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

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Supplemental methods

RNA isolation and qPCR

RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) and subjected to reverse transcription (RT) with the Omniscript-RT kit (Qiagen). All procedures were performed by following the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was performed with a 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA) and SYBR *Premix Ex Taq* II (Takara, Shiga, Japan). Primer sequences were described previously[1].

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-human TRA 1-85 (R&D Systems), anti-human CD45 (Becton-Dickinson, Franklin Lakes, NJ), phycoerythrin (PE)-conjugated anti-human SSEA4 (R&D Systems), anti-human CD34 (Beckman Coulter, Fullerton, CA), anti-human CD45 (Becton-Dickinson), allophycocyanin (APC)-conjugated Annexin V (Becton-Dickinson), and anti-human CD45 (Becton-Dickinson) antibodies were used for flow cytometric analysis. Goat anti-human lactoferrin (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-human MMP9

(gelatinase) (Abcam, Cambridge, UK) antibodies were used for immunocytochemical analysis. Biotinylated horse anti-goat or anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was used as the secondary antibody. Mouse anti-human β-actin (Santa Cruz Biotechnology) and mouse anti-human HAX1 (Becton-Dickinson) antibodies were used for Western blotting. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Santa Cruz Biotechnology) was used as the secondary antibody.

DNA sequencing analysis

Genomic DNA was isolated from iPS cells using the QIAamp DNA blood mini kit (Qiagen). PCR was performed with primers that spanned all exons of HAX1. The PCR product was sequenced directly using the BigDye Terminator v3.1 Cycle Sequencing kit and an ABI 3130xl genetic analyser (Applied Biosystems). Primer sequences were described previously[2].

Teratoma formation

Approximately 2×10^6 iPS cells were injected subcutaneously into the dorsal flank of immunocompromised NOD/SCID/γc^{null} (NOG) mice. Tissue masses were excised 8–10 weeks after injection and fixed with phosphate-buffered saline (PBS)

containing 4% paraformaldehyde (PFA, wt/vol). Paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin.

Karyotyping and short tandem repeat analysis

Chromosomal G-banding analyses were performed at the Nihon Gene Research Laboratories (Miyagi, Japan). For short tandem repeat analysis, genomic DNA was used for PCR with the Powerplex 16 system (Promega, Fitchburg, WI). Analysis was performed using an ABI 3100 sequencer and Gene Mapper v3.5 software (Applied Biosystems).

Cytostaining

Cytospin and cytostaining were performed as described previously[3]. In brief, collected cells were centrifuged onto glass slides using a Shandon Cytospin® 4 cytocentrifuge (Thermo, Pittsburgh, PA) and analyzed by microscopy after May-Giemsa staining. For immunocytochemical analysis, cells centrifuged onto glass slides were fixed immediately with PBS containing 4% PFA (wt/vol). Immunostaining was performed as described previously[3]. For neutrophil elastase staining, cells were transferred to glass slides by centrifugation and fixed. Cells were stained using the

elastase staining kit and the elastase AS-D staining kit (Muto Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions.

Colony-forming assay

The colony-forming assay was performed as described previously[4] with some modifications. On day 16 of culture, adherent cells were treated with Stempro® Accutase® (Gibco, Carlsbad, CA), harvested, and incubated in a new tissue culture dish (Becton–Dickinson) for 10 min to eliminate adherent non-haematopoietic cells[5]. Floating cells were collected and dispersed using 40-mm strainers. Dead cells were eliminated using the Dead Cell Removal kit (Miltenyi Biotec). Live hematopoietic cells were plated at a density of 1×10^4 cells/ml in 35-mm petri dishes (Becton-Dickinson) using 1 ml/dish of MethoCult H4230 semisolid medium (STEMCELL Technologies, Vancouver, BC, Canada) containing stem cell factor (50 ng/mL, R&D Systems), IL-3 (50 ng/mL, R&D Systems), and thrombopoietin (5 ng/mL, kindly provided by Kyowa Hakko Kirin), in the absence or presence of granulocyte colony-stimulating factor (50 ng/mL, also kindly provided by Kyowa Hakko Kirin). Colonies were counted after 14 days of incubation.

Mitochondrial membrane potential ($\Delta\psi_m$) assay

$\Delta\psi_m$ was analysed using the MitoProbe^{TM} DiIC$_1$(5) Assay Kit (Molecular Probes, Carlsbad, CA) according to the manufacturer's instructions.

