL-4 and IL-13. [2,5] M1 macrophages are generally characterized by high production of proinflammatory cytokines, while M2 are characterized by high production of anti-inflammatory cytokines. DCs are the most powerful antigen-presenting cells and have an indispensable role for the activation of T lymphocytes. Because of their ability to mediate communication between innate and acquired immunity, ex vivo expansion of DCs is expected to be a useful source of material for cancer immunotherapies, such as DC-based vaccines. [6–7] Moreover, recent reports of monocyte and/or DC deficiencies highlight the importance of understanding their development in humans. [8] However, there have been technical limitations for tracing the development of human monocytic cells, or for propagating them ex vivo.

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are undifferentiated pluripotent cells that can be propagated indefinitely. [9–11] The development of monocytic cells from these pluripotent cells is of particular interest because it would provide an unlimited source of these cells for clinical applications and the examination of disease pathologies. Although the methods for hematopoietic differentiation from ESCs/iPSCs

using embryonic body or feeder co-culture systems have already been established, [12] these methods usually depend on xenogeneic feeder cells and/or animal- or human-derived serum, and therefore have a relatively poor-reproducibility. For instance, batch-to-batch variability of serum or feeder cells can influence the characteristics of *in vitro* differentiated DCs. [13] Here, we describe a novel serum- and feeder cell-free method that robustly and repetitively produces monocytic lineage cells from human ESCs/iPSCs.

Materials and Methods

Cell Culture

This study used human ESCs (cell line: KhES1) and iPSCs (cell lines: 201B7, 253G4, CIRA188Ai-W2, and CB-A11). [10,14–15] 201B7, 253G4 [10] and CIRA188Ai-W2 [15] were previously described. A human ES cell line KhES1 was kindly provided by Dr. Norio Nakatsuji. Human iPS cell lines 201B7 and 253G4 were kindly provided by Dr. Shinya Yamanaka. CB-A11 was established from cord-blood mononuclear cells by using episomal vectors. [16] These ESCs/iPSCs were maintained on tissue culture dishes coated with growth factor-reduced Matrigel (Becton-Dickinson) in mTeSR1 serum-free medium (STEMCELL Technologies).

Monocytic Lineage Cell Differentiation Method

The monocytic lineage differentiation protocol was modified from a previously established hematopoietic differentiation protocol (Figure 1). [17] The protocol consists of 5 sequential steps by which mature MPs and DCs are differentiated from human pluripotent cells in a stepwise manner. In the first step, primitive streak cells were induced from undifferentiated ESCs/iPSCs, which were then differentiated into hemangioblast-like hematopoietic progenitors in the second step. In step 3, expanded hematopoietic progenitors were committed towards initial myeloid differentiation, and then differentiated into the monocytic lineage in step 4. Finally, $CD14^+$ monocytes were differentiated into either MPs or DCs in step 5. The cytokines used in this study were purchased from R&D systems.

Step 1: induction of primitive streak-like cells from undifferentiated human ES/iPS cells with BMP4. BMP4 is an important molecule for the initial stage of mesodermal commitment of pluripotent stem cells in vitro. [17] Undifferentiated ESCs/iPSCs colonies were disseminated onto a 100 mm culture dish coated with growth factor-reduced Matrigel in mTeSR1 medium at a density of about 30 colonies per dish. Individual colonies were grown to a diameter of approximately 1 mm (Day 0), and BMP4 (80 ng/mL) was added to the mTeSR1 medium.

Step 2: generation of KDR^+CD34^+ hemangioblast-like cells with VEGF, basic FGF and SCF. VEGF and SCF have been reported to be important cytokines for development of hemoangiogenic progenitors. [18–19] In this step, we also added basic FGF which enhances the development of mesodermal hematopoietic progenitors. [18,20] The mTeSR1 medium was replaced by StemPro-34 serum-free medium (Gibco) containing 2 mM glutamax (Invitrogen) on day 4, and then was supplemented with the step-2 cytokine cocktail composed of VEGF (80 ng/mL), basic FGF (25 ng/ml), and SCF (100 ng/mL).

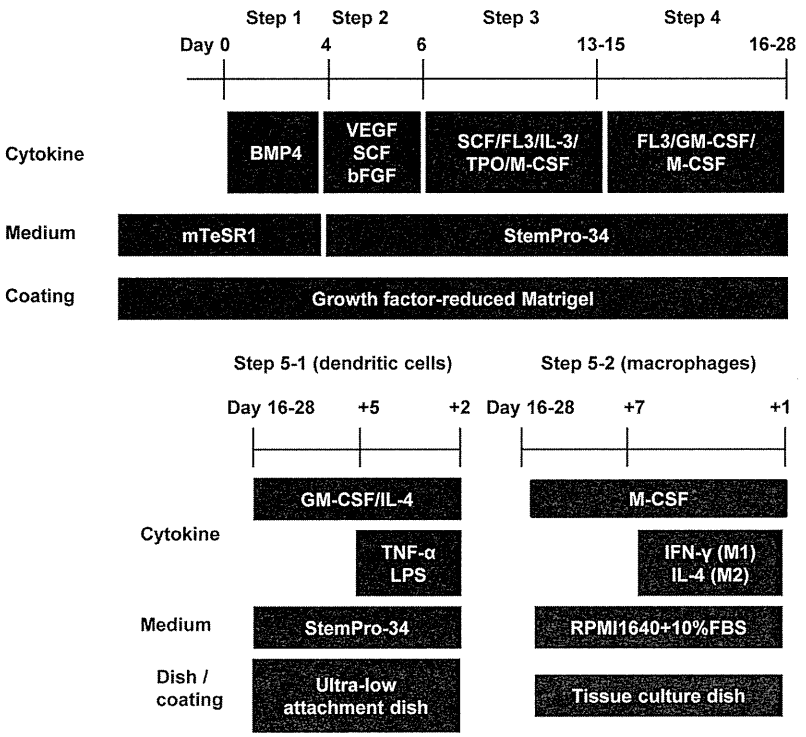

Figure 1. Protocol for monocytic lineage cell differentiation from human pluripotent stem cells. The protocol is composed of 5 steps. CD14-positive cells that are sorted between step-4 are differentiated into dendritic cells by step 5-1 or into macrophages by step 5-2. FL-3: Flt-3 ligand, TPO: Thrombopoietin.
doi:10.1371/journal.pone.0059243.g001

