Supporting Information

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SI Materials and Methods

Cell Culture. Human fibroblast BJ cells from neonatal foreskin (American Type Culture Collection) and HDFs from the facial dermis of a 36-y-old woman (Cell Applications) were maintained in fibroblast growth medium (Cell Applications) or DMEM (Invitrogen) supplemented with 10% FBS. PA6 feeder cells (RIKEN BioResource Center) were grown in α-MEM (Invitrogen) with 10% FBS. Human iPSCs were maintained on MMC-treated MEF feeder cells in primate ESC medium (ReproCELL) supplemented with 4 ng/mL of basic fibroblast growth factor (bFGF; Wako Pure Chemical). Human iPSCs were passaged by 1 mg/mL of collagenase IV (Invitrogen).

The frozen $CD34^+$ fraction of CB cells was obtained from RIKEN BioResource Center. The fresh CB cells were provided by Asagiri Hospital (Kobe, Japan) after written informed consent for research use was obtained. $CD34^+$ cells were purified from mononuclear cells isolated from fresh CB cells with Lymphoprep (Cosmo Bio) using a human CD34 Micro Bead Kit and Auto Macs columns (Miltenyi Biotec) in accordance with the manufacturer's instructions. Frozen $CD34^+$ cells were cultured in hematopoietic culture media [serum free X-VIVO10 (Lonza) containing 50 ng/mL of IL-6 (Peprotech), 50 ng/mL of sIL-6R (Peprotech), 50 ng/mL of SCF (Peprotech), 10 ng/mL of TPO (Peprotech), and 20 ng/mL of Flt3 ligand (R&D Systems)] for 1 d before viral infection. SNL 76/7 feeder cells (European Collection of Cell Cultures) were treated with 100 μL of MMC solution (1 mg/mL; Nacalai Tesque) in 10-cm dishes for 3 h to generate MMC- treated-SNL 76/7 feeder cells (MMC-SNLs). These were seeded on 24-well plates (BD) or 6-well plates or 60 mm dishes in hESC media [DMEM/F-12 (Sigma-Aldrich) containing 20% knockout serum replacement (KSR; Invitrogen), 2 mM L-glutamine (Invitrogen), 1% Nonessential Amino Acids (Invitrogen), 0.1 mM 2-ME (Invitrogen), and 4 ng/mL bFGF (Peprotech)] to feed reprogrammed cells. The medium was changed every day.

Generation of iPSCs from CB Cells. The frozen $CD34^+$ cells (RIKEN BioResource Center) were thawed and cultured for 1 d in hematopoietic culture media in six-well plates at a density of 2×10^4 cells/2 mL/well before infection with SeV. The thawed $CD34^+$ cells (1×10^4), or an equivalent number of freshly isolated $CD34^+$ cells, were transferred to 96-well plates in 180 mL of hematopoietic cell culture media with 20 mL of viral supernatant containing 2 MOI each of five SeV constructs (SeV TS7-*OCT3/4*, -*SOX2*, -*KLF4*, -*c-MYC*, and -*GFP*). The medium was changed the next day, and then infected cells were cultured for another 4 d, after which 1×10^4 infected CB cells were seeded and cultured on the semiconfluent SNL cells in six-well plates in hESC media. After ESC–like colonies had appeared, the growing colonies were harvested with collagenase IV treatment, plated on MEF feeder cells, and cultured in primate ESC medium (ReproCELL) supplemented with 4 ng/mL of bFGF to avoid spontaneous differentiation.

