# 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

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**Abstract:** Induced pluripotent stem cells (iPSC) have been generated from somatic cells by introducing reprogramming factors. Integration of foreign genes into the host genome is a technical hurdle for the clinical application. Here, we show that Sendai virus (SeV), an RNA virus and carries no risk of altering host genome, is an efficient solution for generating safe iPSC. Sendai-viral human iPSC expressed pluripotency genes, showed demethylation characteristic of reprogrammed cells. SeV-derived transgenes were decreased during cell division. Moreover, viruses were able to be easily removed by antibody-mediated negative selection utilizing cell surface marker HN that is expressed on SeV-infected cells. Viral-free iPSC differentiated to mature cells of the three embryonic germ layers *in vivo* and *in vitro* including beating cardiomyocytes, neurons, bone and pancreatic cells. Our data demonstrated that highly-efficient, non-integrating SeV-based vector system provides a critical solution for reprogramming somatic cells and will accelerate the clinical application.

**Keywords:** iPS cell, Sendai virus, transgene-free, reprogramming, human

## Introduction

After the report of Takahashi and Yamanaka[1)] induced pluripotent stem (iPS) cells have been generated from somatic cells by transducing reprogramming factors in mammalian including mouse[1)–4)] and human.[5)–9)] A major limitation of this technology is the integration of viral transgenes into the host genome that includes the risk of tumorigenicity.[3)] Several solutions for this problem have been developed with use of adenovirus vectors[10)] or plasmids[11),12)] for induction of iPSCs, but the risk of integration still remains as far as DNA-type vectors are used.[13)] These methods also suffered from the low efficiency of transdution. Alternative methods using a transposon, or Cre/LoxP system require subsequent excision of transgenes from the host genome.[14)–16)] Use of small molecules that replace the reprogramming genes[17)] may solve the problem but remain unrealized in human cells. Delivery of recombinant reprogramming proteins was able to generate iPSCs, however, the efficiency was very low and needed repetitive induction.[18),19)] Considering that all those problems are derived from each of the fact that reprogramming genes have been introduced and present in the host cells as DNA form and/or the low efficiency of protein expression, it is obvious that RNA virus based, and high efficient expression vector such as Sendai virus can bring an ultimate solution for those problem.

We have developed Sendai virus (SeV) vectors that replicate in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells, which do not go through a DNA phase nor integrate into the host genome.[20)] Since SeV vectors are very efficient for introduction of foreign genes in wide spectrum of host cell species and tissues, moreover, controllable for foreign gene expression,[21)] SeV vectors have been considered for clinical studies of gene therapy for cystic fibrosis,[22),23)] critical limb ischemia[24)] or vaccines for AIDS.[25)]

In this study, we investigated whether or not F-protein deficient (ΔF), non-transmissible SeV vectors with low cytotoxity[26),27)] that allow high-level expression of exogenous genes without integrating

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Abbreviations: MOI: multiplicities of infection; iPSC: induced pluripotent stem cell; EB: embryoid body; IH: immunohistochemistry; SeV: Sendai virus; SeV-iPSC: iPS cell generated by SeV vectors.

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into the host genome, could be used to generate human iPSCs.

## Material and methods

**Cell culture.** Human fibroblast BJ from neonatal foreskin (ATCC, USA), and HDF from facial dermis of 36-year femail (Cell Applications, Inc., USA), were maintained in fibroblast growth medium (Cell Applications, Inc., USA) or DMEM (Invitrogen, USA) supplemented with 10% FBS. PA6 feeder cells (RIKEN BRC, Japan) were grown in alpha-MEM (Invitrogen, USA) with 10% FBS. Human iPSCs were maintained on MMC-treated MEF feeder cells (ReproCELL, Japan) in primate ES medium (ReproCELL, Japan) supplemented with 10 ng/ml bFGF (R&D systems, USA). Human iPSCs were passaged by 1mg/ml collagenase IV (Invitrogen, USA).

**Generation of SeV vectors.** The open reading frames (ORFs) of human OCT3/4 were obtained from NCCIT cDNA and the ORFs of human SOX2, KLF4 and c-Myc genes were amplified from Jurkat cell cDNA by RT-PCR. Those four genes were amplified with *Not* I-tagged gene-specific forward primer and *Not* I-tagged gene-specific reverse primer containing SeV-specific transcriptional regulatory signal sequences[26] listed in Table 2. The amplified fragment was introduced into F-deficient SeV vector. Recovery and propagation of the SeV/ΔF vectors were carried out as follows. Briefly, 293T cells were transfected with template pSeV/ΔF carrying each transgenes and pCAGGS-plasmids each carrying the T7 RNA polymerase, NP, P, F5R, and L gene. The cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and cultured for 1 to 3 days to generate the seed SeV/ΔF vector. Then, the vector was propagated using the LLC-MK2/F7/A cells that were Sendai virus F-expressing LLC-MK2 cells line previously established in MEM containing trypsin (2.5 μg/ml). The vector titers (cell infectious unit (CIU) per ml) of recovered SeV vector were determined by immunostaining using anti-SeV rabbit polyclonal serum described as previously.[26]

**Induction of pluripotent stem cells.** Induction of human iPSCs was similar to previous report[5] except using SeV vectors instead of retrovirus. Alternatively, we tested feeder-free protocol: $2 \times 10^5$ cells per well were plated and infected with SeV vectors at an MOI of 3 on 12 well plate, cultured in DMEM supplemented with 10% FBS. After ES-like colonies appeared, medium was changed to primate ES medium with bFGF and then growing colonies were harvested with collagenase IV treatment, plated on MEF feeder cells to avoid spontaneous differentiation.

**Determining reprogramming efficiency.** Reprogramming efficiency was calculated as the number of iPS colonies formed per number of infected cells seeded. iPS colonies were identified based on ES-like morphology, and alkaline phosphatase staining was used to facilitate the identification of iPS colonies.

**Alkaline phosphatase (ALP) and immunofluorescence staining.** ALP staining was performed with ALP substrate (1 step NBT/BCIP/ Pierce) after fixed with 10% neutral buffered formalin solution (Wako, Japan).

Immunofluorescence staining was performed using the following primary antibodies: Nanog, SSEA1, SSEA4, PDX1, SOX17 (R&D systems, USA), SOX2, TH (Chemicon, USA), TRA-1-60 (BD Pharmingen, USA), TRA-1-81, OCT4, βIII-tubulin (2G10) (Santa Cruz Biotechnology, USA), anti-SeV-polyclonal antibody,[27] anti-HN-monoclonal antibody IL4.1.[28] Samples were analyzed with confocal microscopy (Bio-Rad MRC1024) or FLovel HCD-FL (Flovel, Leica). For FACS, CD34-, CD45-, CD33-PE conjugates and CD66b-FITC conjugate were purchased from BioLegend. TO-PRO3 (Molecular Probes, USA) was used for nuclear staining. 2nd antibodies used here: anti-rabbit IgG, anti-mouse IgG, IgM and ProteinA conjugated with Alexa Fluor 488 (Green) or 568 (Red) were purchased from Molecular Probes.