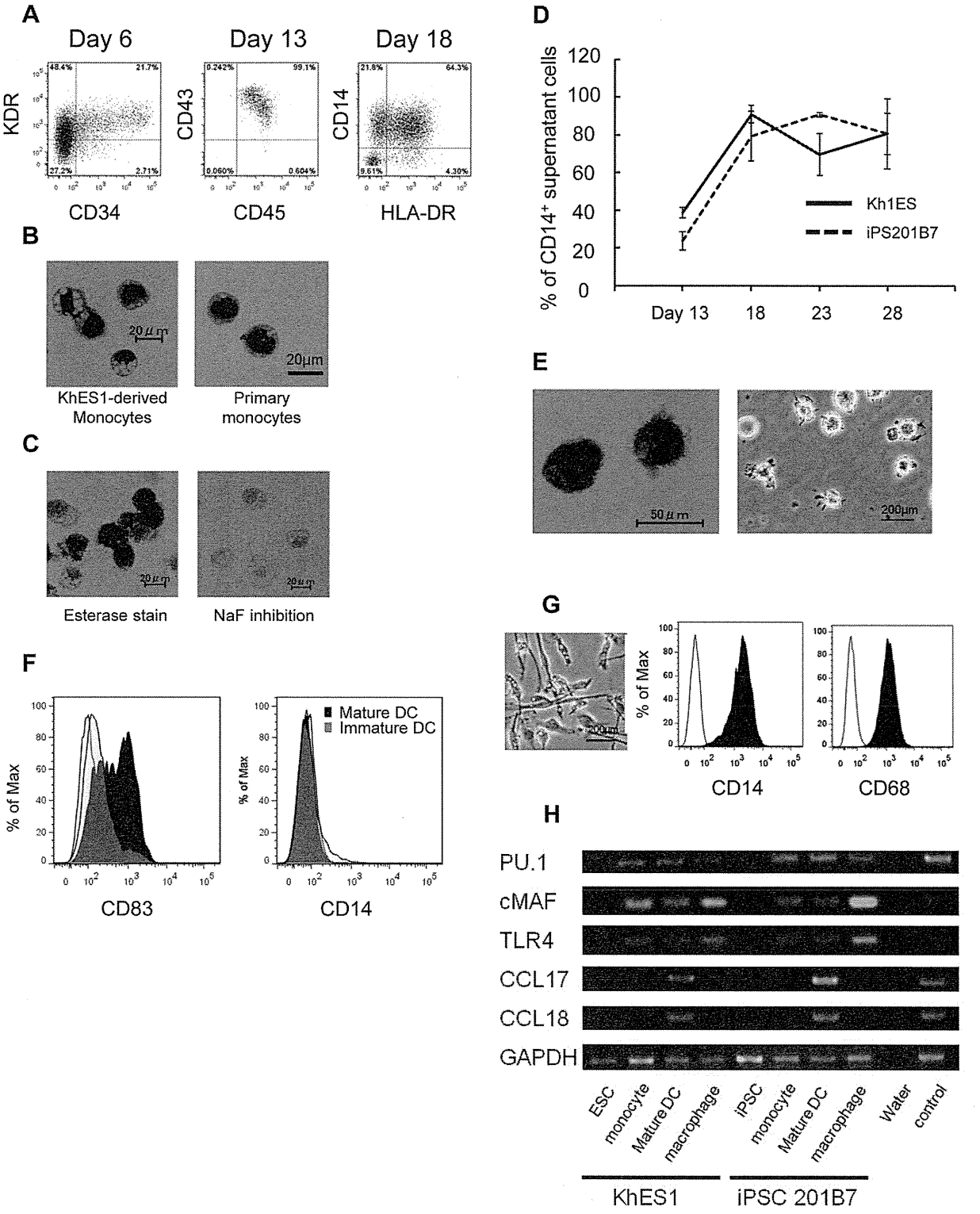

Figure 2. Phenotype analysis and gene expression pattern of monocytic lineage cells derived from pluripotent stem cells. (A) Flow cytometric analysis of monocytic lineage cells derived sequentially from pluripotent stem cells. An analysis of adherent cells on day 6 and supernatant cells on day 13 and 18 is shown. (B) May-Giemsa staining of $CD14^+$ monocyte-like cells derived from KhES1 on day 16 (left) and primary human monocytes (right). (C) Esterase staining for $CD14^+$ monocyte-like cells derived from KhES1 on day 16. (D) The percentage of $CD14^+$ cells within the total floating cells derived from KhES1/iPS-201B7 was evaluated from day 13 to day 28. (E) May-Giemsa staining (left) and phase contrast image (right)

of mature DCs derived from pluripotent stem cells. (F) Flow cytometric analysis of immature/mature DCs derived from pluripotent stem cells. (G) Phase contrast image and flow cytometric analysis of macrophages derived from pluripotent stem cells.(H) RT-PCR analysis of monocytic lineage cells derived from KhES1/iPS-201B7 clones for expression of monocytic lineage marker genes (*PU.1, c-MAF, TLR4, CCL17* and *CCL18*). Peripheral blood monocytes and peripheral blood monocyte-derived mature DCs were used as positive controls.(A–C, E–G) The data from KhES1-derived cells are shown as representative.
doi:10.1371/journal.pone.0059243.g002

Step 3: generation of hematopoietic cells with hematopoietic cytokines. The cytokines in StemPro-34 medium were switched to the step-3 cytokine cocktail composed of SCF (50 ng/mL), IL-3 (50 ng/mL), TPO (Thrombopoietin) (5 ng/mL), M-CSF (50 ng/mL), and Flt-3 ligand (50 ng/mL), on day 6. Thereafter, the medium was changed on day 10.

Step 4: monocytic lineage-directed differentiation with Flt-3 ligand, GM-CSF and M-CSF. The cytokines in StemPro-34 medium were switched to the step-4 cytokine cocktail composed of Flt-3 ligand (50 ng/mL), GM-CSF (25 ng/mL), and M-CSF (50 ng/mL) on day 13–15. The medium was changed every 3–4 days. The $CD14^+$ monocytic lineage-directed cell fraction in supernatant was positively sorted by autoMACS pro (Miltenyi Biotec) with CD14 MicroBeads (Miltenyi Biotec) on days 15–28.

Step 5: differentiation into DCs (step 5-1) and MPs (step 5-2) from $CD14^+$ monocytic lineage-cells. $CD14^+$ cells sorted by autoMACS pro (1.5×10^6 cells per well in a 6-well plate with Ultra-Low Attachment Surface (CORNING)) were cultured in StemPro-34 medium supplemented with GM-CSF (25 ng/mL) and IL-4 (40 ng/mL), with a medium change 4 days later, for differentiation into DCs (step 5-1). LPS (100 ng/mL, InvivoGen) and TNFα (0.2 ng/mL) were added for the last 2 days of the 7 day DC differentiation culture to promote maturation of DCs. $CD14^+$ cells (1.5×10^6 cells per well in a 6-well tissue culture plate) were cultured in RPMI-1640 medium (Sigma) supplemented with 10%