Generation of Sendai Virus Vectors, Retrovirus, and Lentivirus Vectors. The ORF of human *OCT3/4* was obtained from human embryonic carcinoma cell NCCIT cDNA, and the ORFs of human *SOX2*, *KLF4*, and *c-MYC* genes were amplified from Jurkat cell cDNA using qRT-PCR. Those four genes were amplified using NotI-tagged gene-specific forward and reverse primers containing the SeV-specific transcriptional regulatory signal sequences listed in Table S4. The amplified fragment was introduced into an FΔ SeV vector. Recovery and propagation of the SeV/ΔF vectors were performed as follows. First, 293T cells were transfected with template pSeV/ΔF carrying each transgene, as well as pCAGGS plasmids carrying the T7 RNA polymerase and the *NP*, *P*, *F5R*, and *L* genes. Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and cultured for 1–3 d to generate the seed SeV/ΔF vector. The vectors were propagated using LLC-MK2/F7/A cells, an SeV F-expressing LLC-MK2 cell line (1), in MEM containing trypsin (2.5 μg/mL). The vector titers (cell infectious units/mL) of recovered SeV vector were determined by immunostaining using anti-SeV rabbit polyclonal serum as described previously (2). To generate new TS SeV vectors, mutations were introduced into the conventional TS SeV/ΔF vector (3) by oligonucleotide-directed mutagenesis (QuikChange; Stratagene) with the primer pairs listed in Table S4. For retroviral production, the *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* genes were amplified as described above, cloned into the NotI site of a pMX vector, and then transfected to the packaging cell, PLAT-E (licensed from Dr. T. Kitamura, University of Tokyo). The murine cationic amino acid transporter (mCAT) for retrovirus receptors was cloned from NIH/3T3 cells (Table S4) and subcloned into the NotI site of the gene transfer vector pGTV of replication-defective self-inactivating SIV-based lentivirus. The plasmid and three packaging plasmids (encoding gag-pol, rev, and VSV-G env) were transfected into 293T cells to produce a self-inactivating SIV-based lentiviral vector, as described previously (4).

Screening of TS SeV Vectors. Confluent LLC-MK2 cells were transduced with each SeV vector carrying the *GFP* gene and cultured at various temperatures (32, 35, 37, 38, and 39 °C). Green fluorescence was compared at 3 d after infection. To confirm the irreversible inactivation of gene expression by temperature-shift treatment, infected cells were first cultured at 37 °C for 10 d before being split into two aliquots, one cultured at 37 °C and the other cultured at 39 °C, with passaging every 7 d for a total of 28 d. To monitor vector reactivation, after treatment at the nonpermissive temperature of 39 °C for 7 d, infected cells were cultured at 37 °C and passaged every 7 d for a total of 28 d, and *GFP* gene expression was evaluated to confirm the irreversible inactivation of gene expression.

Determining Reprogramming Efficiency. Reprogramming efficiency was calculated as the number of iPSC colonies formed/number of infected cells seeded. The iPSC colonies were identified on the basis of ESC-like morphology, as well as ALP staining.

ALP and Immunofluorescence Staining. Cells were stained for ALP using ALP substrate (1-Step nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Pierce) after fixation with 10% neutral buffered formalin solution (Wako Pure Chemical). Immunofluorescence staining was performed using the following primary antibodies: NANOG (ReproCELL), OCT4 (Santa Cruz Biotechnology), PDX1 (R&D Systems), TH (Chemicon), SSEA4, TRA-1-60, TRA-1-81 (Cell Signaling Technology), βIII-tubulin (2G10; Santa Cruz Biotechnology), anti-SeV polyclonal antibody (MBL), and anti-HN monoclonal antibody IL4.1 (5). Samples were analyzed with a confocal microscope [MRC1024 (Bio-Rad) or BIOREVO BZ-9000 (Keyence)]. TO-PRO3 (Molecular Probes) or DAPI was used for nuclear staining. Secondary antibodies [anti-rabbit IgG, anti-mouse IgG, IgM, and ProteinA conjugated with Alexa Fluor 488 (green) or 568 (red)] were purchased from Molecular Probes. For CB-iPSCs, photomicrographs were taken with a fluorescent microscope (Olympus BX51 or IX71) and a visible light microscope (Olympus

CKX31). Anti-AFP (MAB1368; R&D Systems), vimentin (sc-5565; Santa Cruz Biotechnology), and desmin (M0760; Dako) were used for staining of differentiated tissues.

In Vitro Differentiation of Human iPSCs. Embryoid bodies were generated from clumps of human iPSCs in suspension culture for 6 d in DMEM with 20% FBS, and then grown in adherent culture on gelatin-coated dishes with ascorbic acid to induce differentiation into cardiomyocytes (6). For embryoid body–mediated spontaneous differentiation of CB-iPSCs, DMEM/F12 containing 20% KSR (Invitrogen) was used instead of DMEM with FBS. For differentiation into dopaminergic neurons, small clumps of SeV-generated iPSCs were cocultured with PA6 (stromal cells derived from skull bone marrow; RIKEN BioResource Center) in Glasgow Minimal Essential Medium (Invitrogen) containing 10% KSR (Invitrogen), 1×10^{-4} M nonessential amino acids, and 2-mercaptoethanol for 16 d (7). For the induction of definitive endoderm cells and pancreatic cells, small clumps of iPSCs were cultured on feeder cells with 100 ng/mL of activin A (R&D Systems) in RPMI1640 (Invitrogen) supplemented with 2% FBS for 4 d, followed by an additional 8 d of culture in DMEM/F12 supplemented with N2 and B27 (Invitrogen), nonessential amino acids, 2-mercaptoethanol, 0.5 mg/mL BSA, L-glutamine, and penicillin/streptomycin (8).