***In vitro* differentiation of human iPSCs.** Embryoid bodies were generated from clumps of human iPSCs in suspension culture for 6 days in IMDM with 15% FBS, and then grown in adherent culture on gelatin-coated dish with cytokine cocktails (100 ng/ml SCF, 100 ng/ml Flt3L, 50 ng/ml TPO, 100 ng/ml G-CSF, 20 ng/ml IGF-2, and 100 ng/ml VEGF) to induce lymphoid lineage cells and cardimyocytes.[29] For differentiation to dopaminergic neurons, small clumps of SeV-iPSC were cocultured with PA6 (stromal cells derived from skull bone marrow; RIKEN BRC, Japan) in GMEM (Invitrogen, USA) containing 10% KSR (Invitrogen, USA), $1 \times 10^{-4}$ M non-essential amino acids and 2-mercaptoethanol for 16 days.[30] For induction of definitive endoderm cells and pancreatic cells, small clumps of iPSCs were cultured on feeder cells with 100 ng/ml activin A (R&D

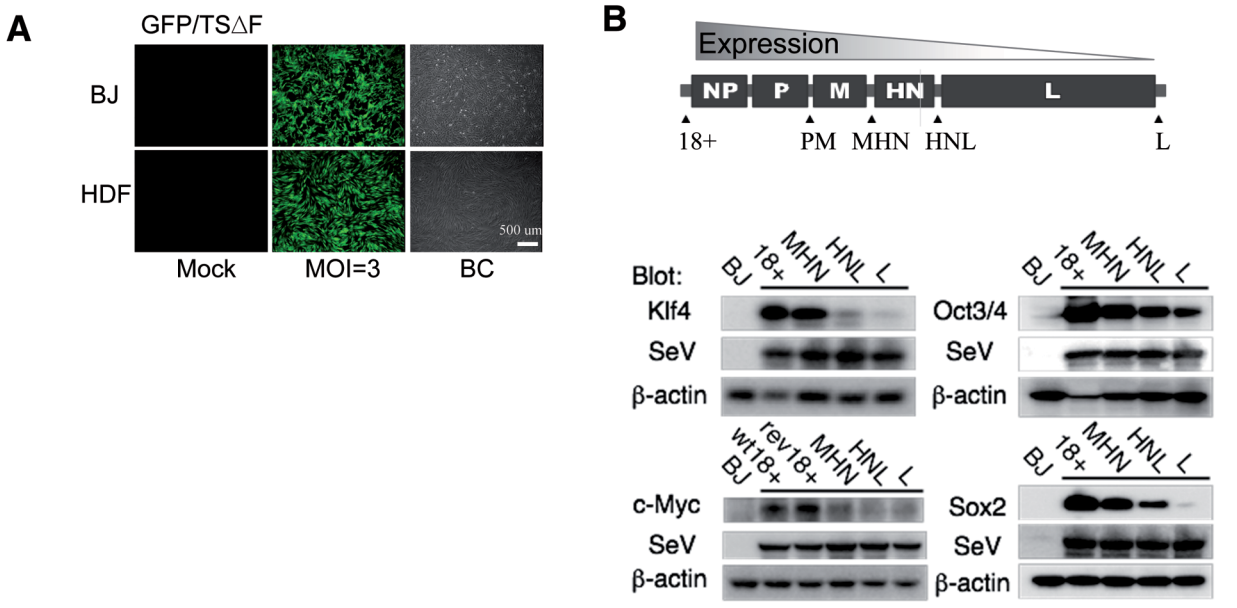



Fig. 1. Expression of exogenous genes in human fibroblasts by SeV vectors. **A.** Efficient induction of *GFP* cDNA by TSΔF/SeV in BJ and HDF at an MOI of 3. BC: bright contrast. **B.** Schematic presentation of SeV vector genomes. Reprogramming genes were inserted at 18+, PM, HN, HNL and Leis (L), respectively. The expression levels of inserted genes decreased depending on the inserted site (polar effect: Refs. 21) as shown by Western blotting on day 3 after infection. Anti-SeV blot was performed to confirm equal infection efficiency of the vectors.

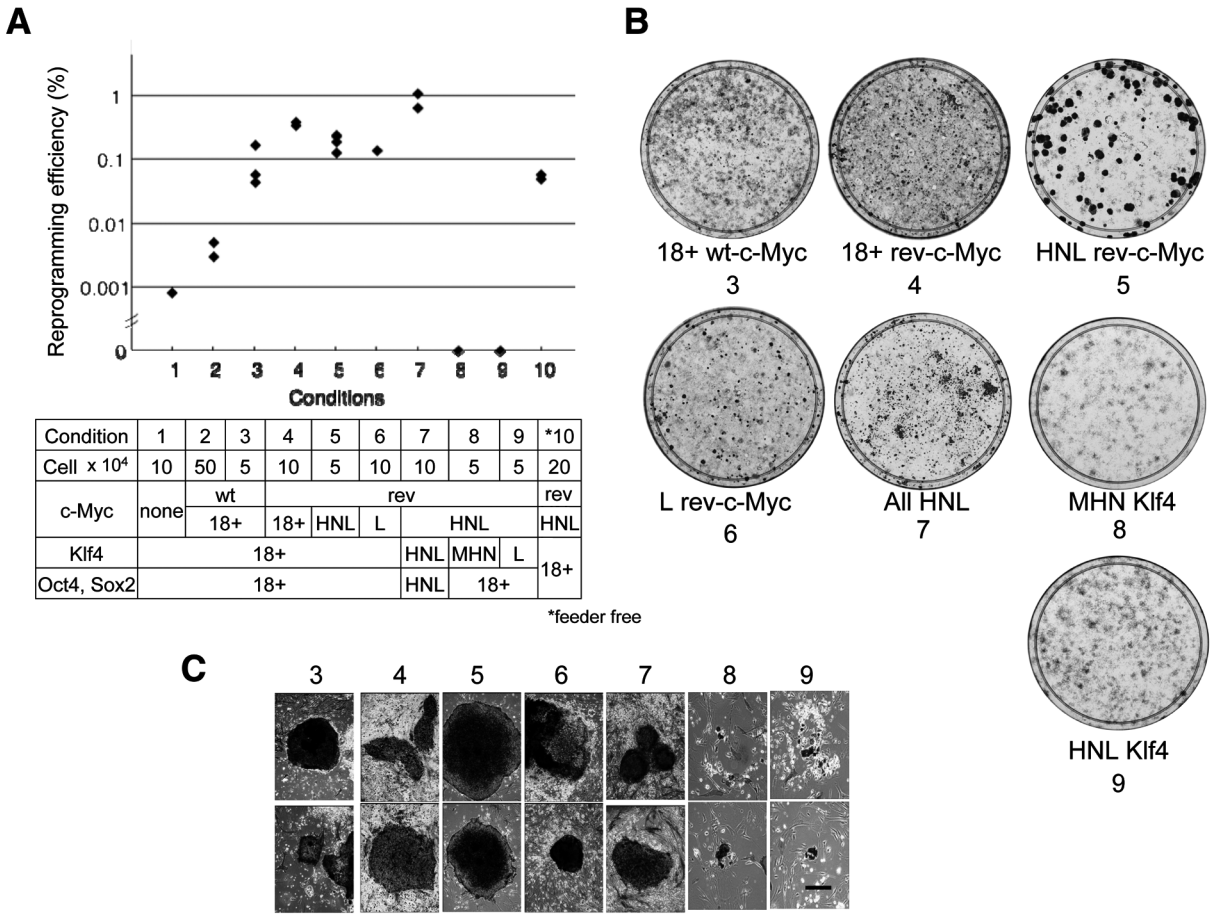
Systems, USA) in RPMI1640 (Invitrogen, USA) supplemented with 2% FBS for 4 days, and followed by additional 8 days culture in DMEM/F12 supplemented with N2 and B27, non-essential amino acids, β-mercaptoethanol, 0.5 mg/ml bovine serum albumin, L-glutamine and penicillin/streptomycin.[31)]

**Teratoma formation by human iPSCs.** Human iPSCs grown on MEF feeder layers were collected by collagenase IV treatment and injected subcutaneously into SCID mice. Palpable tumors were observed about one month after injection. Tumor samples were collected typically in two months, fixed in 10% formalin, and processed for paraffin embedding and hematoxylin-eosin staining following standard procedures.

**Whole-genome expression analysis.** For transcriptional analysis, total RNA was isolated from cells cultured in 6-well dishes using RNeasy Mini Kit. Cyanine labeled antisense RNA were amplified using Quick Amp Labeling Kit from Agilent, hybridized with Gene Expression Hybridization Kit on Whole Human Genome Oligo Microarray (one color, $4 \times 44$K, Agilent, USA) and analyzed by Agilent Microarray Scanner. Data were analyzed by using GeneSpring GX10.0 software (Agilent, USA). Two normalization procedures were applied; first, signal intensities less than 1 were set to 1. Then each chip was normalized to the 50th percentile of the measurements taken from that chip. Each gene was normalized to the median of that gene in the respective controls to enable comparison of relative changes in gene expression levels between different conditions. The microarray data of hES H9 cells[32)] and hiPSCs[5)] were retrived from GEO DataSets (GSM194390 and GSE9561, respectively). These analyses were performed at Bio Matrix Research, Inc.

