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Generation of ICM-Type Human iPS Cells from CD34+ Cord Blood Cells

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1. Introduction

One of the major technical hurdles for clinical application of embryonic stem (ES) cells or induced pluripotent stem (iPS) cells is formation of teratomas by undifferentiated cells after transplantation. In addition, iPS cells have their own safety concerns such as an increased chance of tumorigenicity caused by chromosomal instability or alteration during the reprogramming process (1). Since the first report of mouse iPS cell generation by retroviral vectors (2), several non-integrating vector systems have been examined in pursuit of "safer" iPS cell generation methods. These approaches include adenoviruses (3), Sendai viruses (SeV) (4, 5), Cre-excisable viruses (6), the piggyBac transposition system (7, 8) conventional plasmids (9), the oriP/EBNA1-episomal vector (10), direct protein delivery methods (11, 12) or small molecule delivery methods (13, 14).

A number of cell sources for generating human iPS cells have been reported, including dermal fibroblasts (15), keratinocytes (16), peripheral blood cells (17), adipose tissue (18), and cord blood (CB) cells (19). The three germ layer differentiation potential of these established iPS cells has been demonstrated. However, it is not clear which cell source is best for generating "standard" iPS cells, as differentiation preferences of established iPS cells reflect the epigenetic status of the original cells (called "epigenetic memory") (20).

Recently, several groups reported new insights into two distinct stages of pluripotency in ES cells. These stem cell stages consist of the inner cell mass (ICM) of blastocyst type (ICM type-cells or naïve cells), and epiblast type stem cells (EpiSCs or prime cells) (21). Mouse 129 or C57/BL6 mouse ES cells are the ICM type: "true" pluripotent stem cells representing pre-implantation blastocysts that contribute to chimerism and demonstrate germ line transmission when placed back into blastocysts. They can also be grown in single cell suspension. In contrast, the "EpiSCs" or "prime" ES cells represent post-implantation stage epiblasts. They retain the potential of three germ line differentiation *in vitro*, but are incapable of contributing to chimerism and cannot survive after single cell cloning. Human ES cells or iPS cells seem to correspond to the EpiSCs with respect to colony morphology

and gene expression prolife (22, 23), but can be converted to the naïve stem cell stage by cultivation (24) or constitutive activation of *KLF2/KLF4* genes (25). In this report, we demonstrate an easier and safer reprogramming method for the direct establishment of ICM-type human iPS cells from fresh or frozen CB cells using temperature-sensitive SeV vectors, which facilitates confirmation of removal of the SeV construct at a single cell level.

2. Experimental procedures, materials, and methods

All experimental protocols were reviewed and approved by the ethical committee of the Riken Center for Developmental Biology (CDB), the Foundation for Biomedical Research and Innovation (FBRI), Asagiri Hospital, and the animal experiment committee of FBRI.
Fresh CB was supplied by Asagiri Hospital. $CD34^+$ cells were purified from mononuclear cells (isolated from fresh CB with Lymphoprep TM (Cosmo Bio Co., Tokyo, Japan)) using a human CD34 Micro Bead kit and Auto Macs columns (Miltenyi Biotec) in accordance with the manufacturer's instruction. We also used frozen $CD34^+$ CB cells obtained from Riken RBC (Tsukuba, Japan). $CD34^+$ cells were cultured in hematopoietic culture medium (HC media) [serum free X-VIVO 10 (Lonza, Basel Switzerland) containing 50 ng/mL IL-6 (Peprotech, London UK), 50 ng/mL sIL-6R (Peprotech), 50 ng/mL SCF (Peprotech), 10 ng/mL TPO (Peprotech), 20 ng/mL Flt3-ligand (R&D system, MN)] (4) for one day prior to viral infection. SNL76/7 feeder cells (European Collection of Cell Culture, Salisbury, UK) were treated with 100 μL of mitomycin C solution (1 mg/mL) (Nacalai Tesque, Kyoto, Japan) in 10 cm dishes for three hours to generate mitomycin C treated-SNL 76/7 feeder cells (MMC-SNL). They were seeded on 24-well plates (Becton Dickinson, Tokyo, Japan), or in six-well plates, or in 60 mm dishes in naïve human ES cell culture medium. Fifty mL of naïve human ES cell medium was prepared by mixing 24 mL DMEM/F12 (Invitrogen; 11320), 24 mL Neurobasal (Invitrogen; 21103), 0.5 mL of x100 nonessential amino acids (Invitrogen), 1 mL B27 supplement (Invitrogen; 17504044), and 0.5 mL N2 supplement (Invitrogen; 17502048). The medium also contained 0.5 mg/mL of BSA Fraction V (Sigma), penicillin-streptomycin (final x 1, Invitrogen), 1 mM glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), 1.0 μM PD0325901 (Stemgent), 3.0 μM CHIR99021 (Stemgent), 10 μM forskolin (Sigma) and 20 ng/mL of recombinant human LIF (Millipore; LIF1005). Prime human iPS cells were cultured with prime human ES cell medium [DMEM/F-12 (SIGMA) containing 20% KSR (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% NEAA (Invitrogen), 0.1 mM 2-ME (Invitrogen), and 4 ng/mL bFGF (Peprotech)]. The medium was changed every day. Passage of human ES cell-like cells was previously described (26). The split ratio was routinely 1:3 or 1:4.