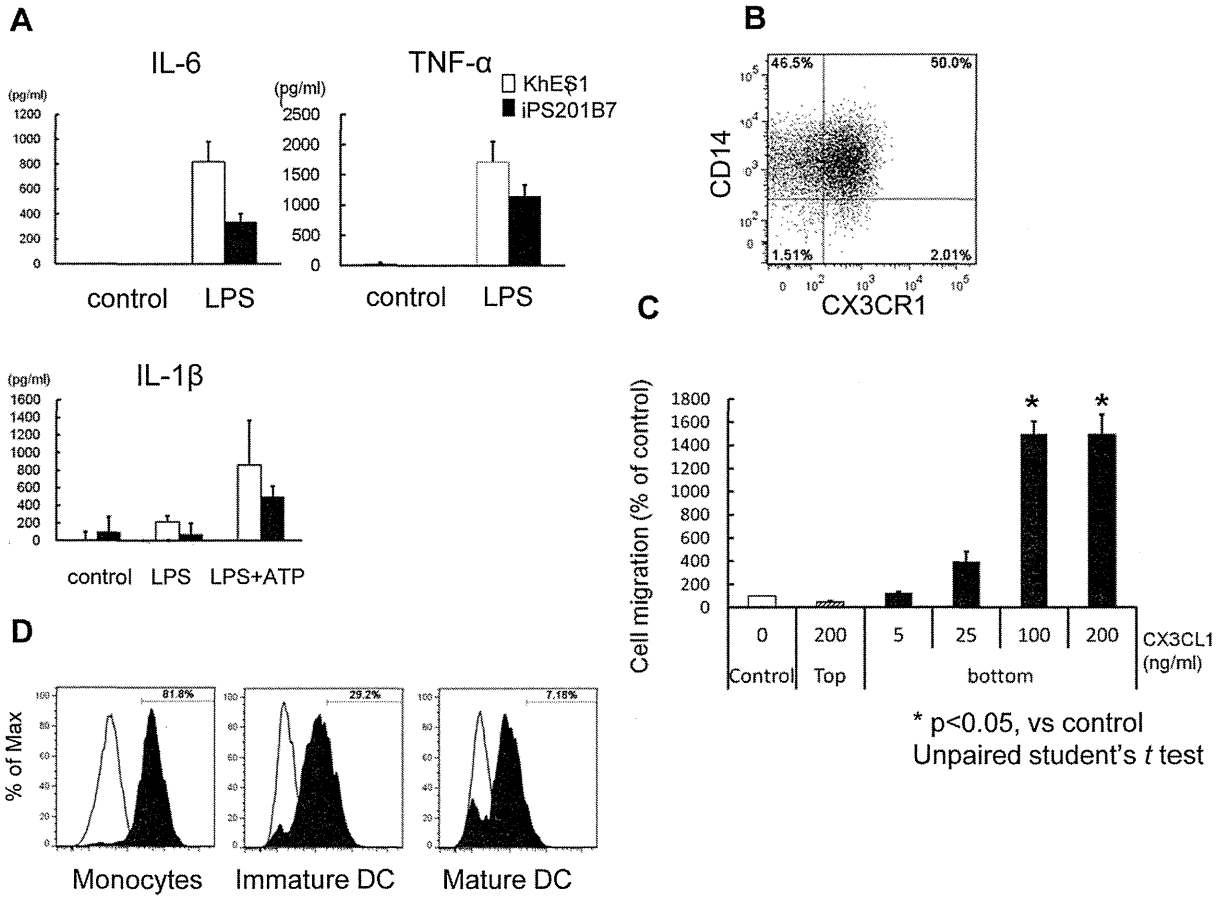

Figure 3. Functional assays for monocytes derived from pluripotent stem cells. (A) The levels of IL-6 and TNFα in supernatants of PS-Mo culture medium 4 hours after LPS stimulation. The levels of IL-1β were measured 4 hours after LPS stimulation with/without an additional 30-minute ATP stimulation. (B) Flow cytometric analysis of CX3CR1 on PS-Mo. (C) Chemotaxis assay of PS-Mo for CX3CL1 (fractalkine) using a trans-well migration assay. After the addition of CX3CL1 into either the bottom or top of the trans-well chamber, PS-Mo were applied and incubated for 5 hours at 37°C. (D) Antigen uptake was evaluated in monocytes, immature DCs and mature DCs derived from pluripotent stem cells by examining the fluorescence intensity of Alexa fluor 488-conjugated ovalbumin 45 minute after incubation at 37°C (black). Control samples (white) were kept on ice. (B–D) The data of KhES1-derived cells are shown as representative. PS-Mo: monocyte derived from pluripotent stem cells.
doi:10.1371/journal.pone.0059243.g003

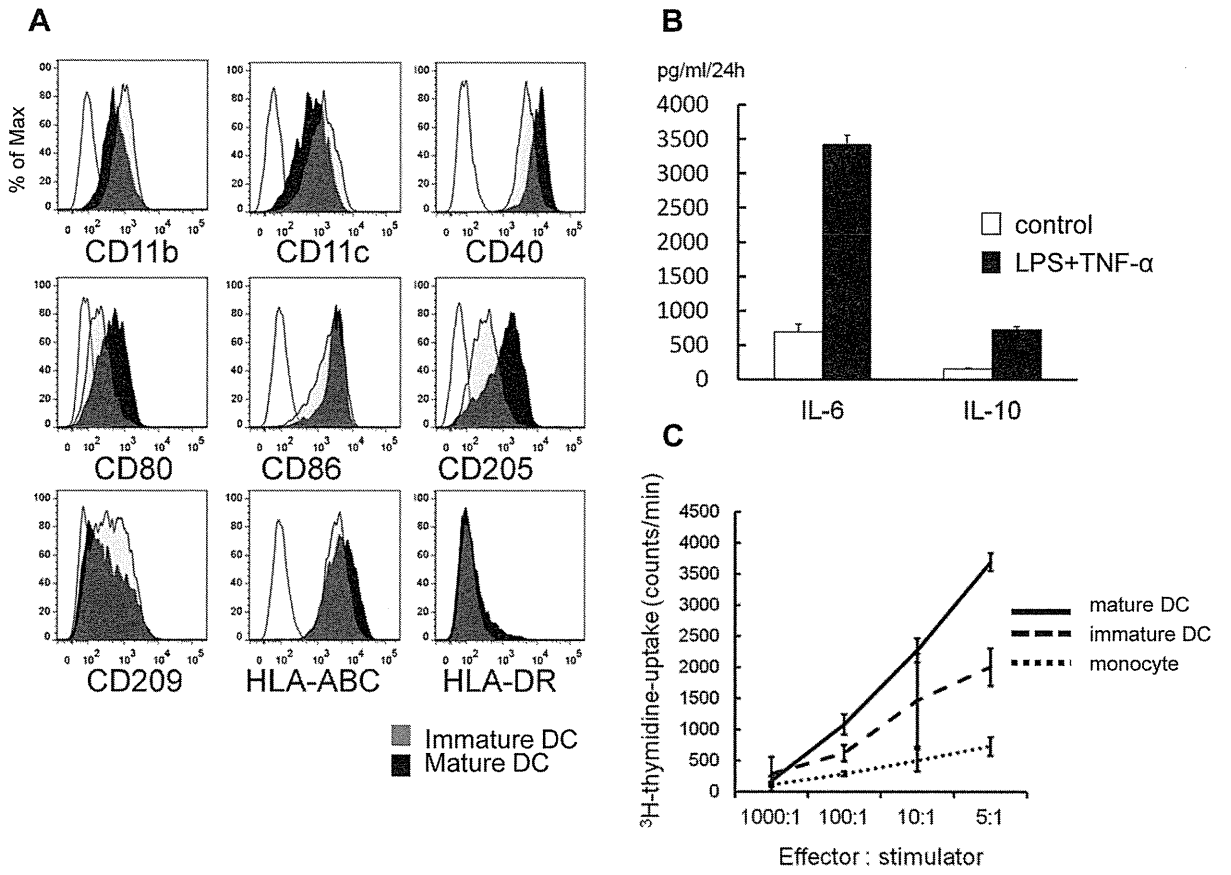

Figure 4. Functional assays for dendritic cells derived from pluripotent stem cells. (A) Flow cytometric analysis of immature/mature DCs derived from pluripotent stem cells. (B) The levels of IL-10 and TNFα in supernatants of culture medium with PS-DCs 24 hours after LPS stimulation. (C) The proliferation of allogeneic naïve T cells (1×10^5 cells per well) co-cultured with 40 Gy-irradiated stimulator cells for 3 days was evaluated. The proliferation of naïve T cells in the last 16 hours was measured by ^{3}H-thymidine uptake. (A–C) The data of KhES1-derived cells are shown as representative.
doi:10.1371/journal.pone.0059243.g004

fetal bovine serum (FBS) and M-CSF (100 ng/mL) for 7 days with a medium change at day 4, for differentiation into macrophages (step 5-2). IFNγ (20 ng/ml) or IL-4 (20 ng/ml) was added for another day to promote differentiation into M1 or M2 macrophages, respectively.