**Detection of telomerase activity.** Telomerase activity was detected with a TRAPEZE telomerase detection kit (Chemicon, USA). The samples were separated by TBE-based 10% acrylamide nondenaturing gel electrophoresis. The gel was stained with ethidium bromide.

**Bisulfite genomic sequencing.** Genomic DNA (1 μg) from BJ, HDF and iPSCs were treated with sodium bisulfite using the BisulFast DNA Modification Kit (Toyobo, Japan) according to the manufacturer's instruction. The promoter regions of Oct4 and Nanog were amplified by PCR using primer sets




| Condition | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | *10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Cell x $10^4$ | 10 | 50 | 5 | 10 | 5 | 10 | 10 | 5 | 5 | 20 |
| c-Myc | none | wt | | rev | | | | | | rev |
| | | 18+ | | 18+ | HNL | L | HNL | | | HNL |
| Klf4 | 18+ | | | | | | HNL | MHN | L | 18+ |
| Oct4, Sox2 | 18+ | | | | | | HNL | 18+ | | |

*feeder free

Fig. 2. Efficient generation of human iPSCs by non-integrating SeV vectors. **A.** The reprogramming efficiency with SeV vectors. iPS colonies were determined by ALP positive and ES-like morphology. Lane numbers correlate with the conditions listed in the column under the figure. Each dot represents one experiment. The bars represent the average efficiency for each condition. **B.** ALP-staining of growing cells on 100 mm dishes. **C.** Typical ALP-positive colonies (scale bar: 100 μm). Numbering of **C** and **B** are correlated with the numbers in the column in **A**. Colonies in conditions 1,2 and feeder free were similar to those of 3 (data not shown).

previously described.[5),9)] The resultant PCR products were cloned into pGEM-Teasy vector (Promega, USA) and sequenced.

**RNA isolation, RT-PCR, and real-time quantitative PCR analysis.** Total RNA was isolated using ISOGEN (Nippon Gene, Japan) and cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, USA). Real-time PCR was performed with the ABI Prism 7700 sequence detection system (Applied Biosystems, USA) and SYBR™ Green PCR Master Mix (Applied Biosystems, USA). RT-PCR was performed with cDNAs using gene-specific primers. Primer sequences are listed in Table 2.

**DNA fingerprinting.** Genomic DNA was isolated from parental fibroblasts, SeV vector-induced iPS clones using the DNeasy kit (Qiagen, Germany). Three variable number of tandem repeats (VNTR) loci, MCT118, D17S1290 and 3'ApoB were amplified by PCR and analysed by 3% agarose gel electrophoresis.

**Southern blot analysis.** 10 μg of genomic DNA was digested with Afl II and Bam HI, or Dra I and Nco I, separated on a 1% agarose gel and blotted onto Hybond N+ membrane (Amersham Biosciences, GE healthcare, USA). Dig-labeled probes were prepared with DIG-High Prime (Roche, Swiss) using full-length PCR products of Oct3/4, Sox2, Klf4 and c-Myc genes, respectively. The membranes were hybridized with the DIG-labeled probes and then incu-

Table 1. Characterization of established clones

| clone | source | c-Myc | marker expression | | | | epigenetics | | pluripotency | | | | | SeV(-) established at |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | RT-PCR | IH | WB | telomerase | bisulte | microarray | EB | teratoma | cardiomyocytes | PA6 | pancreas | |
| XH1 | HDF | 18+ | ✓ | | | | ✓ | | ✓ | ✓ | | | | P(30) |
| 7H5 | | | ✓ | ✓ | ✓ | ✓ | ✓ | | ✓ | −** | −** | −** | | *(P20) |
| 7H2 | | | ✓ | | | | | | ✓ | | | | | |
| 7H8 | | | ✓ | | | | | | ✓ | | | | | |
| 7H9 | | | ✓ | | | | | | ✓ | | | | | |
| 7H10 | | | ✓ | | | | | | ✓ | | | | | |
| 4BJ1 | BJ | | ✓ | ✓ | | | | | ✓ | − | − | − | | ** |
| B1 | | | ✓ | ✓ | ✓ | ✓ | | | ✓ | ± | − | | | ** |
| 5B1 | | | ✓ | | | | | | ✓ | | | | | |
| 7B6 | | | ✓ | | | | | | ✓ | | | | | |
| 7B1 | | ✓ | | ✓ | | | | ✓ | | | | | | |
| HNLs | | HNL | ✓ | ✓ | ✓ | ✓ | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | P17 |
| HNL1 | | | ✓ | | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | P5 |
| HNL2 | | | ✓ | | | | | | ✓ | | | | | |
| HNL4 | | | ✓ | | | | | | ✓ | | | | | |
| HNL5 | | | ✓ | | | | | | ✓ | | − | | | ** |
| HNL6 | | | ✓ | | | | | | ✓ | | | | | |
| HNLp | | | ✓ | | ✓ | | | | ✓ | | | | | *P20 |
| T10 | | | ✓ | | | | | | ✓ | | | | | |
| T11 | | | ✓ | | | | | | ✓ | | | | | |

P: passage number; PA6: doparmigenic neuron on PA6
*anti-HN-negative selection; **Tg/SeV-remained clones (P15-20)
✓: positive; −: negative; ±: no endoderm

bated with the anti-DIG antibody conjugated with alkaline phosphatase conjugate. Signals were visualized with CDP-Star chemiluminescent substrate (Roche, Swiss) and the resulted chemiluminescence was detected using the LAS-1000 (FUJIFILM, Japan).

**Karyotyping.** Cell division was blocked in mitotic metaphase using colcemid-spindle formation inhibitor (karyoMax colcemid solution, Gibco Invitrogen, USA). Nuclear membranes were broken after hypotonic treatment. For the chromosome visualization we used G-band standard staining (Giemsa, Wako, Japan).

## Results

**Expression of exogenous genes in human fibroblasts by SeV vectors.** To test the efficiency of the expression of exogenous genes by SeV vectors in human fibroblast cells, BJ from neonatal foreskin, and HDF from adult facial dermis, were infected with green fluorescent protein (GFP)-carrying non-transmissible ΔF/TS-SeV vector.[26),27)] Almost all the infected cells expressed GFP at low multiplicities of infection (MOI, number of viral particles per cell) of three (Fig. 1A).

We next attempted to test the efficiency of SeV vectors to transduce reprogramming factors for iPSC induction. We cloned the cDNAs for reprogramming factors, Oct3/4, Sox2, Klf4, and c-Myc[1)] into ΔF/TS-SeV vectors at different sites, designated 18+, HMN, PM, MHN and L, to control the expression level (polar effect: 21) (Fig. 1B). Since the abundance of AT-rich region decreased the expression of target