2.1.1 Viral infection and generation of ICM-type iPS cells

Temperature-sensitive Sendai viral vector constructs integrating the four Yamanaka factors (SeV18+*OCT3/4*/TS7, SeV18+*SOX2*/TS7, SeV18+*KLF4*/TS7, and SeV(HNL)c-MYC/TS7) were supplied by DNAVEC Corp. The $CD34^+$ cells were thawed and cultured for one day in HC media in six-well plates at a density of 2×10^4 cells/two mL/well before the 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 μL of hematopoietic cell culture medium with 20 μL of viral supernatant containing two m.o.i. each of the five SeV constructs (SeVTS7-*OCT3/4, -SOX2, -KLF4, -c-MYC, -GFP*). The medium was replaced by fresh medium the following day and infected cells were cultured another four days. At this point, 1×10^4

infected CB cells were seeded and cultured on confluent MMC-SNL cells in six-well plates in human naïve ES cell medium supplemented with PD0325901, CHIR99021, recombinant human LIF (rhLIF) and forskolin under hypoxic conditions (MCO-5M, SANYO Japan, 5% O_2, 5% CO_2 at 37° C). Dome-shaped naive ES cell like-colonies were picked up between fourteen and nineteen days, suspended as single cells, seeded on MMC-SNL and cultured with naïve ES cell medium. The second passage colonies were subjected to heat treatment (38° C for three days) and then passaged again for detection of remaining SeV constructs by RT-PCR and immunostaining with anti-SeV (HN) antibody. SeV-free colonies were transferred to a normal oxygen environment (MCO-5M, SANYO Japan, 20% O_2, 5% CO_2 at 37° C) and cultured on MMC-SNL cells with prime human ES cell medium shown in Fig 1.

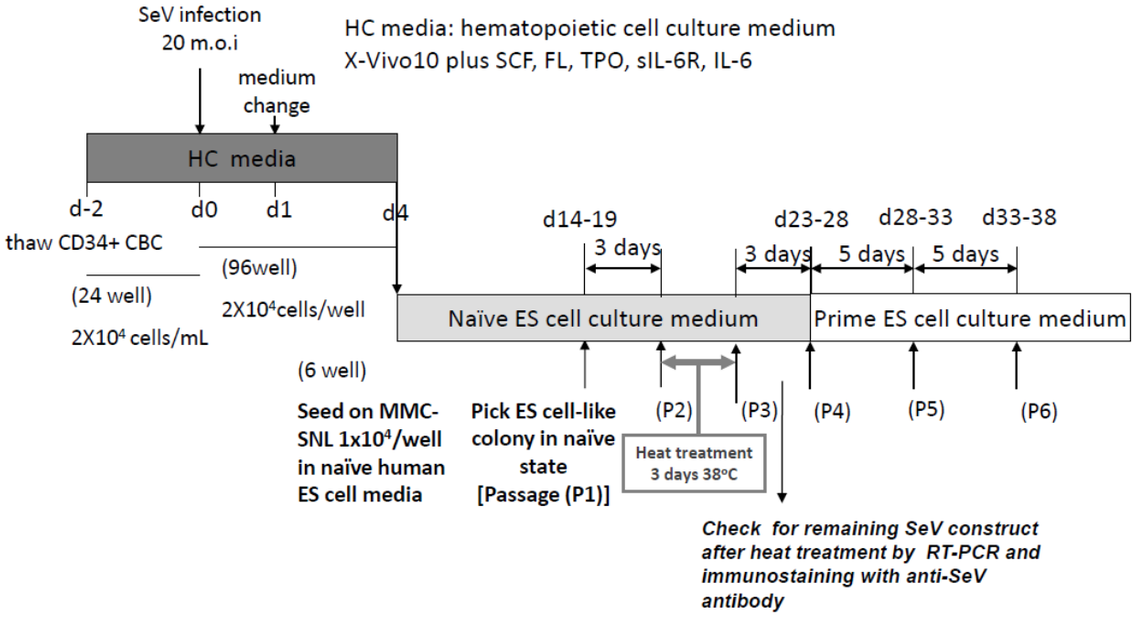

Fig. 1. Schema for generating naive and prime iPS cell from CB cells with SeV vectors.

2.1.2 Optimized culture conditions for naïve iPS cells

1X10⁴ SeV-infected CD34+ CB cells were transferred onto various numbers of pre-seeded MMC-SNL cells in 60 mm dishes (from 1.0 x 10^5 to 2.0 x 10^6). Cells were cultured for 14 days in naïve human ES cell medium either under hypoxic or normoxic conditions. The emergent colonies were fixed and stained for ALP activities. The number of colonies stained positively for ALP activities was scored.