Flow Cytometric Analysis

Flow cytometric analysis data were collected using the MACS Quant™ Analyzer (Miltenyi Biotec) and then analyzed utilizing the FlowJo software package (Treestar). The following antibodies were purchased from BD Biosciences: CD11b-FITC, CD11c-APC, CD34-PE, CD40-PE, CD43-FITC, CD80-PE, CD83-PE, CD86-FITC, CD205-Alexa fluor 647, CD206-FITC, CD209-PE, HLA-ABC-FITC and HLA-DR-FITC. CD14-APC and CD45-APC antibodies were purchased from Beckman Coulter. CD163-APC antibody was purchased from R&D systems. KDR (CD309)-Alexa fluor 647 and CX3CR1-PE antibodies were purchased from Biolegend.

May-Giemsa Staining and Esterase Staining

Cells were seeded onto glass slides by CYTOSPIN 4 (Thermo Scientific) and stained with May-Grunwald and Giemsa staining solution (MERCK) and Esterase staining solution (Muto pure chemicals) following the manufacturer's instructions.

RNA Extraction and RT-PCR Analysis

RNA samples were prepared using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. Typically, 500 ng of total RNA were subjected to reverse transcription (RT) with a Sensiscript-RT kit (Qiagen). RT-PCR was performed for the evaluation of the expression of monocytic lineage marker genes such as *PU.1, MAF, TLR4, CCL17* and *CCL18* using the primers in **Table S1**. [21–22] Peripheral blood monocyte-derived mature DCs/macrophages were generated from peripheral $CD14^+$ monocytes using the step 5-1/5-2 cytokine cocktails in 10% FBS-supplemented RPMI-1640 for use as positive controls.

Cytokine Assay

Concentrations of cytokines (IL-1β, IL-6, IL-10, IL-12p70 and TNFα) in supernatants were analyzed with FlowCytomix kits (Bender MedSystems) following the manufacturer's instructions. The IL-1β, IL-6 and TNFα levels in the culture supernatants of pluripotent cell-derived monocytes (PS-Mo) were analyzed in three settings, (1) culture in RPMI-1640 medium supplemented with 10% FBS and LPS (100 ng/ml) for 4.5 hours, (2) as in (1) but with

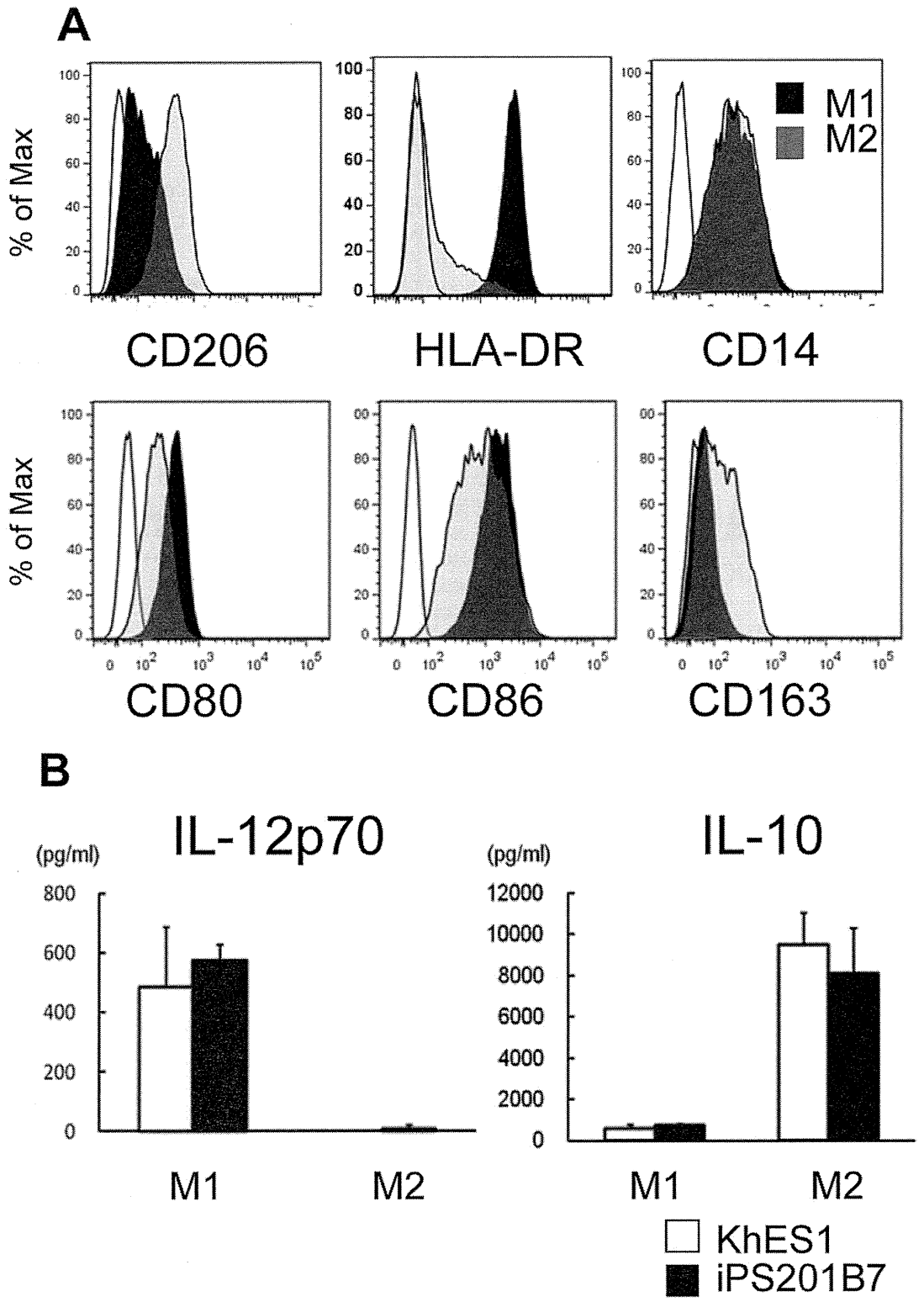

Figure 5. Functional assays for M1/M2 macrophages derived from pluripotent stem cells. (A) Flow cytometric analysis of M1/M2 macrophages derived from pluripotent stem cells. (B) The levels of IL-12p70 and IL-10 in supernatants of culture medium with M1/M2 macrophages derived from pluripotent stem cells 24 hours after LPS stimulation. The data of KhES1-derived cells are shown as representative.
doi:10.1371/journal.pone.0059243.g005

the addition of ATP (1 mM) for the last 30 min, (3) without LPS or ATP for 4.5 hours, to evaluate the production pattern of IL-1β in response to LPS plus ATP. [23].

The levels of IL-6, IL-10, IL-12p70 and TNFα in the supernatants of M1 or M2 macrophage culture were measured 24 hours after LPS (100 ng/ml) stimulation.

Chemotaxis Assay

PS-Mo chemotaxis was evaluated using a trans-well migration assay with 8-μm pore size inserts (BD Biosciences). After CX3CL1 (fractalkine; R&D systems) was added to either the bottom or top of the chamber, serum-starved PS-Mo were loaded onto the inserts which were placed into 24-well plates containing RPMI-1640 and then incubated at 37°C for 5 hours. [24] Cell migration was measured by flow cytometry as previously reported: equivalent amounts of counting beads were added to each sample and the ratios of PS-Mo to the counting beads were calculated. [25].