Teratoma Formation by Human iPSCs. Human iPSCs grown on MEF feeder layers were collected by collagenase IV treatment and injected s.c. or beneath the testicular capsule in SCID mice (SLC Japan). Palpable tumors were observed typically 1 mo after injection. Tumor samples were collected after 2 mo in most cases, fixed in 10% formalin, and then processed for paraffin embedding and H&E staining using standard procedures.

Whole-Genome Expression Analysis. For transcriptional analysis, total RNA was isolated from cells cultured in six-well dishes using the RNeasy Mini Kit (Qiagen). Cyanine-labeled antisense RNA was amplified using the Quick Amp Labeling Kit (Agilent), hybridized with the Gene Expression Hybridization Kit on Whole Human Genome Oligo Microarray (one color, 4 × 44K; Agilent) and analyzed with an Agilent microarray scanner. Data were analyzed using GeneSpring GX10.0 software (Agilent). Two normalization procedures were applied. First, signal intensities <1 were set to 1, and then each chip was normalized to the 50th percentile of the measurements taken from that chip. Each gene was normalized against the median of that gene in the respective control to enable comparison of relative changes in gene expression between different conditions. The microarray data of hES H9, KhES01, BG ESCs, and retroviral-induced human iPSCs (4) were retrieved from GEO DataSets (accession nos. GSM194390, GSM194391, GSM194392, and GSE9561, respectively). These analyses were performed at Bio Matrix Research. These data have been deposited in GEO DataSets (accession no. GSE24240). For CB-iPSC analysis, total RNA from several established iPSC lines, KhES-3 cells (a gift from the Yamanaka laboratory, Kyoto University), $CD34^+$ CB cells, or CB mononuclear cells were purified using the Qiagen RNeasy Mini Kit and hybridized with Gene Chip Human Genome U133 Plus 2.0 Array (Affymetrix) according to the manufacturer's instructions (accession no. of the data, GSE25090).

Bisulfite Genomic Sequencing. Genomic DNA (1 μg) from BJ cells, HDF cells, and iPSCs was treated with sodium bisulfite using the BisulFast DNA Modification Kit (Toyobo) in accordance with the manufacturer's instructions. The promoter regions of *OCT4* were amplified by qRT-PCR using the primer sets listed in Table S4. The resultant PCR products were cloned into pGEM-Teasy vector (Promega) and sequenced.

RNA Isolation and qRT-PCR Analysis. Total RNA was isolated using ISOGEN (Nippon Gene), and cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). qRT-PCR was performed with cDNAs using the gene-specific primers listed in Table S4 (30 cycles) with the 7500 Fast Real-Time PCR system (Applied Biosystems) and TaqMan Gene Expression Master Mix (Applied Biosystems). SeV genomic transcript levels were normalized against those of β-actin, and relative transcription ratios were calculated. TaqMan Human Endogenous Control (Applied Biosystems) was used to detect β-actin.

Detection of Integrated Transgenes by qPCR. Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) with RNaseA treatment. Quantitative PCR was performed with a 7500 Fast Real-Time PCR system (Applied Biosystems) and TaqMan Gene Expression Master Mix (Applied Biosystems). Gene-specific primers for *SOX2*, *KLF4*, and *c-MYC* were designed to detect single copies of the genes on the chromosome, whereas the primers for *OCT4* were designed to detect six copies (Table S4).

DNA Fingerprinting. Genomic DNA was isolated from parental fibroblasts and SeV vector–induced iPSC clones using the Qiagen DNeasy Kit. Three variable number tandem repeat loci—MCT118, D17S1290, and 3′ApoB—were amplified by PCR and analyzed by 3% agarose gel electrophoresis.

Karyotyping. Chromosomal G-band analysis was performed by Nihon Gene Research Laboratories.