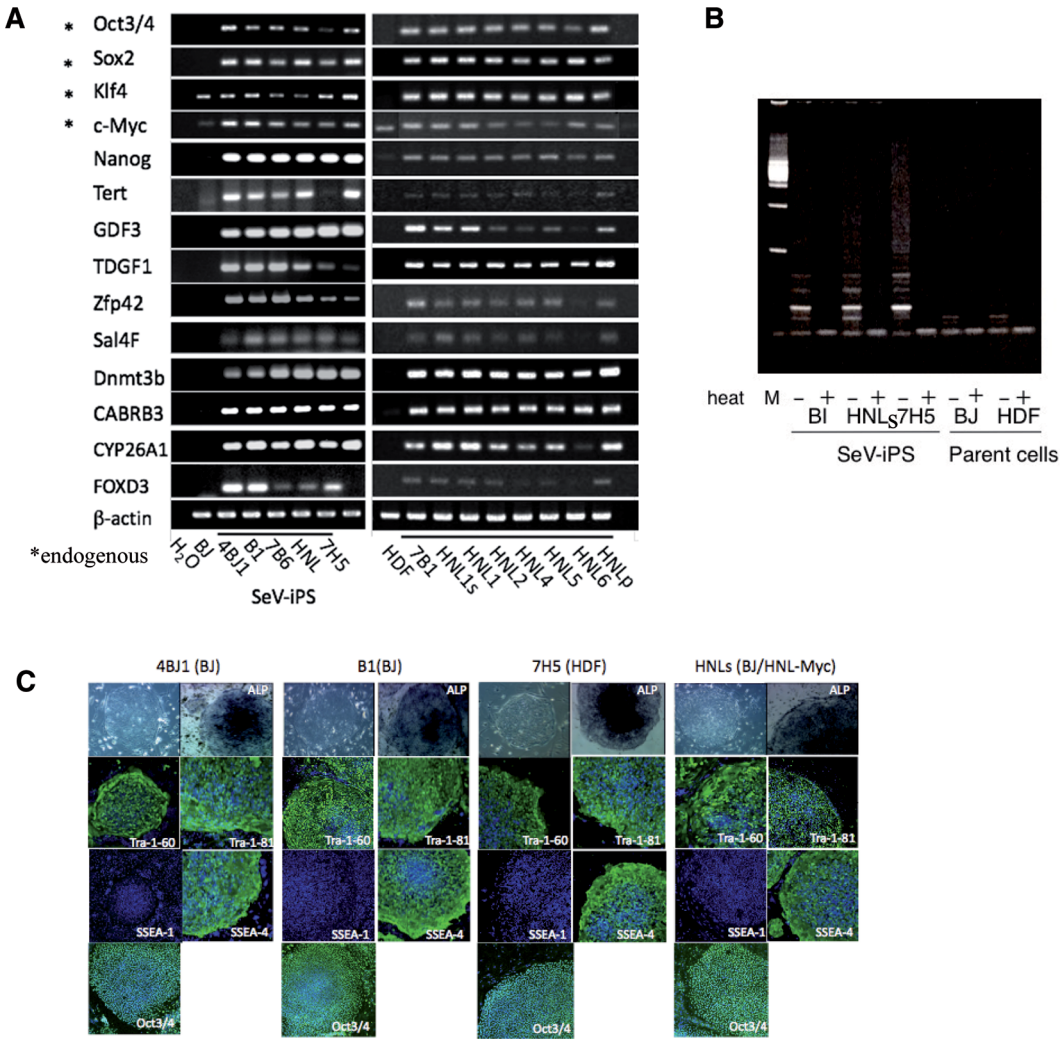



Fig. 3. Expression of hES markers and telomerase activity in SeV-iPSC. **A.** RT-PCR anaysis of human ES cell-marker genes. Primers used for Oct3/4, Sox2, Klf4 and c-Myc were designed to detect the expressions of endogenous genes, but not of transgenes. Cont: PCR without cDNA. **B.** Telomerase activity of human SeV-iPSC. Telomerase activity was detected by the TRAP method.[5] Heat-inactivated samples (+) were used as negative controls. **C.** Immunofluorescence staining of established clones with human ES cell-markers (Tra-1-60, Tra-1-81, SSEA-4 and Nanog). SeV-iPS colonies were positive for ALP and negative for SSEA-1 as in hES cells. Nuclei were stained with TO-PRO3 (blue).

genes in SeV (Stuttering sequence: 23), silent mutations of the codons without replacement of amino acids were also prepared for wt c-Myc (designated rev-c-Myc). Expressions of these genes in human fibroblast cells by these SeV vectors were confirmed by Western blotting (Fig. 1B). Expressions of the inserted genes decreased depending on the location of insertions: insertion at 18+ showed maximum expression while insertion at L lead to minimum expression. Anti-SeV blot showed the equivalent level of infection of each SeV vector (Fig. 1B).

**Optimization of SeV transduction for generating human iPSCs.** Above results suggest that SeV vector that allows sustained expression of transgenes in a relatively controlled manner would be suitable for introducing reprogramming factors for iPSC formation. We next determined optimum conditions for applying SeV vectors to induce iPSC. Induction of human iPSC was carried out in a similar mannner described previously.[5] In brief, $1 \times 10^6$ cells of BJ or HDF on 100 mm dish were infected with Oct3/4, Sox2, Klf4 and/or c-Myc by a series of SeV

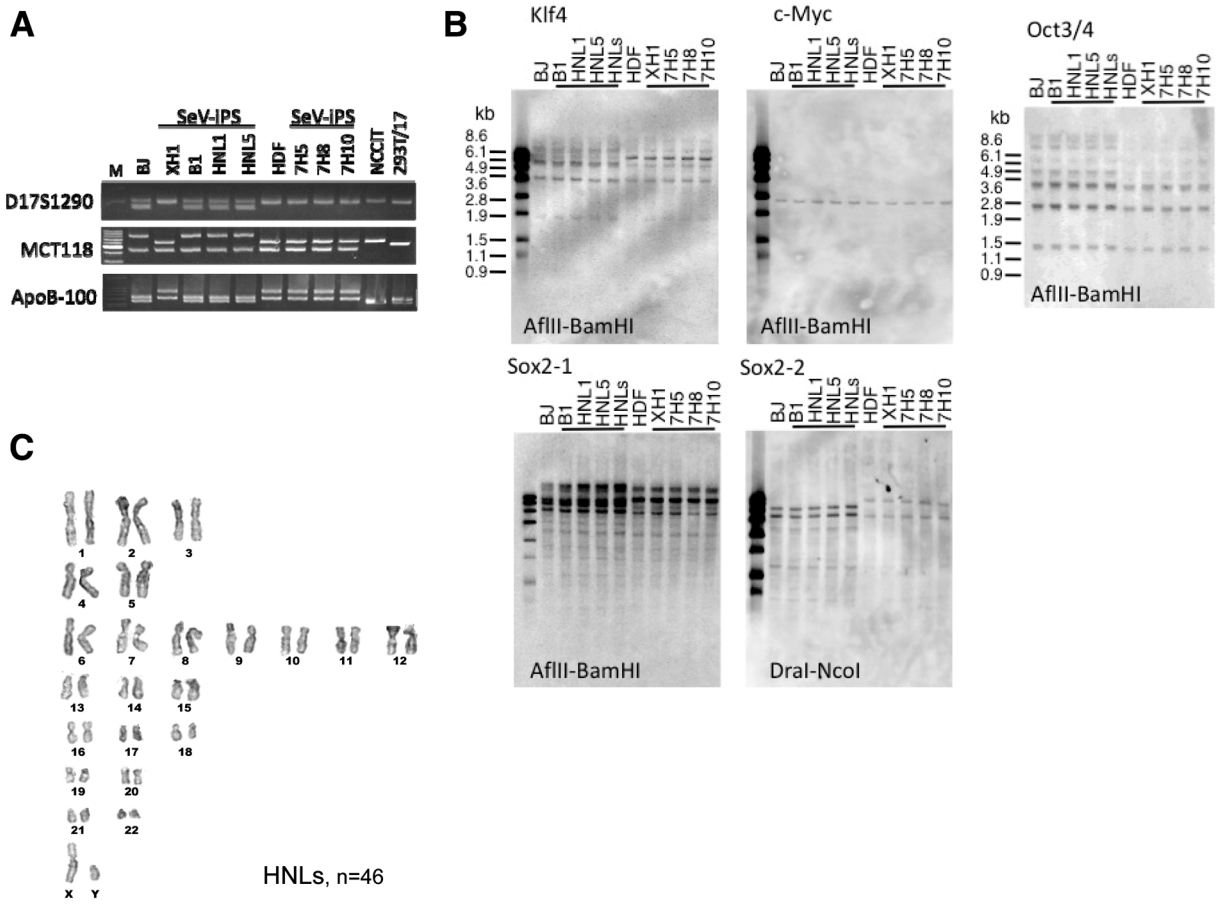



Fig. 4. Genomic Southern blot, karyotyping and fingerprinting of SeV-iPSC. **A.** DNA fingerprinting of SeV-iPS clones. PCR analysis of three variable number of tandem repeats (VNTR) loci of D17S1290, MCT118 and ApoB-100 using genomic DNA from the SeV-iPS clones confirmed that these clones were originated from human fibroblasts BJ or HDF. B1, HNL1 and HNL5 were derived from BJ; XH1, 7H5, 7H8 and 7H10 were from HDF. **B.** Viral transgenes were not detected from the host genome as analyzed by genomic Southern blot. **C.** Karyotyping of SeV-iPSC. Viral-free SeV-iPSC HNLs at passage 34 were used for karyotyping.