The naïve ES cell-like colonies were picked up 14 to 19 days after seeding on SNL in naïve ES cell medium under 5% O_2 culture conditions. These cells were subjected to heat treatment at 38 °C for three days at passage two in the naive state. After heat treatment, prime ES cell-like colonies were passaged (passage three) and checked for residual SeV constructs by RT-PCR and immunostaining with anti-SeV antibody. Then, the virus-free cell clumps from passage three were cultured in prime human ES cell medium under 20% O_2 culture conditions. Viral-Free (VF) iPS cell colonies were passaged two or three times and then tested for further appraisal of differentiation potential of the reprogrammed cell clones. We tried to induce pluripotency in adherent cells derived from CD34+ cells in Table 1.

Cell source	Vector	Infected cell numbers	Infectivity	Substrate on cuture plate	Numbers of ES-like colony	iPS cell clones characterized
Fresh $CD34^+$ CB	SeV	$1.0x10^4$	20	MMC-SNL cells	5	5

Clone #	RT-PCT (undifferentiation)	RT-PCT (differentiation)	IHC	Teratoma	Karyotype
#24	✓				
#30	✓		✓		
#35	✓	✓	✓	✓	
#36	✓				
#37	✓	✓	✓	✓	✓

✓: performed

Table 1. Efficiency of induction of iPSC clones from cord blood cells with SeV vectors.

2.1.3 Alkaline phosphatase and immunohistological staining

Naive ES cell like- and prime ES cell like-colonies were stained with leukocyte alkaline phosphatase kit (VECTOR, Burlingame, CA) in accordance with the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde followed by immunostaining with a series of antibodies. Nuclei were stained with DAPI (1:1000, SIGMA). Photomicrographs were taken with a fluorescent microscope (Olympus BX51, IX71, Tokyo) and a visible light microscope (Olympus CKX31).

Expression of CD34 and CD45 in mononuclear cells (MNC) from CB was determined by flow cytometry (middle). $CD34^+CD45^{low+}$ cells (0.2%) and $CD34^-$ $CD45^+$ cells were fractionated by cell sorting and both were infected with SeV carrying four factors and GFP. Phase contrast microscopic and fluorescence photographs of $CD34^+$ cells (right) and $CD34^-$ mononuclear cells (left) the day after infection are shown in lower panels.

We found that the GFP^+ population was selectively found in the $CD34^+$ fraction the day after SeV infection (Fig. 2). This fraction corresponds to hematopoietic stem cells or progenitors, as reported elsewhere (27).

2.1.4 Determination of SeV construct in naïve ES cell-like cells

The remaining SeV constructs in naïve ES cell-like colonies were determined by RT-PCR and immunostaining. Using four temperature-sensitive Sendai viral constructs (SeV TS7) integrating Yamanaka's transcription factor quartet (*c-MYC, KLF4, OCT3/4 and SOX2*), we were able to generate ES cell-like colonies from $CD34^+$ $CD45^{low+}$ CB cells. The protocol for generating iPS cells from CB cells with temperature-sensitive SeV vector is shown in Fig. 1. Naïve ES cell-like colonies were generated by culturing cells in naïve human ES cell medium under hypoxic conditions (5% O_2). Merged dome-like colonies were picked up three weeks after SeV infection and subjected to heat treatment at 38^oC to reduce the amount of residual SeV constructs. Remaining SeV constructs were detected by RT-PCR and immunostaining with anti-SeV antibody. Then, the cell clumps of "naïve" virus-free cell clones were transferred to conventional prime human ES cell medium and cultured under normoxic