1. Li HO, et al. (2000) A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. *J Virol* 74:6564–6569.
2. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M (2009) Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad, Ser B, Phys Biol Sci* 85:348–362.
3. Inoue M, et al. (2003) Nontransmissible virus-like particle formation by F-deficient Sendai virus is temperature-sensitive and reduced by mutations in M and HN proteins. *J Virol* 77:3238–3246.
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5. Nagai Y, Takakura A, Irie T, Yonemitsu M, Gotoh B (2011) Evolution of Sendai virus: The journey from mouse pathogen to a state-of-the-art tool in virus research and biotechnology. *The Paramyxoviruses*, ed Samal SK (Horizon Scientific Press, Norwich, UK).
6. Guo XM, et al. (2006) Creation of engineered cardiac tissue in vitro from mouse embryonic stem cells. *Circulation* 113:2229–2237.
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8. D'Amour KA, et al. (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol* 23:1534–1541.
9. Takahashi K, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872.

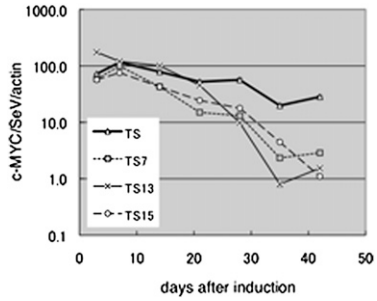

Fig. S1. qRT-PCR of exogenous c-MYC expression by SeV vectors in transduced cells performed during reprogramming and subsequent expansion of established iPSCs as described in Fig. 2C, but using the primers listed in Table S4.

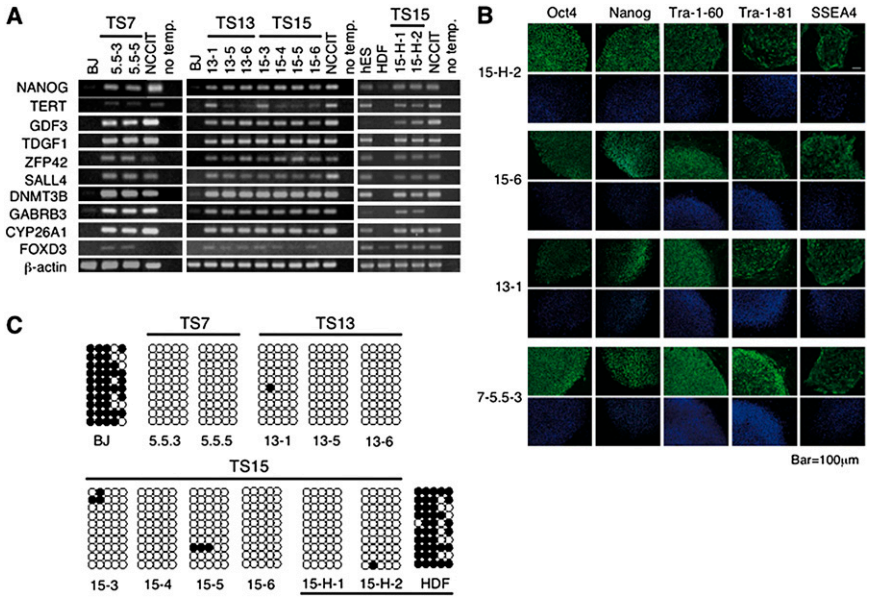

Fig. S2. Expression of hESC markers and epigenetics of established iPSCs. (*A*) qRT-PCR analysis of hESC marker genes in established iPSCs. BJ and HDF, parental fibroblasts; NCCIT, embryonic carcinoma line and hESC (KhES01) used as a positive control; no temp, without template. (*B*) Expression of hESC markers determined by immunostaining (green). Nuclei were stained with DAPI (blue). (*C*) Bisulfite sequencing of the *OCT3/4* promoter region (−2330 to −2110) in iPSCs. Open circle, demethylated; closed circle, methylated. 15-H-1 and 15-H-2 were derived from HDF cells; the others were derived from BJ cells.