Antigen Uptake Assay

The antigen uptake capacity of monocytic lineage cells was evaluated as previously described. [26] Briefly, the cells were collected and stored on ice for 10 min. PS-Mo, pluripotent cell-derived immature DCs (PS-imDCs) and pluripotent cell-derived mature DCs (PS-mDCs) (5×10^4 cells) were incubated with Ovalbumin Alexa fluor 488 Conjugate (Molecular Probes) at 0.1 mg/ml at 37°C or on ice for 45 min. Ice-cold FACS buffer was added in order to stop the reaction, samples washed twice, and the fluorescence intensity analyzed by flow cytometry.

Mixed Leukocyte Reactions

Allogeneic naïve T cells (1×10^5 cells per well) were purified from umbilical cord blood mononuclear cells using naïve $CD4^+$ T cell isolation kits (Miltenyi Biotec) and then co-cultured with 40 Gy-irradiated stimulator cells (PS-Mo, PS-imDC, and PS-mDC) in 96-well round bottomed culture plates for 3–5 days. ^{3}H-methylthymidine (25 uCi/ml, Moravek Biochemicals and Radiochemicals) was added to the culture medium of 10% FBS-supplemented RPMI-1640 for the last 16 hours. The cells were harvested onto a filter mat (Perkin Elmer) and the ^{3}H methylthymidine uptake determined using a scintillation counter (MicroBeta TriLux, Perkin Elmer).

Ethical Considerations

This study was approved by the Ethics Committee of Kyoto University and written informed consent was obtained from each healthy volunteer.

Statistics

Data are presented as the mean ± S.D. and the statistical significance of the differences between cultures were evaluated by Student's *t*-test.

Results

Differentiation of ESCs/iPSCs into Dendritic Cells and Macrophages via Monocyte-like Cells

A KDR^+CD34^+ hemangioblast-like population was detected in adherent cell clusters on day 6 (steps 1,2), and around 95% of supernatant cells were $CD43^+CD45^+$ hematopoietic cells on days 13–15 (step 3; **Figure 2A**). [17] Floating cells were recovered every 3–4 days in step 4 (**Figure S1**); the majority of these cells were $CD14^+$ monocyte-like cells (**Figure 2A**). These pluripotent cell-derived monocytes (PS-Mo) were similar to peripheral blood monocytes in morphology (**Figure 2B**). PS-Mo are positive for Esterase staining which was inhibited by NaF (**Figure 2C**). The percentages of PS-Mo in floating cells were constantly about 50–90% between day 18–28 (**Figure 2D and Figure S2A**). The yield of PS-Mo per 100 mm culture dish starting with about 30 colonies was $1.3\times10^6\pm0.3\times10^6$ at each step-4 medium exchange.

To derive DCs, PS-Mo were purified by magnetic sorting, and differentiated into $CD14^-CD83^-$ immature DCs (PS-imDCs) with the step 5-1 cytokine cocktail in 5 days (**Figure 2E**). PS-imDCs were stimulated with LPS and TNFα for an additional 2 days, which further differentiated them into $CD14^-CD83^+$ mature DCs (PS-mDCs) (**Figure 2F**). The differentiation efficiency of mature DCs from PS-Mo was comparable to that from primary monocytes (7.7%±0.9% vs. 16.5%±1.0%, $p=0.20$, unpaired Student's *t*-test). PS-Mo also had the potential to differentiate into macrophages (PS-MPs) with the step 5-2 cytokine cocktail. PS-MPs are morphologically comparable to primary monocyte-derived macrophages and they express typical surface markers such as CD14 and CD68 (**Figure 2G and Figure S3A,B**).

We confirmed that PS-Mo, pluripotent cell-derived DCs (PS-DCs), and PS-MPs expressed monocytic lineage-specific genes (**Figure 2H and Figure S2B**). [22,27] Collectively, by using this protocol, sufficient numbers of monocytic cell lineage cells can be obtained from a small number of human ESCs/iPSCs.

Functional Assays for Monocytes Derived from ESCs/iPSCs

Next, we evaluated the functional activity of pluripotent cell-derived monocytic lineage cells. PS-Mo robustly produced the pro-inflammatory cytokines IL-6 and TNFα after LPS stimulation (**Figure 3A, Figure S3C**). Secretion pattern of IL-1β from PS-Mo with two stepwise signals LPS and ATP were similar to primary monocytes (**Figure 3A, Figure S3D**). [23,28].

PS-Mo expressed CX3CR1, implying chemotactic responses to CX3CL1 (fractalkine) (**Figure 3B**). PS-Mo migration in trans-well assays increased with increasing doses of CX3CL1 in the lower compartment of the chamber (**Figure 3C**). This phenomenon was not due to chemokinesis, but chemotaxis, because CX3CL1 in the top compartment could not induce PS-Mo migration into the lower compartment of the chamber. [24] We next compared the antigen uptake ability of PS-Mo, PS-imDCs, and PS-mDCs by incubating them with Ovalbumin Alexa fluor 488 Conjugate. [26] PS-Mo had the highest ability to take up antigen and as DCs matured they lost their ability to endocytose antigens (**Figure 3D**).

Functional Assays for DCs Derived from ESCs/iPSCs

For evaluating functions of PS-DCs, we first confirmed that patterns of expression of cell surface markers on PS-imDCs/mDCs were comparable to those on primary dendritic cells (**Figure 4A, Figure S4A**). When stimulated with LPS and TNFα, PS-DCs also produced almost comparable amounts of pro-inflammatory and anti-inflammatory cytokines (**Figure 4B, Figure S4B**).

To test the ability of PS-DCs to activate naïve T cells, we next co-cultured allogeneic naïve T cells with PS-DCs and PS-Mo. As shown in Figure 4C, PS-mDCs had the most potent capacity to stimulate allogeneic T cell proliferation and this dose-response relationship was comparable to that observed with PB-DCs (Figure S4C).

Functional Assays for Macrophages Derived from ESCs/iPSCs

Using this technique, we obtained morphologically typical macrophage-like cells that adhered firmly to the culture dish. To test whether these PS-MPs possessed functional plasticity like primary macrophages, we tried to polarize them into M1 or M2 state by treating them with IFNγ or IL-4, respectively. PS-MPs exhibited typical surface markers that were characteristic of primary M1 or M2 macrophages (**Figure 5A, Figure S5A**). The M1 cytokine pattern is typically IL-12^{high} and IL-10^{low}, whereas the M2 pattern is IL-12^{low} and IL-10^{high}. [5] Pluripotent cell-derived M1 and M2 macrophages (PS-M1/M2) also exhibited cytokine profiles that were comparable to those generated from primary monocytes (**Figure 5B, Figure S5B**).

Discussion

We have established a novel differentiation system for monocytic cells from human ES and iPS cells. Since macrophages and dendritic cells are usually obtained *in vitro* from monocytes, the most important point of the evaluation is to establish whether monocytes differentiated from ESCs/iPSCs are functionally comparable to primary monocytes. In several functional assays, PS-Mo indeed proved to be comparable to primary monocytes, and importantly, PS-DCs and PC-MPs from PS-Mo were also functionally comparable to their primary counterparts.