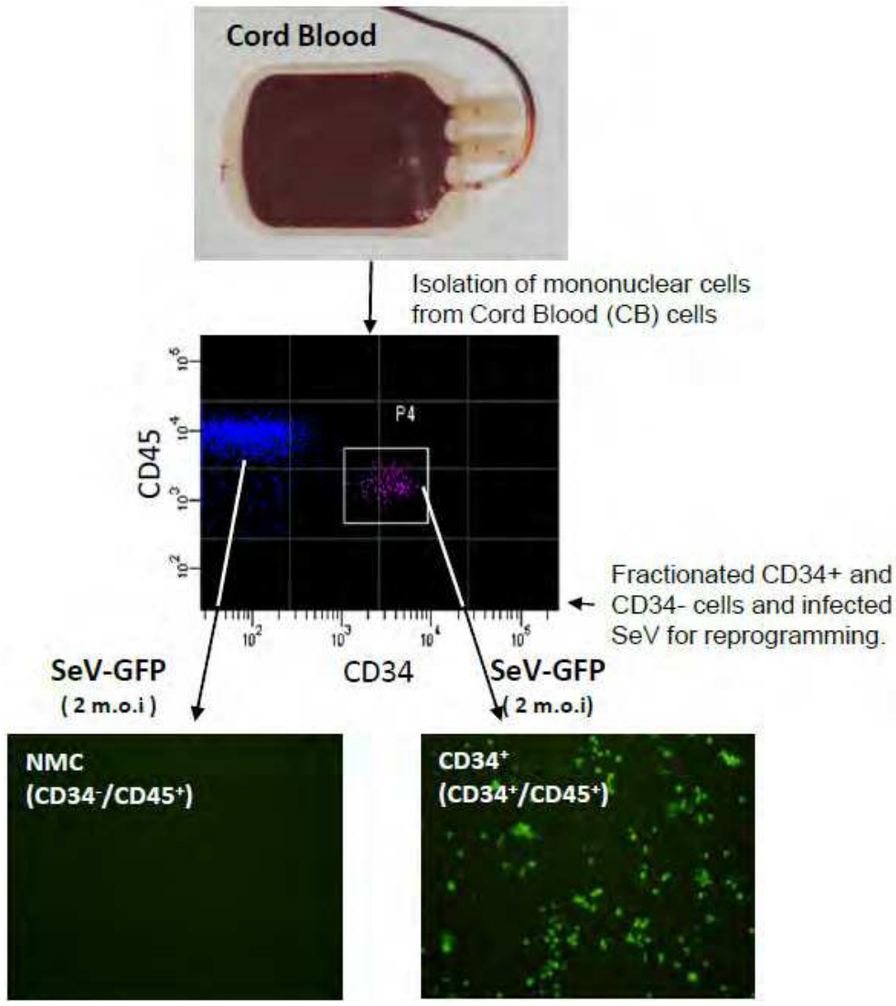

Fig. 2. SeV selectively infects the CD34+ fraction of CB cells.

conditions (20% O_2) to convert cells to "prime" virus-free ES cell-like cells. We cannot maintain the naïve state for more than five passages due to the instability of pluripotency in naïve culture conditions and the tendency for spontaneous differentiation. In contrast, pluripotency in the prime state (like conventional human ES cells) was stable and we could maintain prime ES or iPS cells for more than 50 passages. Therefore, further appraisal of the differentiation potential of the reprogrammed cells was done in the prime state (Fig. 4A,B).
Naïve ES cell-like clones from a single cell suspension were examined. Like mouse ES cells, emergent dome-like colonies (P = 1) started to express SSEA-1 in the naïve stage (Fig. 3C, lower left), but its expression ceased after shifting to the prime state (Fig. 3C, lower right). Expression of pluripotency-related molecules in the prime state was examined by immunostaining with a set of antibodies (Table 2). The presence of SeV constructs in the naïve reprogrammed cells was examined by RT-PCR at the single cell level (Fig. 3D). Heat-treated naïve clones that were free of SeV constructs under hypoxic conditions (5% O_2) were transferred to prime culture with a normoxic atmosphere (20% O_2). These virus-free ES cell like-clones were expanded in conventional prime human ES cell culture for further appraisal of the differentiation potential.

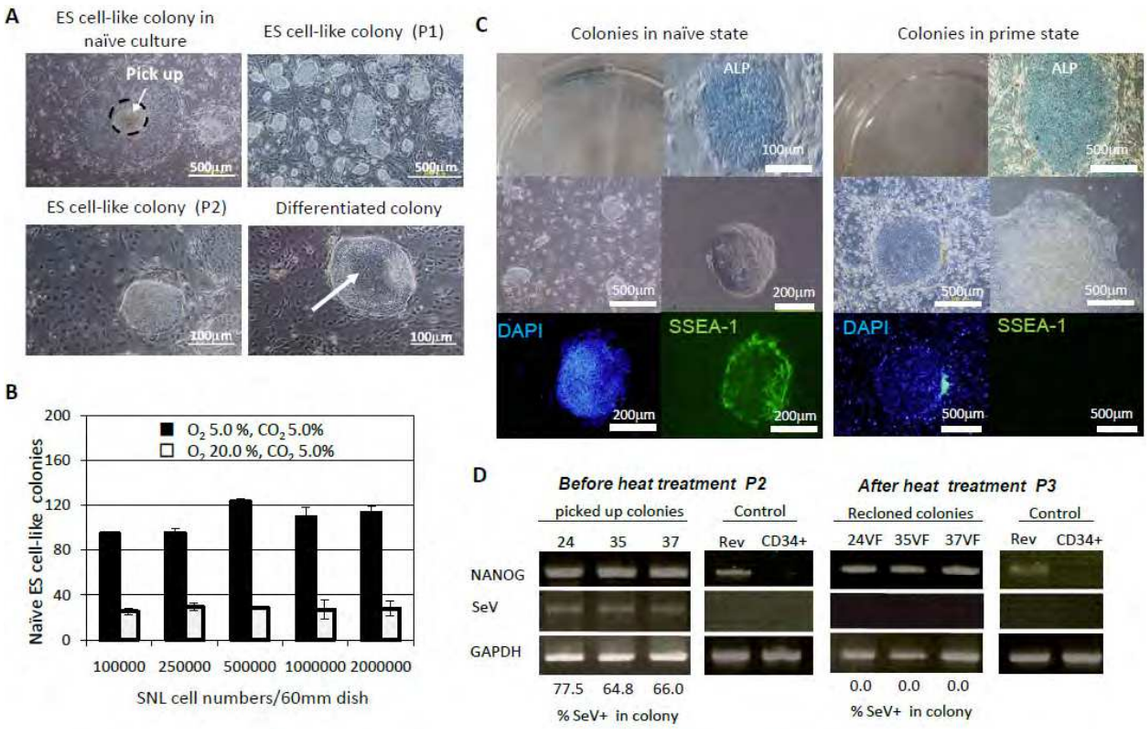

Fig. 3. Elimination of SeV constructs as determined by RT-PCR and generation of naïve or prime iPS cells.