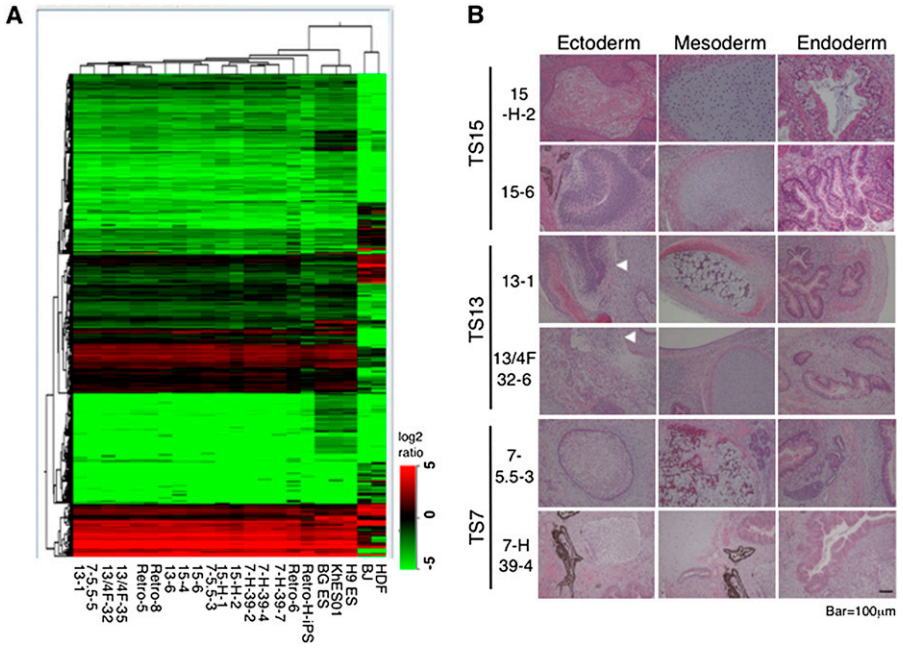

Fig. S3. (*A*) Global gene expression analysis of SeV-generated iPSCs. Comparison of gene expression profiles of parental fibroblasts (HDF and BJ), hESCs (H9, KhES01, and BG, retrieved from GEO DataSets GSM194390, GSM194391, and GSM194392, respectively), retroviral-induced human iPSCs [retro-H-iPS from HDF cells (9) (GSE9561) and retro-5, -6, and -8 (P21) from BJ cells], and SeV-generated vial/factor-free iPSC lines from HDF cells [15-H-1 (P20), 15-H-2 (P17; MYC/TS15), 7-H-39-2, 7-H-39-4, and 7-H-39-7 (P18; 4F/TS7, temperature- shifted at 39 °C)] and BJ cells [7-5.5-3 (P21), 7-5.5-5 (P27; MYC/TS7), 13-1 (P28), 13-6 (P30; MYC/TS13), 15-4, 15-6 (P32; MYC/TS15), 13/4F-32, and 13/4F-35 (P10: 4F/TS13)]. All genes (n = 8,828) showing at least fourfold differential expression between hESCs and fibroblasts were ordered by this ratio and hierarchically clustered by sample (using uncentered correlation and average linkage). All $\log_2$ ratios are relative to fibroblasts. The expression profile of viral/factor-negative human iPSCs is more highly correlated with hESCs than the expression profile of retroviral factor-carrying human iPSCs, retro-H-iPS, and retro-6, but is similar to the expression profile for retro-5 and retro-8. (*B*) Pluripotency of SeV-generated iPSCs. Teratoma sections of SeV-generated iPSCs clones were stained with H&E at 6 wk after injection into NOD/SCID mice. Teratomas were differentiated from SeV-generated iPSCs (TS15: 15-H-2 and 15-6; TS13: 13-1 and 13/4F-32-6; TS7: 7-5.5-3 and 7-H-39-4) and contained multiple tissues derived from three germ layers: ectoderm (keratinocytes, neural tissues, and epithelial tissues), mesoderm (cartilage, bone, and muscle), and endoderm (gut-like structures with paneth granular cells characteristic of intestinal tissue, seen in several panels). Arrowheads indicate the ectoderm-derived tissues from multiple tissues.