vectors at an MOI of 3. One week after infection, cells were collected and re-plated on mitomycin C-treated MEF feeder cells. The next day, medium was changed to primate ES medium supplemented with 10 ng/ml bFGF. The conditions to generate SeV-iPSC performed were summarized in the column in Fig. 2A. Reprogramming efficiency was calculated as the number of alkaline phosphatase (ALP)-positive, ES-like colonies formed per number of infected cells seeded (Fig. 2B). Typical colonies were shown in Fig. 2C. Reduction in numbers of seeded cells on MEF improved the induction efficiency (Fig. 2A, lanes 2, 3). AT-revised c-Myc also enhanced the efficiency depending on the expression level of c-Myc (rev-c-Myc: lanes 4–6). All genes inserted at HNL showed the maximum efficiency, around 1% (lane 7). The efficiency of reprogramming decreased approximately 100 fold when c-Myc was absent (lane 1), comparable to the result of retroviral induction without c-Myc.[33] When Klf4, but not Oct3/4 and Sox2, were moved to the other sites than 18+ or HNL, no colonies were observed (lanes 8, 9), suggesting that the balance of expression among reprogramming factors Oct3/4, Sox2 and Klf4 is a critical factor in efficient induction of reprogramming.

We were also able to induce iPSCs by SeV vectors without feeder cells: (Fig. 2A, lane 10). Taken together, the efficiency of iPSC induction by SeV vectors with c-Myc significantly surpassed those of retroviral transduction (0.02%: Ref. 5).

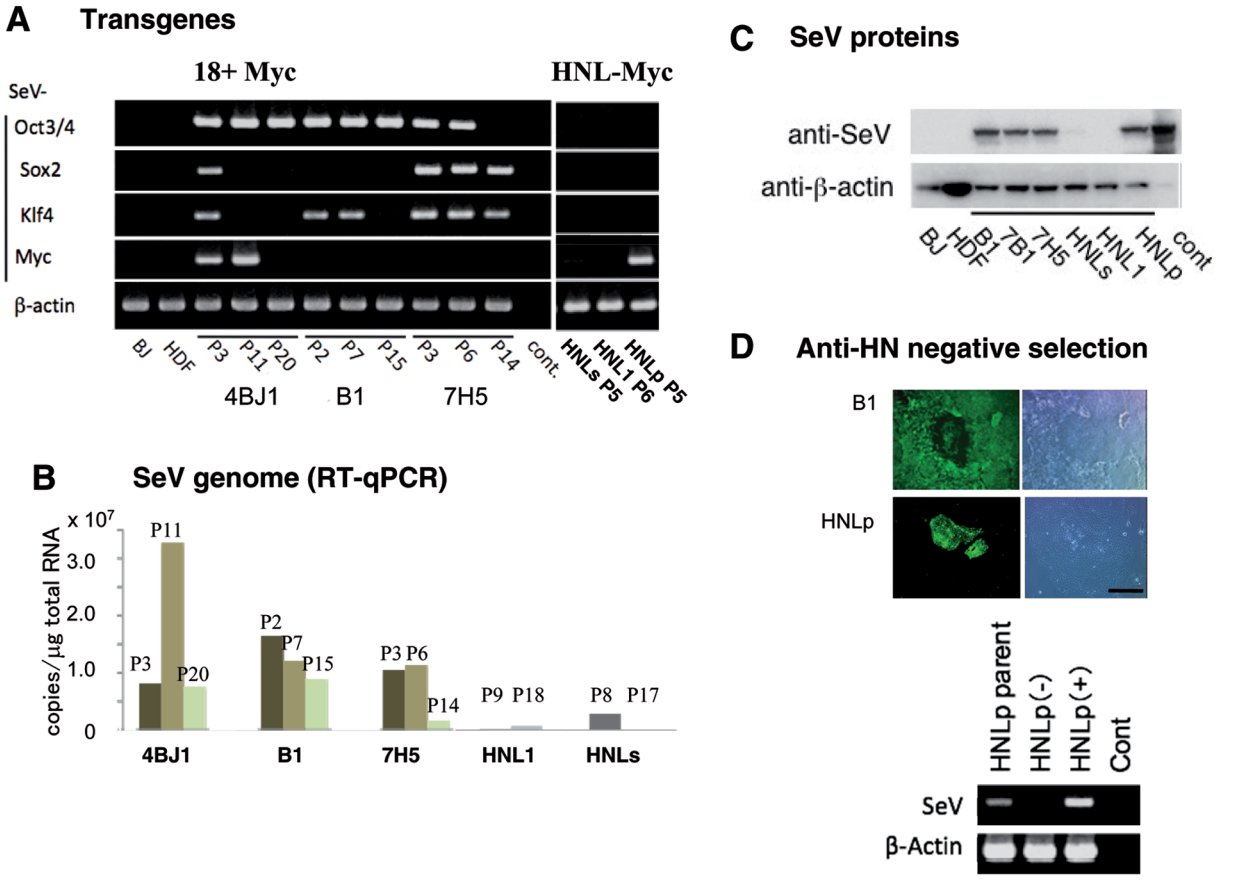



Fig. 5. SeV vectors were diluted and lost during cell growth. **A.** Kinetics of transgene expression determined by RT-PCR using combination of specific primers for SeV and transgenes. cont: no template. **B.** Kinetics of SeV genome expression during cell growth by real time quantitative PCR. Pn means passage numbers. **C.** SeV-derived protein expression determined by Western blotting with anti-SeV polyclonal antibody. Passage numbers are correlated with A. cont: positive control from SeV-infected LLC-MK2 cells. Viral proteins in HNLs were slightly existed at this time (P8), but those of HNL1 were completely lost later at P17 as well as HNL1 at P9 (**B**). **D.** Anti-SeV-immunostaining revealed that SeV distribution was heterologous in iPS colonies (Upper). SeV could be removed by anti-HN-antibody mediated negative selection using anti-mouse IgG1-conjugated IMag-beads. Anti-HN antibody separated SeV-negative population (−) and SeV-enriched population (+).

**Human iPSCs generated by SeV vectors (SeV-iPSCs) express hES Markers.** In order to determine how many ES-like colonies fulfill the more stringent criteria for iPSCs,[34] we randomly picked up ES-like colonies and established 20 clones and the results are as summarized in Table 1. To our surprise, ES-like morphology was sufficient to select SeV-generated iPSCs that fulfill typical phenotype of human ES cells. All clones expressed pluripotent markers,[34] including endogenous Oct3/4, Sox2, Nanog, GDF3, TDGF1, Zfp42, Sal4F, Dnmt3b, CABRB3, CYP26A1, FOXD3, and telomerase reverse transcriptase (hTERT), at the comparable level to the expression by human embryonic carcinoma cell line, NCCIT (Fig. 3A). SeV-iPSC also expressed surface markers such as SSEA4 and TRA-1-60 and -81 (Fig. 3C), high telomerase activity (Fig. 3B) and proliferated more than six months and over 30 passages (Table 1). DNA fingerprinting analysis[8] confirmed their fibroblast origin (Fig. 4A). Taken together, SeV-vector system enables a highly efficient method to generate *bona fide* iPSC.

**SeV vectors were diluted and disappeared during cell growth.** SeV vectors used here usually replicated constitutively in the cytoplasm of infected cells.[26] Thus we next analyzed the level of vector RNA in iPSCs by RT-PCR using SeV-specific primers (Table 2).