A: Emerging naïve ES cell–like colony. Dome-like colonies emerged and were picked up (top left). Naïve ES cell-like colonies were seeded on MMC-SNL cells (passage one: P = 1, top right). Naïve ES cell-like colony (P = 2, lower left). Cells in the center of the naïve colony (white arrow) started to differentiate at later passages (P = 6, lower right). **B**: The efficiency of generation of naïve ES cell-like colonies under hypoxic (black) and normoxic (white) conditions. The number of MMC-SNL cells seeded on 60 mm dishes and the number of ES cell-like colonies which emerged are scored on the X-axis and Y-axis, respectively. **C**: Staining of naïve ES cell-like colonies (left panels) and prime ES cell-like colonies (right panels). ALP staining of colonies on MMC-SNL (top left and right), phase contract observations of colonies on MMC-SNL (middle left) or Matrigel (middle right), colonies stained with DAPI (lower left) or immunostained with anti-SSEA-1 antibody (lower right). **D**: Detection of SeV construct in heat-treated clones by PCR. Picked colonies #24, #35, and #37 were subject to heat treatment (passage 2: P = 2) and subcloned. Subclones were named #24VF, #35VF, or #37VF (passage 3: P = 3). iPS cells generated from CB by retrovirus (ReV) and parent $CD34^+$ CB cell were used as negative controls. % SeV^+ in colony is the area positively stained with anti-SeV antibody divided by the total area of the colony calculated by two value recognition software (Adobe Photoshop). There was no difference in the frequency of emerging dome-shaped ES cell-like colonies in the naïve state from freshly isolated $CD34^+$ cells and from frozen $CD34^+$ cells.

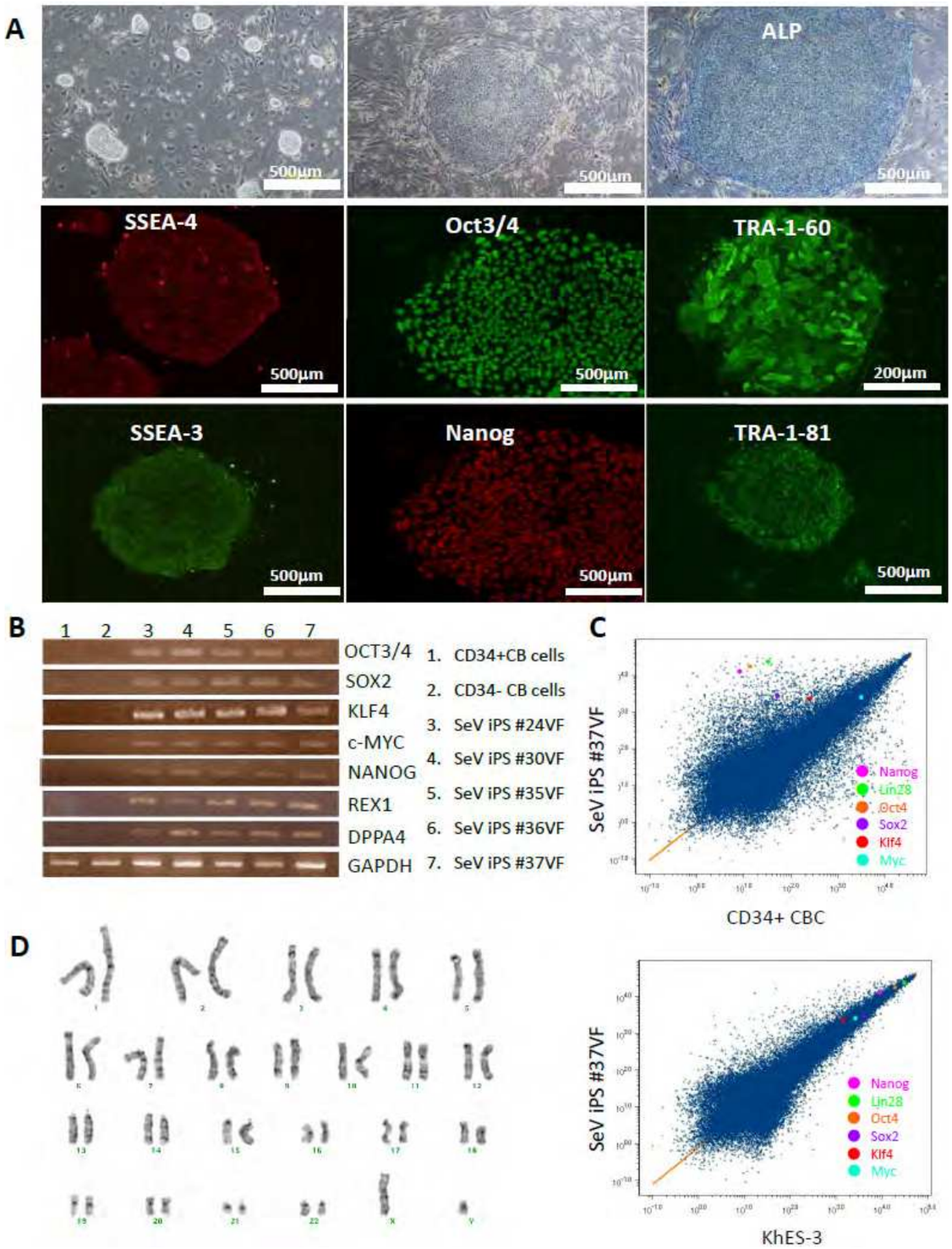

Fig. 4. Expression of pluripotency-associated genes and molecules in established SeV-free iPS cell clones.