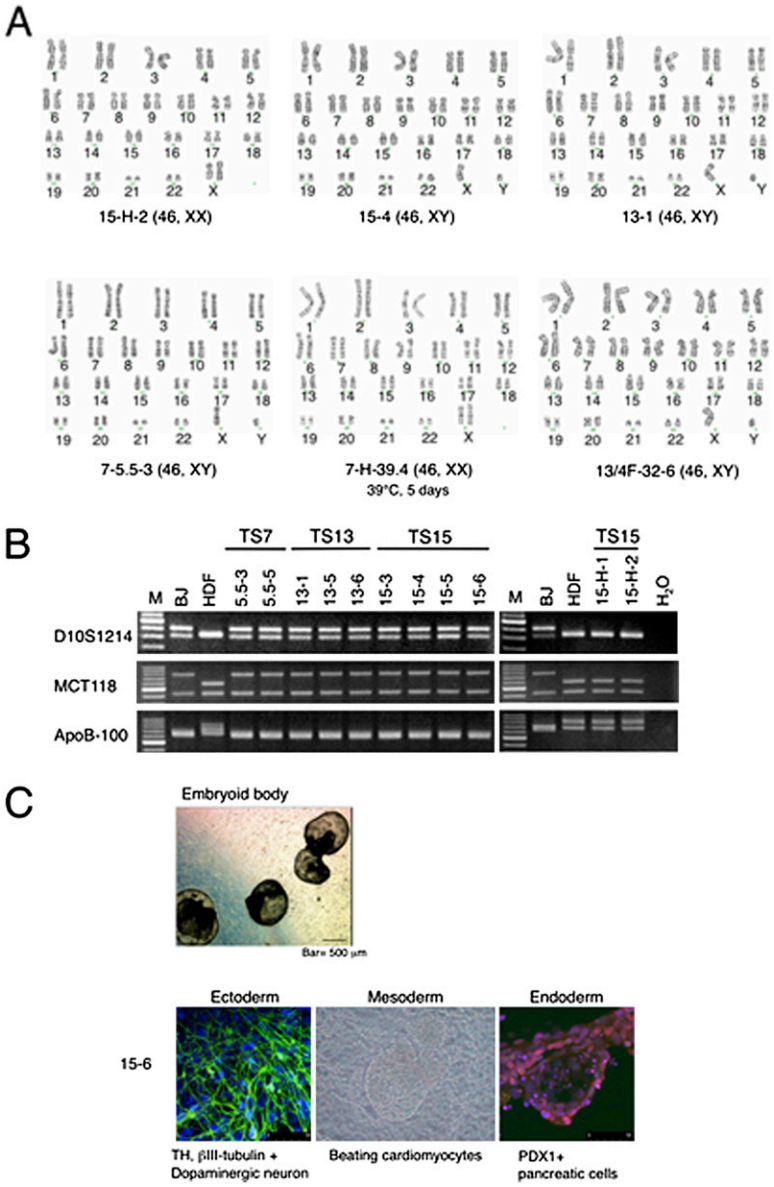

Fig. S4. (*A*) Karyotyping of SeV-generated iPSCs. Viral-free SeV-generated iPSCs were used for karyotyping, and these cells were found to be karyotypically normal. The 15-H-2 and 7-H-39.4 were generated from HDF cells (female facial skin), and the others were generated from BJ cells (male neonatal foreskin). The iPSCs tested were generated with MYC/TS15 [15-H-2 (P13); 15-4 (P22)], MYC/TS13 [13-1 (P22)], 4F/TS13 [32-6 (P23)], MYC/TS7 [7-5.5-3 (P26)], and 4F/TS7 [7-H-39.4 (P16)] that had been treated at 39 °C for 3 d to remove viral vectors. Other iPSCs tested [MYC/TS15: 15-H-1 (P11), 15-6 (P23); 4F/TS13: 13-32-6 (P23), 13-35-2 (P23); MYC/TS7: 7-5.5-5 (P22)] and temperature-shifted iPSCs [H-39-2 (P16); H-39-7 (P16)] were also karyotypically normal (Table S3). (*B*) Fingerprinting of SeV-generated iPSCs. PCR analysis of three variable number tandem repeat loci of D17S1290, MCT118, and ApoB-100 using genomic DNA from the SeV-generated iPSC clones confirmed that these clones originated from human fibroblasts, namely BJ and HDF cells. (*C*) In vitro differentiation of viral/factor-free SeV-generated iPSCs. (*Upper*) Viral/factor-free SeV-generated iPSCs formed cystic embryo bodies in suspension culture. (*Lower*) In vitro differentiation to three germ layers. Putative dopaminergic neurons coexpressing tyrosine hydroxylase (TH) were generated cocultured with PA6 cells, beating cardiomyocytes, and pancreatic cells expressing PDX1.