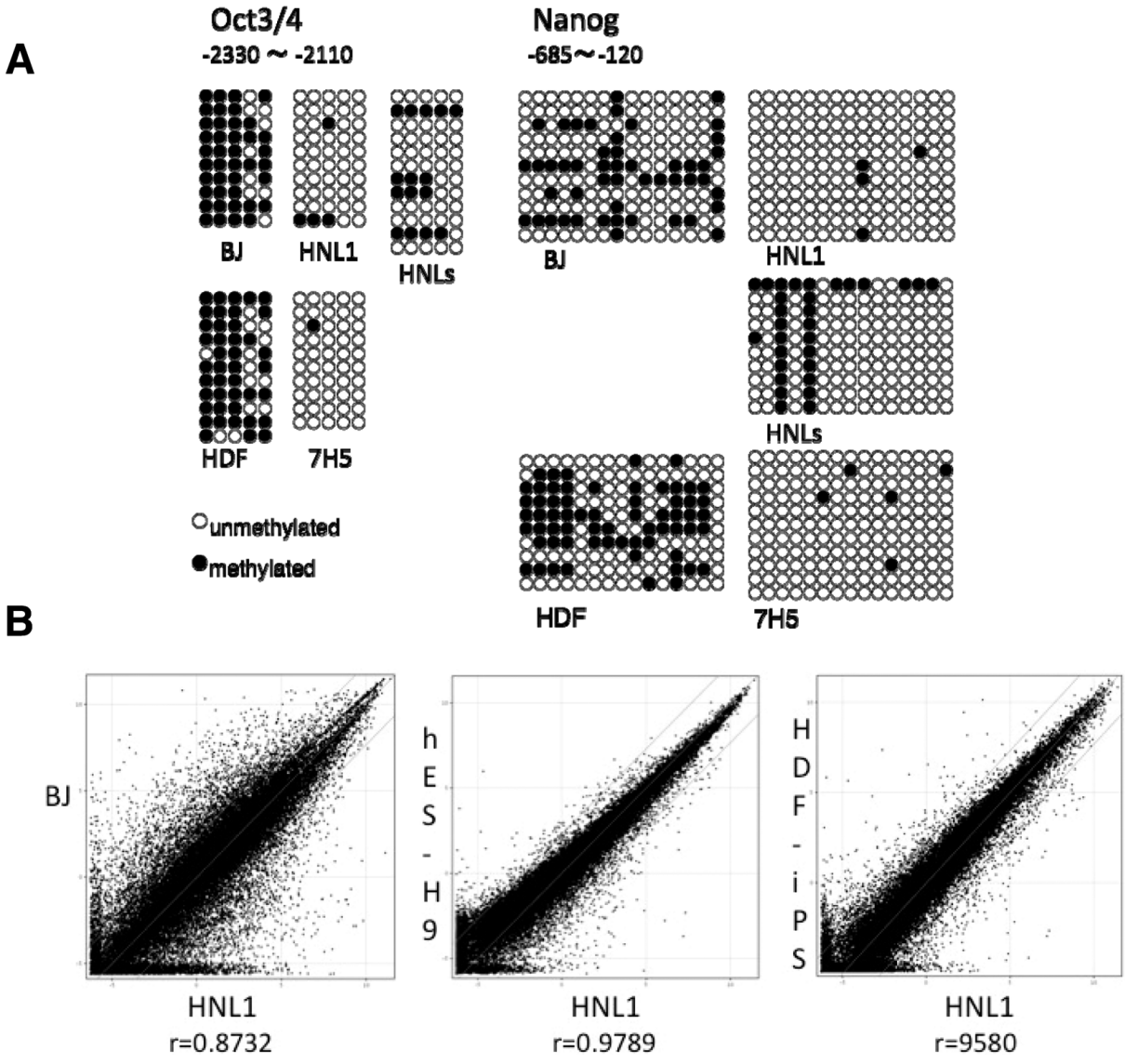



Fig. 6. DNA methylation and global gene expression profiles of SeV-iPSC. **A.** Methylation analysis of Oct3/4 and Nanog promoter regions in SeV-iPSC. **B.** The global gene-expression patterns were compared between SeV-iPSC (HNL1) and BJ, human ES cells (H9)[32] and HDF-iPSC[5] with microarrays. The lines indicate the diagonal and 5-fold changes between two samples.

Transgenes were expressed as early as three days after transduction (Fig. 1), and continued to be present after iPSCs were established (Fig. 5). However, transgenes and vectors tend to decrease over time. Fig. 5A showed our analyses on three clones that were generated with c-Myc inserted at 18+ site. Transgenes were lost at variable extent among these clones. In 4BJ1 and B1 (BJ-iPSC), Oct3/4 transgene persisted over 20 and 15 passages, whereas, in 7H5 (HDF-iPSC), Sox2 and Klf4 persisted longer. Among clones derived form vectors with c-Myc insertion at HNL, c-myc transgene usually persisted longer than other transgenes (Fig. 5A, right panel). This may be due to the difference of vector replication and growth advantage of c-Myc expressing cells. However, even from those clones derived from HNL-myc vectors, we could obtain two clones that lost all viral genomes (HNLs, HNL1).