A: Phase contrast images of a representative naive ES cell-like colony on MMC-SNL (P = 1: top left), after heat-treatment and recloning of a prime clone (SeV iPS #37VF, top middle) and its

ALP staining (top right). The expression of SSEA-4, Oct3/4, TRA-1-60, SSEA-3, Nanog and TRA-1-81 in the same prime clone (SeV iPS #37VF) was detected by immunohistochemistry. Alexa 594 (red) and Alexa 488 (green) conjugated secondary antibodies were used to visualize expression. **B**: Endogenous gene expression determined by RT-PCR. Sample description, pluripotency-associated genes, and lanes are indicated. $CD34^+$ and $CD34^-$ CB cells were used for controls. **C**: Gene expression comparison of SeV iPS #37VF vs $CD34^+$ CBC (upper panel) and SeV iPS #37VF vs human ES cell line KhES-3 (lower panel). Expression levels of pluripotency-related genes are marked in the panels. **D**: Karyotyping of SeV iPS #37VF.

Antibodies	supplier	Cat No	Dilution
anti-Oct4	Santa Cruz	sc-5279	1/ 100
anti-TRA-1-81	Chemicon	MAB4381	1/ 200
anti-TRA-1-60	Chemicon	MAB4360	1/ 200
anti-SSEA-3	Chemicon	MAB4303	1/ 200
anti-SSEA-4	Chemicon	MAB4304	1/ 200
anti-Nanog	Reprocell	RCAB0003P	1/ 1000
α-fetoprotein(AFP)	R&D	MAB1368	1/ 100
vimentin	Santa Cruz	sc-5565	1/ 200
α-smooth muscle actin(SMA)	SIGMA	A-2547	1/ 400
desmin	Dako	M0760	1/ 50
beta-III tubulin	SIGMA	T4026	1/ 200
GFAP	Santa Cruz	sc-6170	1/ 50
anti-SSEA-1	Santa Cruz	sc-21702	1/100
anti SeV HN	DNAVEC	IL4.1	1/100
Alexa Fluor 488 goat anti mouse	Invitrogen	A11001	1/ 1000
Alexa Fluor 594 rabbit anti mouse	Invitrogen	A11005	1/ 1000
Alexa Fluor 594 goat anti rabbit	Invitrogen	A11037	1/ 1000
DAPI	Invitrogen	D1306	5ug/ml

Table 2. List of antibodies used for immunostaining

2.2 Characterization of virus-free ES cell-like clones

2.2.1 Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was purified with RNeasy Mini kit (QIAGEN), according to the manufacturer's instructions. One μg of total RNA was used for reverse transcription reactions with PrimeScript RT reagent kit (TAKARA, Japan). PCR was performed with EXTaq (TAKARA, Japan). Total RNA from cell clones was extracted with the RNeasy minikit (QIAGEN). q-RT-PCR was performed with an ABI PRISM 7000 (Life Technologies Japan) using SYBR Premix EX Taq™ (TAKARA, RR041A) in accordance with the manufacturer's instructions. Primers are listed in Table 3.

2.2.2 Gene Chip analysis and karyotyping

Total RNAs from several established iPS cell clones, human ES cell line KhES-1, $CD34^-$ CB cells and $CD34^+$ CB cells were purified with RNeasy Mini kit (QIAGEN) and hybridized with human Gene Chip (U133 plus 2.0 Array Affymetrix) according to the manufacturer's