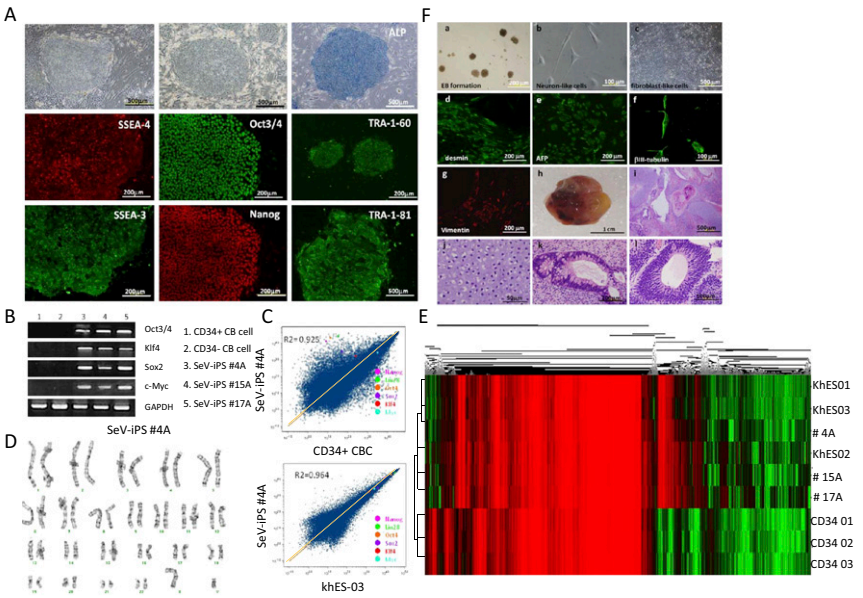

Fig. S5. Characterization of ESC-like colonies generated from CD34$^+$ CB cells. (*A*) Phase-contrast images of a representative ESC-like colony on feeder cell SNL before (clone SeV iPSC 4; *Top*, *Left*) and after heat treatment (clone SeV iPSC 4A; *Top*, *Middle*). The SeV iPSC 4A clone was stained for ALP activity and with antibodies against pluripotency-related molecules, including SSEA-4, OCT 3/4, TRA-1-60, SSEA-3, NANOG, and TRA-1-81, as indicated. Alexa Fluor 594–conjugated (red) and Alexa Fluor 488–conjugated (green) secondary antibodies were used to visualize the expression. (*B*) Endogenous gene expression of pluripotency-related molecules was determined by qRT-PCR. Sample description, tested genes, and lanes are as indicated. CD34$^+$ and CD34$^-$ CB cells were used as controls. (*C*) Gene expression comparison of SeV iPSC 4A and CD34$^+$ CB cells (CD34 01, parent of Sev iPSC 4A; *Upper*) and SeV iPSC 4A and hESC KhES-03 (*Lower*). (*D*) Karyotype of SeV iPSC 4A (also see Table S3). (*E*) Heat map showing gene expression profiles of SeV iPSC clones SeV iPSC 4A, SeV iPSC 15A, and SeV iPSC 17A; parent CD34$^+$ CB cells (CD34 01, CD34 02, and CD34 03); and hESCs KhES01, KhES02, and KhES03. (*F*) In vitro differentiation potential of SeV iPSC 4A. Bright-field images of embryoid bodies were generated after 8 d of culture (a). Embryoid bodies were transferred to gelatin dishes. Phase-contrast images of neuron-like (b) and fibroblast-like (c) are shown. Differentiated cells were stained with antibodies against desmin (d), AFP (e), βIII-tubulin (f), and vimentin (g) to identify specific cell lineages. The in vivo differentiation potential of SeV iPSC 4A was determined by a teratoma formation assay (h). H&E staining of a teratoma (i) showed cartilage cell-like tissue (j, mesoderm), gut-like epithelium (k, endoderm), and immature neural tissue-like tissue (l, ectoderm).

Table S1. Efficiency of induction of iPSCs from fibroblasts and removal of vectors with various SeV vectors

Table S1

Table S2. Efficiency of induction of iPSC clones from cord blood cells with several SeV vectors

Table S2

Table S3. Summary of characterization of established iPSC clones with various SeV vectors

Table S3

Table S4. Primer sequences used for PCR

Table S4