Lentiviral transduction of HAX1-iPS cells

Total RNA was extracted from peripheral blood mononuclear cells obtained from a healthy volunteer, and RNA was reverse transcribed using the Omniscript-RT kit (Qiagen). The human *HAX1* gene was amplified by PCR using KOD-Plus v2 (TOYOBO, Osaka, Japan) and the following primers: 5'-CCGCGGCCGCCCACCATGAGCCTCTTTGATCTCTTCCGGGGCTTT-3'(sense) and 5'-CGCGGATCCCTACCGGGACCGGAACCAACGTCCCAGGAA-3' (antisense). The PCR product was gel-purified, digested with *Not*I and *Bam*HI, and cloned into the *Not*I/*Bam*HI-digested pBlueScript II plasmid. The isolated clone (pBlueScriptII-HAX1) was verified by DNA sequencing. To construct pCSII-EF-IEGFP, the pGCDNsamIRESEGFP vector[6] was digested with *Hind*III. After creating blunt ends, the vector was further digested with *Not*I, and the resultant fragment containing the internal ribosome entry site (IRES) and enhanced green fluorescent protein (EGFP) cassette was inserted into *Not*I/*Hpa*I-sites of the

pCSII-EF-MCS plasmid (kindly provided by Dr. Hiroyuki Miyoshi, The RIKEN BioResource Center, Ibaraki, Japan). To generate pCSII-EF-HAX1-IEGFP, the *Not*I/*Bam*HI fragment of pBlueScriptII-HAX1 containing the *HAX1* sequence was cloned into *Not*I/*Bam*HI-sites of the pCSII-EF-IEGFP plasmid. Viral supernatants were collected as previously described (see http://www.brc.riken.jp/lab/cfm/Subteam_for_Manipulation_of_Cell_Fate/Protocols.html). For lentiviral transduction, the lentiviral supernatant was added to human iPS cell cultures in the presence of 4 μg/mL polybrene, followed by incubation at 37°C in an atmosphere of 5% CO_2 (vol/vol) for 24 hours. These cells were expanded, GFP-positive cells were sorted with a FACSAriaII flow cytometer (Becton-Dickinson), and cultured on mitomycin-C-treated SNL feeder cells in the presence of Y-27632[7] (10 mg/mL) for 24 hours. Single cell-derived GFP-positive colonies were then expanded, and these cell lines were used for subsequent experiments.

Western blot analysis

iPS cell extracts were subjected to SDS-PAGE, and proteins were transferred onto Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked, incubated with primary antibodies, washed, and incubated with corresponding

secondary antibodies. Immunoreactions were visualized using an ECL Western Blotting Detection system (GE Healthcare, Waukesha, WI).

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Supplemental Figure Legends

Supplemental Figure 1. Validation of HAX1-iPS cell lines.

(A) A quantitative RT-PCR assay for the expression of *OCT3/4*, *SOX2*, *KLF4*, and *cMYC* in HAX1-iPS, control ES, and iPS cells, and patient-derived fibroblasts. Copy numbers of genes derived from 1 ng mRNA are indicated. One primer set detected both the transgene and endogenous gene (Total), and the other primer set detected only the transgene (Transgene) (n = 3; bars represent SDs). (B) Karyotype analysis of HAX1-iPS cells. All HAX1-iPS cell lines showed normal karyotypes. (C) Short tandem repeat analysis. All HAX1-iPS cell lines showed the same pattern as fibroblasts. (B-C) Representative data (HAX1 3F5) are shown.