Primers			Size (bp)
hOCT3/4-F1165	GAC AGG GGG AGG GGA GGA GCT AGG	undifferentiated ES cell (endo)	144
hOCT3/4-R1283	CTT CCC TCC AAC CAG TTG CCC CAA AC		
hSOX2-F1430	GGG AAA TGG GAG GGG TGC AAA AGA GG	undifferentiated ES cell (endo)	151
hSOX2-R1555	TTG CGT GAG TGT GGA TGG GAT TGG TG		
hMYC-F253	GCG TCC TGG GAA GGG AGA TCC GGA GC	undifferentiated ES cell (endo)	328
hMYC-R555	TTG AGG GGC ATC GTC GCG GGA GGC TG		
hKLF4-F1128	ACG ATC GTG GCC CCG GAA AAG GAC C	undifferentiated ES cell (endo)	397
hKLF4-R1826	TGA TTG TAG TGC TTT CTG GCT GGG CTC C		
DPPA4-F	GGAGCCGCCTGCCCTGGAAAATTC	undifferentiated ES cell	408
DPPA4-R	TTT TTC CTG ATA TTC TAT TCC CAT		
REX1-F	CAG ATC CTA AAC AGC TCG CAG AAT	undifferentiated ES cell	306
REX1-R	GCG TAC GCA AAT TAA AGT CCA GA		
NANOG-F	CAG CCC CGA TTC TTC CAC CAG TCC C	undifferentiated ES cell	391
NANOG-R	CGG AAG ATT CCC AGT CGG GTT CAC C		
hGAPDH F	AAC AGC CTC AAG ATC ATC AGC	control	337
hGAPDH R	TTG GCA GGT TTT TCT AGA CGG		
hBRACHYURY-F1292	GCC CTC TCC CTC CCC TCC ACG CAC AG	mesoderm	274
hBRACHYURY-R1540	CGG CGC CGT TGC TCA CAG ACC ACA GG		
hPAX6-F1206	ACC CAT TAT CCA GAT GTG TTT GCC CGA G	ectoderm	317
hPAX6-R1497	ATG GTG AAG CTG GGC ATA GGC GGC AG		
hSOX17-F423	CGC TTT CAT GGT GTG GGC TAA GGA CG	endoderm	608
hSOX17-R583	TAG TTG GGG TGG TCC TGC ATG TGC TG		
SeV vector F15204	GGATCACTAGGTGATATCGAGC	SeV vectors	193
SeV vector R15397e	CATATGGACAAGTCCAAGACTTC		

Table 3. List of primers used to detect pluripotency-associated genes in reprogrammed cells.

instructions. Karyotyping of established iPS cells was reported by Nihon Gene Research Laboratories, Inc. (Sendai, Japan).

The expression of pluripotency-related molecules in the prime stage such as SSEA-4, SSEA-3, TRA-1-60, TRA-1-81, Oct3/4 and Nanog were detected by immunostaining (Fig. 4A). Endogenous expression of pluripotency-related genes was determined by RT-PCR (Fig. 4B). Total gene expression profiles of the established iPS clone SeV iPS #37VF are compared with human ES cell line KhES-3 or CD34+ cord blood cells (Fig. 4C). Karyotype of the established iPS cell clone SeV iPS #37VF is presented (Fig. 4D).

2.3 Differentiation assays of virus-free iPS cells *in vitro* and *in vivo*

2.3.1 *In vitro* differentiation assay

Established human ES cell-like clones were harvested using collagenase IV. Cells were transferred to six-well ultra-low attachment plates (Corning) and cultured in human prime ES cell medium without bFGF to form embryoid bodies (EB). The medium was changed every other day. The resulting EBs were transferred to gelatin-coated plates after eight days and cultured in the same fresh medium for another eight days. Three cell lines were tested for differentiation potential on gelatin coated dishes after EB formation (Fig. 5a). All of these