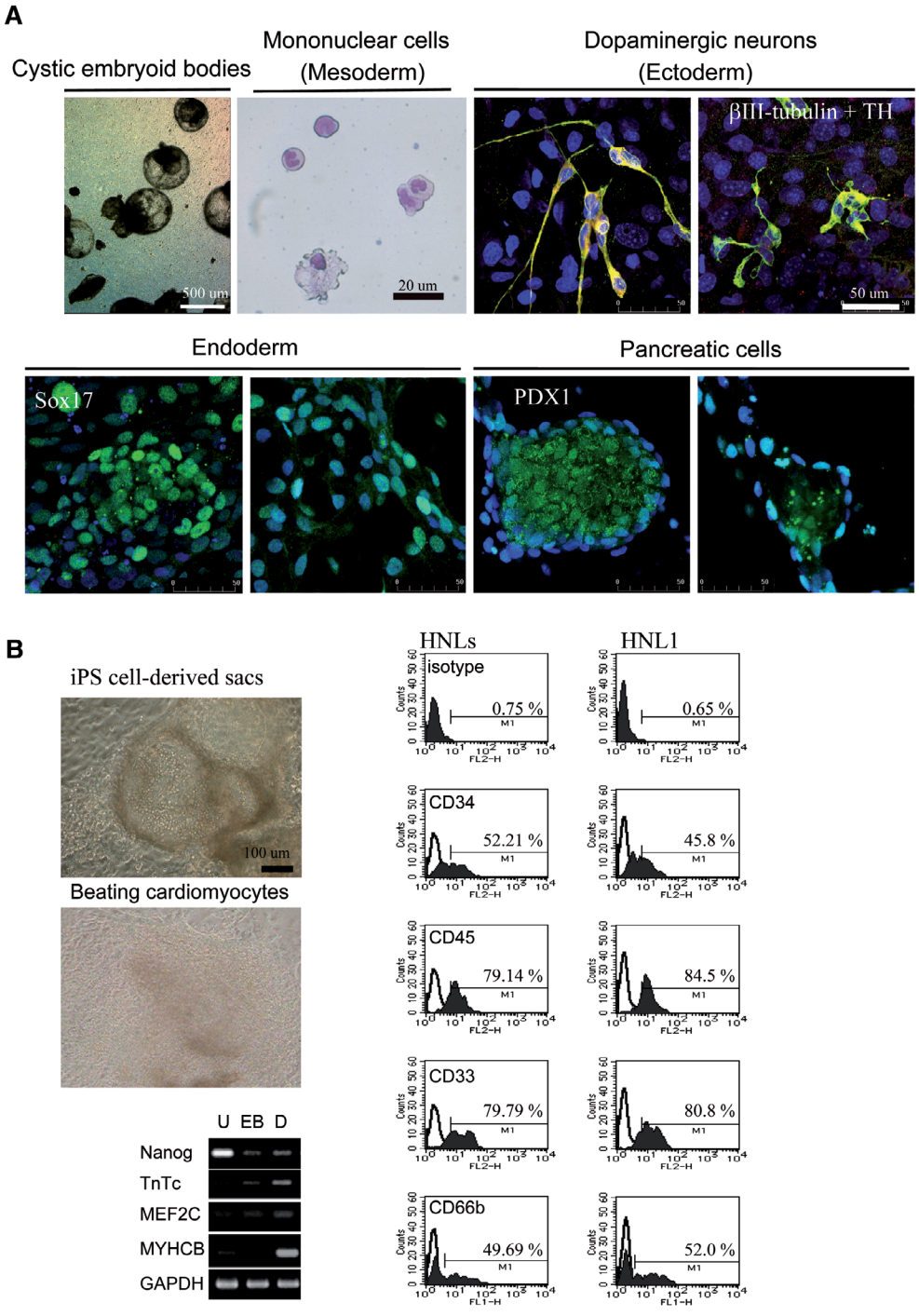



Fig. 7. *In vitro* differentiation of transgenes-free SeV-iPSC. **A.** *In vitro* differentiation of mononuclear cells (mesoderm: shown with Wright-Giemsa staining) via embryoid bodies,[29] putative dopaminergic neurons co-expressing tyrosine hydroxylase (TH)(ectoderm),[30] definitive endoderm (Sox17), and pancreatic cells (PDX1).[31] **B.** *In vitro* differentiation of cardiomyocytes and mononuclear cells from SeV-iPSC via embryo bodies using cytokine cocktails (SCF, Flt3L, TPO, G-CSF, IGF-2, and VEGF).[29] In the adherent culture, hematopoietic sac-like structures filled with mononuclear cells (left, upper) and beating colonies (left, middle) emerged.[29] FACS analysis shows differentiation of SeV-iPSC to mononuclear cells (neutrophil, monocyte, and macrophage), expressing CD34, CD45, CD33, and neutrophil-specific marker CD66b (right). RT-PCR analysis shows that pluripotent marker (Nanog) was decreased and various cardiomyocytes-specific differentiation markers (TnTc, MEF2C, MYHCB) were increased after differentiation (left, lower). U: undifferentiated; EB: embryoid body; D: differentiated.

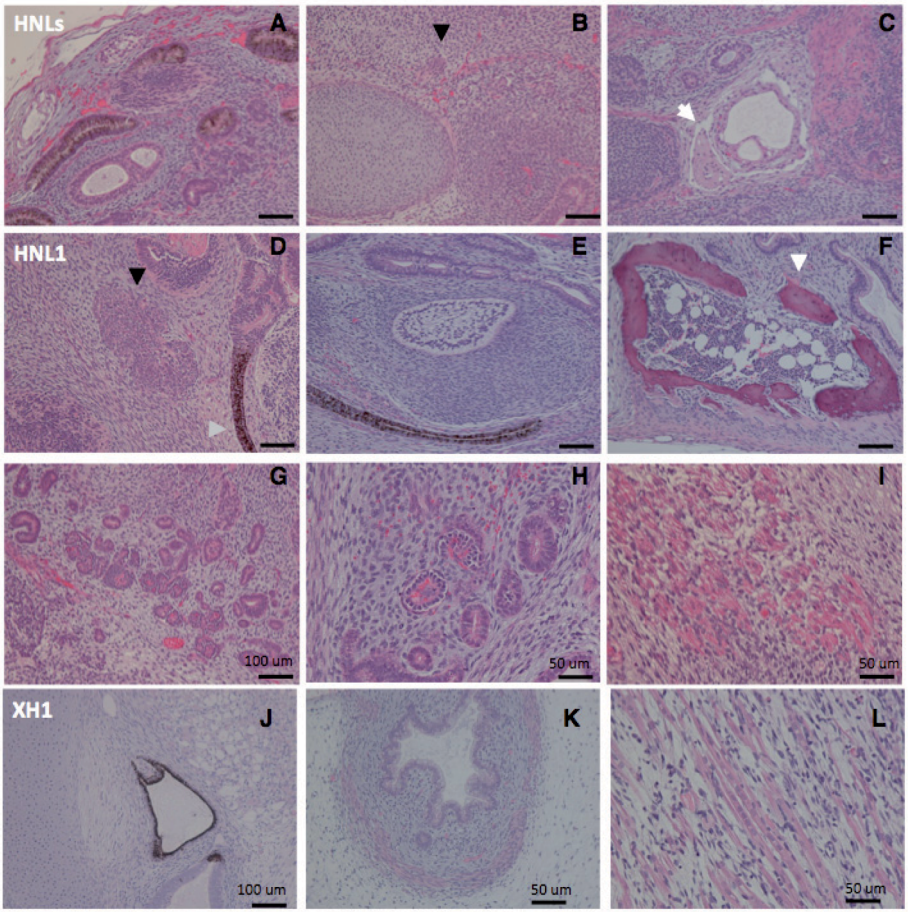



Fig. 8. *In vivo* pluripotency of transgenes-free SeV-iPSC. Hematoxylin and eosin staining of teratoma sections of SeV-iPS clones (6 weeks post-injection into SCID mice). Tissues were differentiated from Tg-free human neonatal fibroblast BJ-derived HNLs (A to C), HNL1 (D to I), and adult fibroblast HDF-derived XH1 (J to L) containing multiple tissues derived from three germ layers: glandular structures (A, G, K), cartilage (B, J), bone (C, F, white arrows) and bone marrow-like structure (F), epithelium (J, D), transitional epithelium (E), population of secreting-like cells (B, D, indicated by black arrows), muscle (C, I, K, L), and glomerulus of kidney-like tissue (H).

Table 2. List of primer sequences for PCR

| For cDNA cloning | | |
|---|---|---|
| Gene | 5′ | 3′ |
| *Oct3/4* | CACCATGCTTGGGGCGCCTTCCTTCC | CATCGGAGTTGCTCTCCACCCCGAC |
| | CCCGCCGTATGAGTTCTGTGG | GCCGCGGCCGCGTTATCAGTTTGAATGCATGGGAGAGCCCAG |
| | GCCGCGGCCGCACCATGGCGGGACACCTGGCTTC | GCCGCGGCCGCGTTATCAGTTTGAATGCATGGGAGAGCCCAG |
| *Sox2* | CAAAGTCCCGGCCGGGCCGAGGGTCGG | CCCTCCAGTTCGCTGTCCGGCCC |
| | GATGTACAACATGATGGAGACGGAGC | GTCACATGTGTGAGAGGGGCAGTG |
| *Klf4* | CCACATTAATGAGGCAGCCACCTGGC | GCAGTGTGGGTCATATCCACTGTCTG |
| | GATGGCTGTCAGCGACGCGCTGCTCCC | GTTAAAAATGCCTCTTCATGTGTAAGGCGAG |
| *c-Myc* | AACCAGCAGCCTCCCGCGACG | AGGACATTTCTGTTAGAAGGAATCG |
| | GATGCCCCTCAACGTTAGCTTCACC | GTTACGCACAAGAGTTCCGTAGCTG |