Although complete M1/M2 macrophage polarization still requires aserum-containing medium, the present results prove that the current method can precisely manipulate macrophages that have the potential to differentiate into M1/M2 macrophages. The cytokine profiles of PS-M1/M2 were also comparable to those of primary M1/M2 macrophages. The expression patterns of surface markers in PS-DCs after LPS stimulation and of PS-MPs after M1/M2 polarization were almost identical to those of DCs/MP derived from primary monocytes. However, the level of IL-10 in PS-DCs after stimulation was higher than that in primary DCs and the expression levels of HLA-DR in PS-DCs/MP were low in comparison with those in DCs/MP derived from primary monocytes. Therefore, further improvement of culture conditions such as the use of a modified medium and cytokine cocktail will be needed.

Several embryonic body methods and feeder cell co-culture methods for PS-DCs/MP differentiation have already been reported. [7,27,29–30] These methods show relatively poor-reproducibility because of the use of xenogeneic feeder cells and/or serum. In an earlier report which describes a protocol that can derive macrophages and dendritic cells from human iPSCs in feeder- and serum-free manner, [7] the authors did not fully characterize the monocytes and noted that PS-DCs/MP were generated only from two of the five iPSC clones tested. The current culture system simply propagated progenitor cells in 2-dimensional cultures without passage or sorting, and floating PS-Mo and PS-DCs/MP could be obtained repetitively from all five ESC/iPSC clones tested (**Figure S2 and S6**). These monocytic cells derived from disease- or patient- specific iPSC would be useful tools for the examination of disease pathologies and for drug discovery in immunological disorders such as autoimmune diseases, immunodeficiencies and autoinflammatory syndromes. However, even in our protocol, there are subtle clonal variations of timing of differentiation such as the day of step 3 to 4 switching which is determined by the emergence of CD43^{+}CD45^{+} cells (day 13–15, data not shown). Fine adjustment of the protocol for each ESC/iPSC clone seemed to further improve the yield of monocytes.

iPSC technology is overcoming immunological and ethical concerns in regenerative medicine using human pluripotent cells. Furthermore, a number of disease-associated iPSCs generated from patients with immunological disorders have been reported. [15,31–34] Because patient- or disease-specific iPS cells will be an important resource for unraveling human immunological disorders, a robust and simple hematopoietic differentiation system that can reliably mimic in vivo hematopoiesis is necessary for this purpose. Our simple and robust protocol to produce monocytic cells is therefore expected to be useful for regenerative medicine and studies of immunological disorders.

Supporting Information

Figure S1 Image of floating hematopoietic cells derived from iPS cells Phase contrast image of floating hematopoietic cells derived from iPS-201B7 at day 21 (step 4).
(PDF)

Figure S2 Phenotype analysis and gene expression pattern of monocytic lineage cells derived from 3 additional pluripotent stem cell lines. (A) The percentage of CD14+ cells within the total floating cells derived from 3 iPSC clones (253G4, CIRA188Ai-W2, and CB-A11) was evaluated from day 13 to day 28. (B) RT-PCR analysis of monocytic lineage cells derived from 253G4, CIRA188Ai-W2, and CB-A11 clones for expression of monocytic lineage marker genes (c-MAF, TLR4, and CCL17). Peripheral blood monocytes and peripheral blood monocyte-derived mature DCs were used as positive controls.
(PDF)

Figure S3 Characteristics of primary monocytes and macrophages. (A) Phase contrast image and (B) flow cytometric analysis of macrophages derived from primary monocytes. (C) The levels of IL-6 and TNF-α in supernatants of primary monocyte culture medium 4 hours after LPS stimulation. (D) The levels of IL-1β were measured 4 hours after LPS stimulation with/without an additional 30-minute ATP stimulation.
(PDF)

Figure S4 Characteristics and functional assays of dendritic cells derived from primary monocytes. (A) Flow cytometric analysis of immature/mature DCs derived from primary monocytes. (B) The levels of IL-10 and TNF-α in supernatants of culture medium with primary-DCs 24 hours after LPS stimulation. (C) The proliferation of allogeneic naïve T cells (1×105 cells per well) co-cultured with 40 Gy-irradiated stimulator cells for 3 days was evaluated. The proliferation of naïve T cells in the last 16 hours was measured by 3H-thymidine uptake.
(PDF)

Figure S5 Characteristics and functional assays of M1/M2 macrophages derived from primary monocytes. (A) Flow cytometric analysis of M1/M2 macrophages derived from primary monocytes. (B) The levels of IL-12p70 and IL-10 in supernatants of culture medium with M1/M2 macrophages derived from primary monocytes 24 hours after LPS stimulation.
(PDF)

Figure S6 Replication assays for 3 additional pluripotent stem cell lines. (A) Phase contrast image (left) and May-Giemsa staining (right) of mature DCs derived from iPSC clones. (B) Phase contrast image of macrophages derived from iPSC clones. (C) Flow cytometric analysis of immature/mature DCs and macrophages derived from iPSC clones.
(PDF)

Table S1 Primers for RT-PCR.
(PDF)

Acknowledgments

We are grateful to Y. Sasaki, Y. Jindai, K. Kobayashi, M. Yamane, and S. Nakamura for technical assistance. We would also like to thank N. Takasu and Y. Takao for administrative assistance.

Author Contributions

iPSC establishment: MDY IA. Conceived and designed the experiments: MDY AN HG TH TN MKS. Performed the experiments: MDY ST SN YM TT JI FHO. Analyzed the data: MDY AN TY KO TN MKS. Wrote the paper: MDY AN TY MKS.

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iPS細胞を用いた神経疾患病態研究

Neurological disease modeling using induced pluripotent stem cells

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Key Words: iPS細胞，神経疾患研究，疾患モデリング，先制医療，アルツハイマー病，Aβオリゴマー

はじめに

神経疾患は多岐に及び，これまでに電気生理学的検査・神経放射線学的検査などの臨床的な手法を用いて疾患概念が確立され，診断法・治療法の開発が進歩してきた。一方で疾患の首座である中枢神経系は，再生が難しいため，限られた場合を除いて生検材料を得ることができない。そのため直接的な病態解明には限界があった。これまで病態解明のための研究は，遺伝性疾患の遺伝学的解析，死後病理組織・遺伝子改変動物・細胞モデルなどの生化学的・組織学的解析を中心として進められており，間接的にしか患者の病態を解析することが出来なかった。

2007年にヒト人工多能性幹細胞（induced Pluripotent Stem cells: iPS細胞）の作製技術が開発され[1]，この業績が評価され2012年山中伸弥教授がノーベル賞を受賞するなど，生物研究全般に多大な影響を与えた。神経疾患領域においても，iPS細胞技術により患者自身の体細胞から中枢神経系組織を含めた疾患の標的細胞を入手することが可能となり，これまでにない全く新たな医療開発が始まっている。

本稿では，現在までのiPS細胞技術を用いた神経疾患研究について紹介するとともに，最近我々が報告したアルツハイマー病の研究について論じる。さらに，今後期待されるiPS細胞技術を用いた医療応用研究の展望について述べる。

iPS細胞の樹立

1）ヒト多能性幹細胞の歴史

ヒト由来の代表的な多能性幹細胞である胚性幹細胞（Embryonic Stem cells: ES細胞）は，1998年に初めて樹立された[2]，本邦においても2003年に初めて樹立されて以来現在までに5クローンが樹立された。ヒトES細胞はその未分化状態を保ったまま，ほぼ無限に増殖を繰り返すことが可能であり，心筋細胞・肝細胞・神経細胞などへの*in vitro*における分化誘導法研究法が進められてきた。特に神経・精神疾患領域においては，生検材料などの生きたヒト研究検体を得ることが出来なかったため，ヒトES細胞から分化誘導して得られる神経細胞には大きな期待が寄せられている。最近では，健常者由来のヒトES細胞株に，病的変異を有する疾患遺伝子を導入することで神経疾患研究への応用が始まっているが[3]，孤発例を含めた多様な神経・精神疾患をカバーすることは困難である。