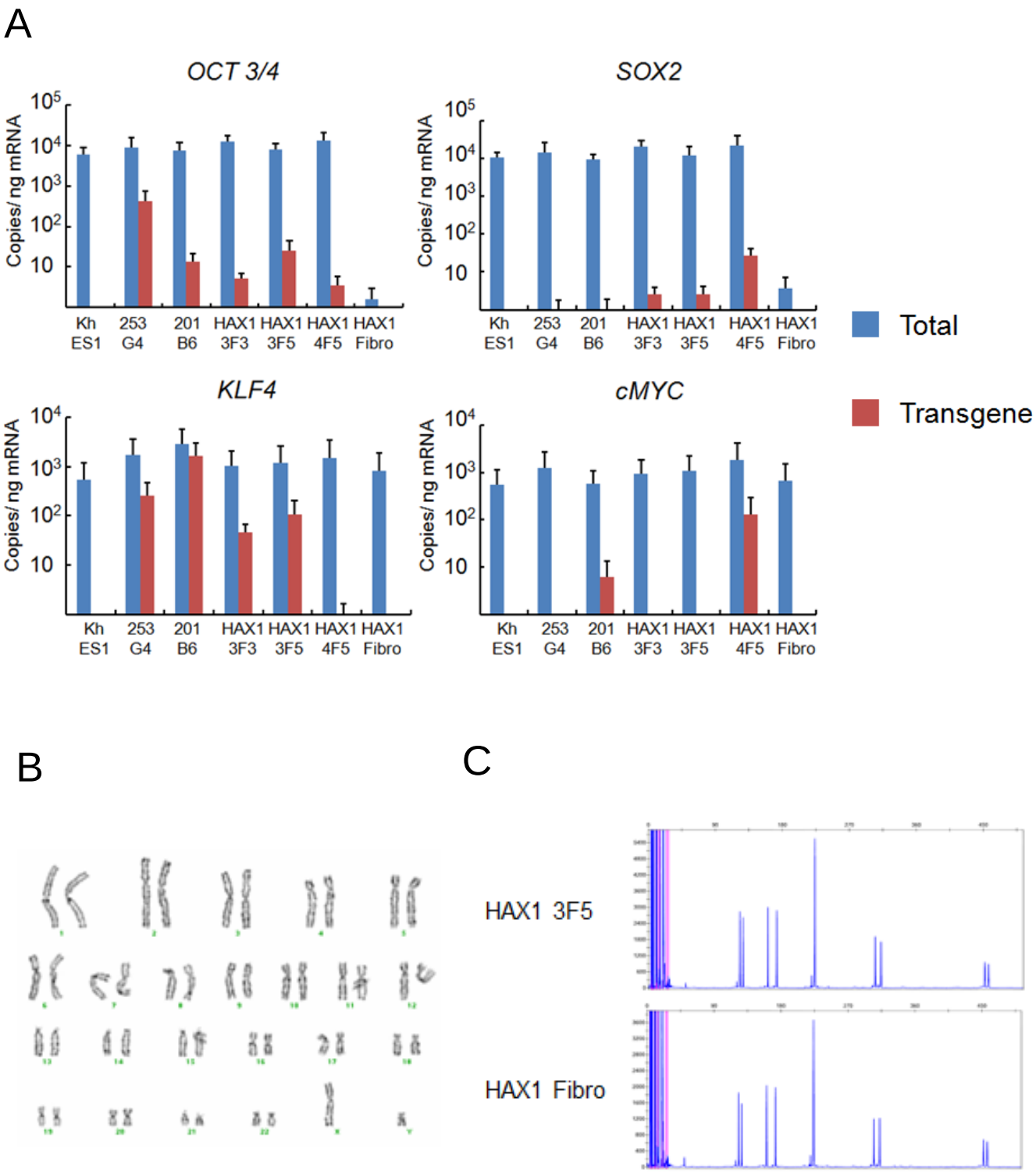
Supplemental Figure 2. System to differentiate neutrophils from human iPS cells in the absence of serum and feeder cells.

A culture system was used to differentiate neutrophils from human iPS cells in the absence of serum and feeder cells. iPS cell colonies were cultured on Matrigel-coated cell culture dishes in serum-free medium. The indicated cytokines were present in the medium on the indicated days.

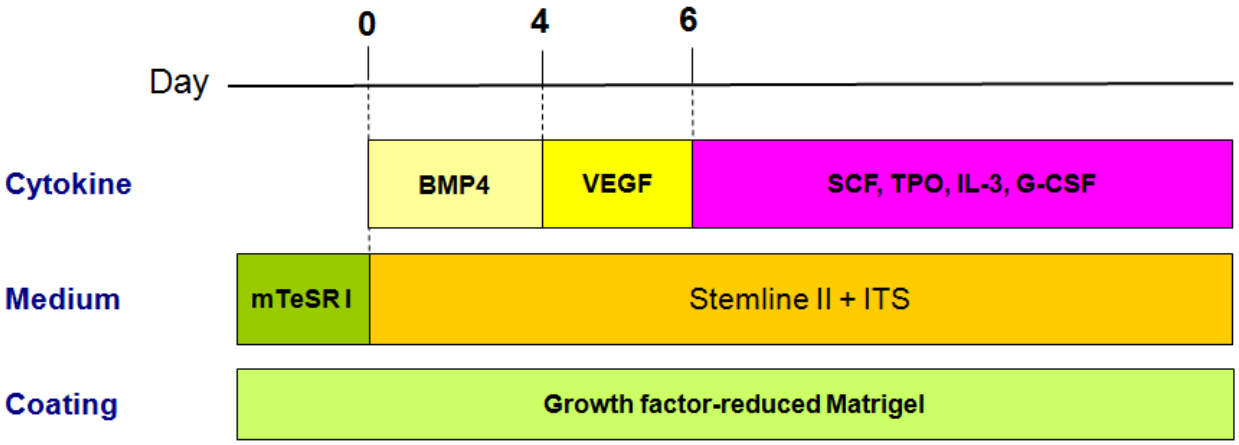
Supplemental Figure 3. Mitochondrial membrane potential assay of undifferentiated iPS cells.

$\Delta\psi_m$ was analysed in undifferentiated iPS cells (n = 3; bars represent SD).

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3