Table 2. Continued

| For plasmid constraction of SeV vector | | |
|---|---|---|
| Notl-*Klf*-4F(5′) | ATTGCGGCCGCGACATGGCTGTCAGCGACGCGCTG | |
| Notl-*Klf*-4R(3′) | ATTGCGGCCGCGATGAACTTTCACCCTAAGTTTTTCTTACTACGGTTAAAAATGCCTCTTCATGTGTAAGGCGAGGTGGTC | |
| Notl-*c-Myc* F(5′) | ATTGCGGCCGCATGCCCCTCAACGTTAGCTTCAC | |
| Notl-*c-Myc* R(3′) | ATTGCGGCCGCGATGAACTTTCACCCTAAGTTTTTCTTACTACGGTTACGCACAAGAGTTCCGTAGCTGTTCAAGTTTGTGTTTC | |
| Notl-*Sox2* F(5′) | ATTGCGGCCGCATGTACAACATGATGGAGACG | |
| Notl-*Sox2* R(3′) | ATTGCGGCCGCGATGAACTTTCACCCTAAGTTTTTCTTACTACGGTCACATGTGTGAGAGGGGCAGTGTGCCGTTAATGGCCGTG | |
| Notl-*Oct3/4* F(5′) | ATTGCGGCCGCCCCATGGCGGGACACCTGGCTTC | |
| Notl-*Oct3/4* R(3′) | ATTGCGGCCGCGATGAACTTTCACCCTAAGTTTTTCTTACTACGGTCAAAGCGGCAGATGGTCGTTTGGCTGAACACCTTC | |
| For RT-PCR | | |
| Gene | 5′ | 3′ |
| *Oct3/4* (SeV-Tg) | CCCGAAAGAGAAAGCGAACCAG (Oct3/4) | AATGTATCGAAGGTGCTCAA (SeV) |
| *Sox2* (SeV-Tg) | ACAAGAGAAAAAAACATGTATGG (SeV) | ATGCGCTGGTTCACGCCCGCGCCCAGG (Sox2) |
| *Klf4* (SeV-Tg) | ACAAGAGAAAAAAACATGTATGG (SeV) | CGCGCTGGCAGGGCCGCTGCTCGAC (Klf4) |
| *c-Myc* (SeV-Tg 18+) | ACAAGAGAAAAAAACATGTATGG (SeV) | TCCACATACAGTCCTGGATGATGATG (c-Myc) |
| *c-Myc* (SeV-Tg HNL) | TAACTGACTAGCAGGCTTGTCG (SeV) | TCCACATACAGTCCTGGATGATGATG (c-Myc) |
| *Oct3/4* (3′UTR:endo) | AGTTTGTGCCAGGGTTTTTG | ACTTCACCTTCCCTCCAACC |
| *Sox2* (3′UTR:endo) | GGGAAATGGGAGGGGTGCAAAAGAGG | TTGCGTGAGTGTGGATGGGATTGGTG |
| *Klf4* (3′UTR:endo) | GACAGTGGATATGACCCACACTGCC | GATAGAAGATCCAGTCACAGACC |
| *c-Myc* (3′UTR:endo) | ATGTCCTGAGCAATCACCTATG | AAGTTCTTTTATGCCCAAAGTCC |
| *Nanog* | TACCTCAGCCTCCAGCAGAT | TGCGTCACACCATTGCTATT |
| *Tert* | TGCCCGGACCTCCATCAGAGCCAG | TCAGTCCAGGATGGTCTTGAAGTCTG |
| *TDGF1* | ATGGACTGCAGGAAGATGGCCCGC | TTAATAGTAGCTTTGTATAGAAAGGC |
| *Zfp42* | TTGGAGTGCAATGGTGTGAT | TCTGTTCACACAGGCTCCAG |
| *Sal4F* | AAACCCCAGCACATCAACTC | GTCATTCCCTGGGTGGTTC |
| *Dnmt3b* | GCAGCGACCAGTCCTCCGACT | AACGTGGGGAAGGCCTGTGC |
| *CABRB3* | CTTGACAATCGAGTGGCTGA | TCATCCGTGGTGTAGCCATA |
| *CYP26A1* | AACCTGCACGACTCCTCGCACA | AGGATGCGCATGGCGATTCG |
| *FOXD3* | GTGAAGCCGCCTTACTCGTAC | CCGAAGCTCTGCATCATGAG |
| *β-actin* | CAACCGCGAGAAGATGAC | AGGAAGGCTGGAAGAGTG |
| For Bisulfite sequencing | | |
| Gene | 5′ | 3′ |
| *Nanog* | GGAATTTAAGGTGTATGTATTTTTTATTTT | AACCCACCCTTATAAATTCTCAATTA |
| *Oct3/4* (1) | AATAGATTTTGAAGGGGAGTTTAGG | TTCCTCCTTCCTCTAAAAAACTCA |
| For Fingerprinting | | |
| Gene | 5′ | 3′ |
| *MCT18* | GAAACTGGCCTCCAAACACTGCCCGCCG | GTCTTGTTGGAGATGCACGTGCCCCTTGC |
| *ApoB-100* | ATGGAAACGGAGAAATTATG | CCTTTCTCACTTGGCAAATAC |
| *D17S1290* | GCCAACAGAGCAAGACTGTC | GGAAACAGTTAAATGGCCAA |
| For DIG-labeling PCR | | |
| Gene | 5′ | 3′ |
| *KLF4* | TGACCCATCCTCCGGAGTCAGTG | GGGGATGGAAGCCGGGAGGAAGCGG |
| *c-Myc* | TGCCACGTCTCCACACATCAGC | GTTACGCACAAGAGTTCCGTAGCTG |
| *Sox2* | GATGTACAACATGATGGAGACGGAGC | GTCACATGTGTGAGAGGGGCAGTG |
| *Oct3/4* | CCCGAAAGAGAAAGCGAACCAG | GTCAGTTTGAATGCATGGGAGAGCCCAGAG |
| For cardiomyocytes | | |
| Gene | 5′ | 3′ |
| *TnTc* | ATGAGCGGGAGAAGGAGCGGCAGAAC | TCAATGGCCAGCACCTTCCTCCTCTC |
| *MEF2C* | TTTAACACCGCCAGCGCTCTTCACCTTG | TCGTGGCGCGTGTGTTGTGGGTATCTCG |
| *MYHCB* | CTGGAGGCCGAGCAGAAGCGCAACG | GTCCGCCCGCTCCTCTGCCTCATCC |
| *GAPDH* | ATCACTGCCACCCAGAAGACT | ACCAGGAAATGAGCTTGACAA |

These clones were free of viral genome and proteins (Fig. 5B, C), carried unaltered host genome (Fig. 4B), and karyotypically normal (Fig. 4C). Moreover, we found that SeV-distributions were heterologous in passaged SeV-iPS colonies (Fig. 5D). Thus, antibody against HN protein, a major protein expressed on the surface of SeV-infected cells,[20] enables us to remove HN positive cells from these heterologous populations (Fig. 5D, lower panels). The established SeV-negative clones tested here were listed in Table 1. From all clones, the virus can be removed by anti-HN-antibody-mediated negative selection theoretically, because SeV vectors were decreased in all clones tested (data not shown).

We selected these viral-free clones, HNL1 and HNLs, for further studies.

**DNA methylation and global gene expression profiles of human SeV-iPSCs.** Reprogramming of methylated sites of genome is a signature of pluripotent cells. Thus, we analyzed the methylation states of CpG dinucleotides in the Oct3/4 and Nanog promoter regions by bisulfite genomic sequencing.[5),9)] The result showed that Oct3/4 and Nanog promoter regions were demethylated in SeV-iPSC contrary to parental fibroblasts (Fig. 6A). We also compared the gene expression profile of our SeV-iPSC with that of human H9 ES cell line and HDF iPSC line that were reported previously.[5)] Our data of HNL1 is markedly similar to that of H9 ES cell lines (Fig. 6B).

**Pluripotency of SeV-iPSCs *in vitro* and *in vivo*.** Differentiation potential of viral-free SeV-iPS clones were analyzed either by *in vitro* embryoid-body culture or *in vivo* teratoma formation according to the methods described previously.[29)–31)] As shown in Fig. 7A and B, we were able to induce representative mature cell lineages derived from all three germ layers; hematopoietic cells and beating cardiomyocytes for mesoderm lineage, Sox17 or PDX1 positive cells for endoderm lineage, and beta III- and tyrosine hydroxylase-positive neuron for ectodermal linage.

We further tested the pluripotency of SeV-iPSC by teratoma formation. Viral-free-iPSCs, HNL1 and HNLs from neonatal BJ and XH1 from adult HDF cells were injected subcutaneously into the flanks of SCID mice. Histological examination of the teratomas revealed the presence of a set of representative tissues that were originated from the three embryonic germ layers, including neural epithelium, muscle, cartilage, bone, and glandular structures (Fig. 8). Remained expression of transgenes seemed to affect pluripotency (Table 1). From the results of *in vitro* and *in vivo* differentiation experiments, viral-free SeV-iPSC showed pluripotency.

## Discussion

SeV based vectors have been used as a safe method for gene therapy,[22)–25)] as SeV per se has no risk of being integrated in the host genome. In this study, we further showed that SeV is an ideal vector for generating human iPSC that fulfills the latest criterion for iPSC. Firstly, as emphasized repeatedly, SeV vector allows expression of transgenes without risk of modification of host genome. Secondly, the efficiency of iPSC generation by gene transduction with SeV vectors is significantly higher than that by other methods especially without any transfection drugs. Finally, it is easy to select iPSC that depleted viral genome from the cytoplasm. Hence, resulting viral-free iPSCs become genetically intact and carries the same genome DNA as the original cells. We emphasize that the method described here has a significant advantage over presently available methods for its safety, efficiency and convenience. Nonetheless, iPS research is entering the new stage by development of methods for epigenetic reprogramming without genetic modification.

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