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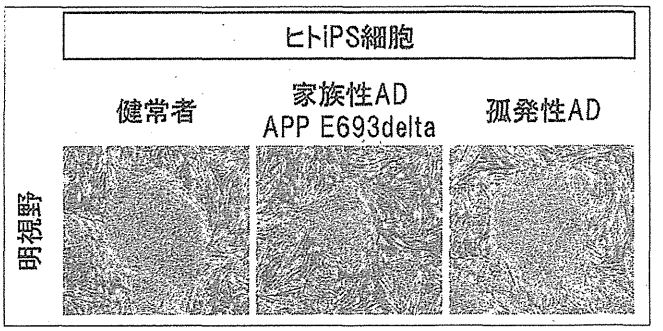

図1　健常者およびアルツハイマー病患者由来のiPS細胞

2）iPS細胞技術の開発

ES細胞の研究が進む一方で，卵子への核移植を用いたヒト体細胞の遺伝子情報を持つ幹細胞樹立（クローン技術）が試みられてきたが，これまでのところヒトでは成功していない（アカゲザルでは2007年にクローン胚由来のES細胞が樹立されている[4]）。

一方で2006年にマウスで，2007年にはヒトで，ES細胞特異的に発現している遺伝子を体細胞に導入することによりES細胞類似の多能性幹細胞樹立がなされ，iPS細胞と命名された[1]。具体的には，ヒト成人線維芽細胞に，レトロウィルスベクターを用いてES細胞特異的遺伝子である*Oct3/4*・*Sox2*・*Klf4*・*c-Myc*を導入することで，ほぼ無限に増殖し，内・中・外胚葉それぞれへの分化能力を有する細胞株が樹立された。

その後も，より効率的なiPS細胞の樹立法研究が展開されるとともに，幹細胞の未分化性維持・遺伝子発現特性の解析，さらには癌研究など広範な研究領域に大きなインパクトを与えている。

■ iPS細胞を用いた神経・精神疾患モデリング

2008年に初めて神経疾患を含む患者由来のiPS細胞株樹立が報告される[5]，2009年からは樹立のみならず神経疾患の*in vitro*病態再現が報告され始めた。まず脊髄性筋萎縮症患者から樹立したiPS細胞由来運動神経数減少と，核ジェム数の減少が報告された[6]。そしてinhibitor of κ light polypeptide gene enhancer in B-cells, kinase complex-associated protein（*IKBKAP*）遺伝子のスプライシング異常が原因である家族性自律神経失調症患者から樹立したiPS細胞由来自律神経細胞数の減少と，遊走能の低下，さらにはKinetinによる*IKBKAP*遺伝子のRNAスプライシング異常の改善が示された[7]。さらに，遺伝性パーキンソン病（*LRRK2*変異[8]，αシヌクレイン重複[9]），遺伝性筋萎縮性側索硬化症（*VAPB*変異[10]），レット症候群（*MECP2*変異[11, 12]），筋萎縮性側索硬化症（*TARDBP*変異）[13]など，非常に多岐にわたる神経疾患のiPS細胞を用いた病態再現が報告されるようになった。2011年には，遺伝性ではなく孤発性の統合失調症患者からのiPS細胞樹立，分化誘導した神経細胞で神経突起・シナプス密度の減少が報告された[14]。

この様に，患者由来iPS細胞を用いた，神経・精神疾患研究が近年急速に広がっており，今後も状況が継続すると予測される。

■ iPS細胞を用いたアルツハイマー病疾患モデリングと先制医療開発の試み

アルツハイマー病（Alzheimer's Disease: AD）は進行性の記銘力低下・見当識障害を中心とする，神経変性疾患である。ADは加齢が大きな発症リスクと成るため，高度高齢化社会を迎えつつある我が国の医療においても非常に重要な疾患といえる。ADの病理学的特徴としては，脳内に老人斑といわれるタンパク質の沈着が見られる。この老人斑の主成分は，アミロイド前駆蛋白（Amyloid Precursor Protein: APP）から二段階の酵素切断により切り出されるアミロイドベータ蛋白（Amyloid beta: Aβ）であることが知られていた。近年まではAPPの過剰発現やノックアウトなど遺伝子操作を施した培養細胞ラインやマウスを用いた，疾患モデリングを元に病態解明が進んできた。そして近年発展の著しいヒトiPS細胞樹立と神経系細胞への分化誘導技術を用いることで，人工的な遺伝操作をせずと

もプレセニリン1[15]や*APP*重複[16]が原因の遺伝性ADの病態再現モデルも報告され始めた。Aβはその線維状凝集体や可溶性凝集体（Aβオリゴマー）の形状をとることにより神経細胞傷害毒性を呈することが知られていたが，その詳細機序はほとんどわかっていなかった。そこで我々は家族性ADの中でも，細胞内にAβが蓄積するタイプの変異である*APP* E693delta変異を有する家族性ADの患者からiPS細胞を樹立し（図1），解析を試みた[17]。

APP E693delta変異を有する家族性AD患者iPS細胞を，新たに開発した方法を用いて大脳皮質神経細胞へ分化誘導させると，細胞外に分泌されるAβが健常人由来の細胞に比べて著しく少なく，細胞質内にAβオリゴマーの蓄積が確認できた（図2）。さらに細胞質内Aβオリゴマーの蓄積が，小胞体ストレスと酸化ストレスという細胞ストレスを生じさせ，細胞死を誘導することを見出した。そしてこの小胞体ストレスをターゲットとして，複数種の化合物を用いて評価したところ，ドコサヘキサエン酸を低濃度培地に添加すると小胞体ストレスを緩和し，細胞死を抑制した。このドコサヘキサエン酸の添加濃度を上げると逆に小胞体ストレスが増加し，至適濃度の存在が示唆された。さらに我々は複数の孤発性AD患者からもiPS細胞を樹立し同様に大脳皮質神経細胞へ分化させた解析を

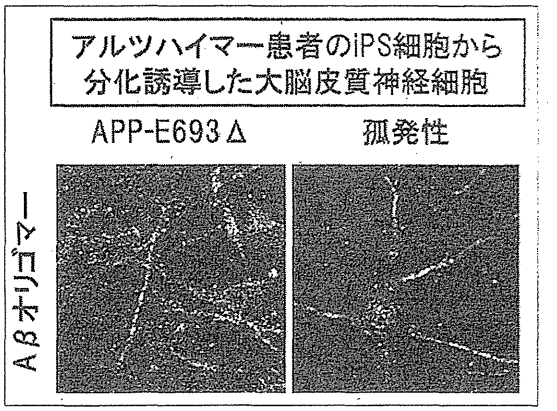

図2　アルツハイマー病患者由来の神経細胞にAβオリゴマーが蓄積する

行った結果，一人の孤発性AD患者由来神経細胞において，*APP* E693delta変異が無いにもかかわらず細胞質内Aβオリゴマーの蓄積（図2）と細胞ストレスが生じていることを見いだした。

以上のことから，臨床症状から孤発性ADとして診断される患者群において，背景にひそむ病態は一元的ではなく，個々の患者の病態特性に応じた治療戦略が重要である可能性が考えられた。iPS細胞技術は，疾患の病態解明・治療法開発のみならず，『病気の原因や進行に関する研究知見を基に，細胞内の異常を早期に診断し，病気を発症する前から

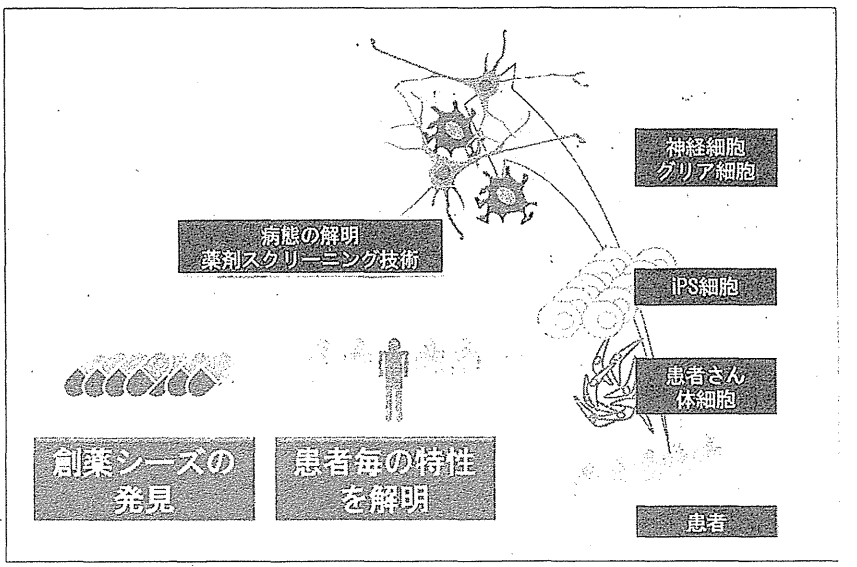

図3　iPS細胞を用いた先制医療の開発

治療を開始することで，病気の発症を遅らせる，もしくは防ぐことを目指す先制医療』に役立つ可能性が考えられた（図3）。

■克服するべき課題と展望

ヒトiPS細胞を用いた神経研究は今後さらに加速するものと予想されるが；2013年現在までのiPS細胞を用いた神経疾患研究は，既存の疾患概念を再現することにとどまる報告が多い。これは，iPS細胞の樹立自体に半年を超える時間と実験エフォートを要することや，樹立したiPS細胞の性質がクローン間差をはらむことなどが背景にあると考える。これらの問題を解決する一つの手段として，iPS細胞の樹立法は短期間・高効率の樹立法が次々と報告されるとともに[18]標準化が進みつつある。またクローン間差の問題については，多能性を安定保持し分化指向性に偏りのないiPS細胞を選別するための評価法に関する検討が進んでいる[19]など，近い将来課題は克服されるであろう。

■おわりに

ヒトiPS細胞の樹立方法開発から，わずか6年で神経疾患領域を含めた疾患研究応用が広がりを見せている。ヒトiPS細胞を用いた疾患研究は，原因遺伝子の過剰発現やノックアウトでは検討が難しかった孤発例の検討や生理的遺伝子発現量における病態解明・治療薬開発に繋がる可能性が高く，今後の発展が期待される。

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再生医学

iPS細胞を用いた神経・精神疾患治療薬の開発

Drug discovery for neurological and psychiatric disorders using iPS cells

iPS細胞を用いた神経・精神疾患研究では，患者の体細胞から作製したiPS細胞を神経細胞やグリア細胞に分化誘導して疾患標的細胞を作製し，それを用いた創薬や移植医療への応用が進められている．本稿では，神経・精神疾患患者由来iPS細胞を用いた創薬研究に関する現在の進歩状況について述べる．

iPS細胞を用いた創薬スクリーニング

これまで，種々の神経・精神疾患においてiPS細胞を用いた疾患研究が行われ，*in vitro*での疾患表現型の再現が報告されている．そのなかで，薬剤投与による疾患表現型の改善を示した研究を表に記した(表1)．

Ebertらは脊髄性筋萎縮症(Spinal muscular atrophy：SMA)患者由来iPS細胞から作製した脊髄運動神経細胞において，その数および細胞体の大きさが低下し，Survival of motor neuron 1(SMN)蛋白が減少していることを示した．さらに，SMN蛋白の発現を上昇させることが知られていたバルプロ酸やトブラマイシンが，SMA患者のiPS細胞由来神経系細胞のSMN蛋白発現を上昇させることを示した[1]．Leeらは I-κ-B kinase complex-associated protein(IKBKAP)遺伝子の異常によって生じる家族性自律神経失調症(Familial dysautonomia：FA)患者由来iPS細胞から作製した神経堤細胞において，変異型IKBKAPが増加していることを見出し，カイネチンが神経堤細胞の変異型IKBKAPを減少させることを示した[2]．Marchettoらはレット症候群患者由来iPS細胞から作製した神経細胞においてシナプスが減少していることを示し，これがinsulin-like growth factor-1(IGF-Ⅰ)およびゲンタマイシンによって改善することを示した[3]．また，Yagiらは家族性Alzheimer病患者由来iPS細胞から作製した神経細胞において，Aβ42/Aβ40産生比が増加しており，γセクレターゼ調節薬(compound W)がAβ42/Aβ40産生比を低下させることを示した[4]．Cooperらはパーキンソン病患者由来iPS細胞から作製した神経細胞がミトコンドリア機能障害に関連する細胞脆弱性を呈し，Coenzyme Q10，rapamycin，GW5074により改善することを示した[5]．これら5つの疾患研究では，他のモデルでの研究結果からすでにその効果が予想されていた薬剤あるいは既知の標的酵素に対する化合物を用いてiPS細胞由来標的細胞の疾患表現型の改善を示し，疾患iPS細胞が創薬プラットホームになることを提示した．

また，統合失調症[6]やチモシー症候群[7]の患者から作製したiPS細胞由来の神経細胞を用いた研究でも，薬剤投与による表現型の改善が示されている．

一方，iPS細胞を用いた疾患解析からあらたな治療薬シーズが見出されている．著者らはTAR DNA-binding protein-43(TDP-43)変異を有する筋萎縮性側索硬化症(Amyotrophic lateral sclerosis：ALS)患者iPS細胞から作製した運動神経細胞において，TDP-43 mRNAの増加，ストレスに対する脆弱性，神経突起の短縮を示した．ALS由来運動神経細胞の遺伝子発現解析の結果から，TDP-43

表1　疾患iPS細胞を用いた薬剤スクリーニング

疾患	薬剤	改善を示した疾患表現型	Reference
脊髄性筋萎縮症	バルプロ酸，トブラマイシン	SMN蛋白の減少	Ebert, 2009[1]
家族性自律神経失調症	カイネチン	変異型IKBKAPの増加	Lee, 2009[2]
レット症候群	IGF-Ⅰ	シナプスの減少	Marchetto, 2010[3]
Alzheimer病	Compound W	Aβ42/Aβ40産生比の増加	Yagi, 2011[4]
統合失調症	ロキサピン	neuronal conectivityの低下	Brennand, 2011[6]
チモシー症候群	ロスコビチン	TH陽性神経細胞の増加	Pasca, 2011[7]
パーキンソン病	Coenzyme Q10, rapamycin, GW5074	ミトコンドリア機能障害に関連した細胞脆弱性	Cooper, 2012[5]
筋萎縮性側索硬化症	アナカルジン酸	TDP-43 mRNAの増加，ストレス脆弱性，神経突起短縮	Egawa, 2012[8]
家族性自律神経失調症	SKF-86466	正常型IKBKAPの減少，神経マーカーの発現低下	Lee, 2012[9]