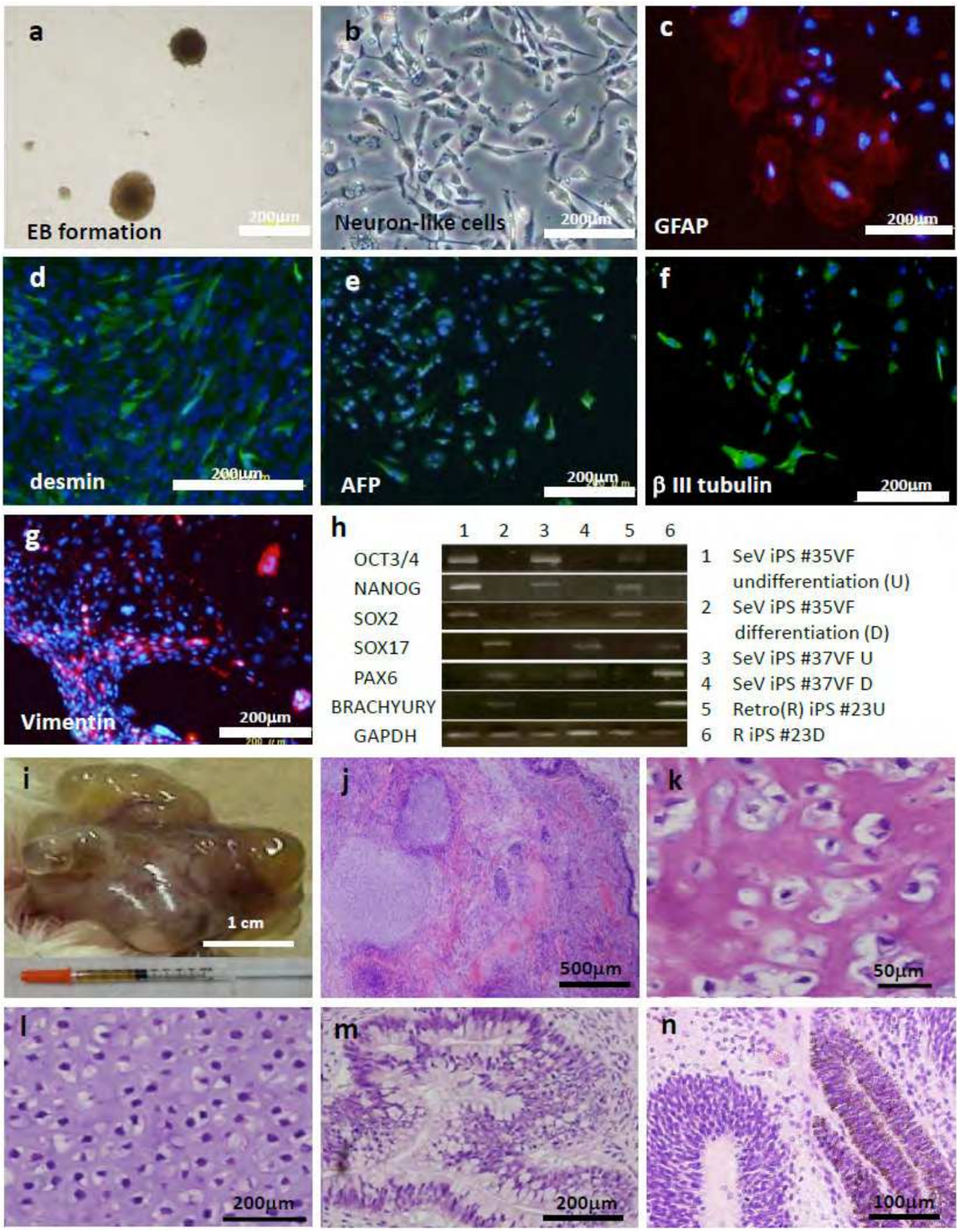

Fig. 5. *In vitro* and *in vivo* differentiation potentials of established iPS cell lines.
(**a-g**) Embryoid body-mediated differentiation of established iPS cells. All images shown are from cells derived from clone SeV iPS #35VF. Bright field images of embryoid bodies generated after eight days of culture (**a**). Embryoid bodies were transferred to gelatin dishes

and differentiated for a further eight days to induce either un-directed or guided differentiation (**b-g**). Phase contrast images of neuron-like cells (**b**) after differentiation on gelatin. Cells were fixed and stained with antibodies against GFAP (**c**), desmin (**d**), AFP (**e**), βIII-tubulin (**f**), and vimentin (**g**) to determine lineage-specific differentiation potential. (**h**) RT-PCR for lineage-specific differentiation of established iPS cell lines, SeV clones #35VF and #37VF. Retrovirally generated iPS cell clone R#23 from CD34+ CB cells was used for a control. (**i**) Teratoma formed from SeV iPS #35VF was injected into testis capsule. Teratoma had a cystic structure. The content of cysts is shown in the 1 mL syringe. (**j**) Hematoxylin and eosin staining of teratoma derived from iPS cells at low magnification. Histology showed derivatives of all three embryonic germ layers including bone-like (**k**: mesoderm), cartilage-like (**l**: mesoderm), gut-like epithelium (**m**: endoderm) and neural rosette-like (**n**: ectoderm) tissue.

clones were able to give rise to cells from all three germ layers as evidenced by cell morphology (Fig. 5b) and immunocytochemistry (Fig. 5c-g). Upon differentiation, the presence of gene expression characteristic of all three germ layers was determined by RT-PCR (Fig. 5h).

2.3.2 *In vivo* differentiation assay

One million iPS cells were injected beneath the testicular capsule of SCID mice (SLC Japan) for teratoma formation. Tumor formation was observed 60 - 80 days after cell transplantation. Tumor tissues were fixed with 4% formalin followed by hematoxylin and eosin staining. Two lines were tested for teratoma formation and both cell lines formed teratomas with a cystic structure (Fig. 5i). HE staining of teratoma tissues (Fig. 5j - 5n) showed differentiated tissues corresponding to all three germ layers.

3. Conclusions

Reprogramming of somatic cells with SeV vector without DNA integration is advantageous, as it reduces the chance for tumorigenicity caused by random genomic integration. Advantages of using SeV vector over other non-integrating reprogramming methods such as using adenovirus, episomal plasmid vectors, conventional plasmid vectors, or small molecule delivery systems include superior reprogramming capability with potent protein expression potential (13). The remaining concern in using SeV vector is how we can confirm the removal of potent SeV vectors from reprogrammed cells. In this report, we used the temperature-sensitive SeV vector TS7 to reduce the number of SeV-infected cells. In addition, we made use of a single cell cloning technique in the naïve state to confirm the absence of SeV vector constructs in the reprogrammed cells at a single cell level. Therefore, this cloning technique provides an ultimate solution for RNA virus vector-based reprogramming methodology.

The benefits of reprogramming somatic cells in the naïve state are not limited to a single cell cloning technology. It may provide answers to interesting questions like whether "standard" human iPS cells, having the correct epigenetic memory, can be generated by reprogramming somatic cells in the naïve state. Accumulation of epigenetic information before and after transferring to the naïve state would provide an answer to this question.

Several reports showed that iPS cells can be preferentially generated from the CD34+ fraction of CB cells and peripheral blood cells with retroviral vectors (19, 26). In our experiment, we also showed that the SeV TS7-GFP vector selectively infects freshly isolated

$CD34^+$ $CD45^{low+}$ cells and is able to reprogram this fraction. These data suggested that the use of SeV would facilitate the effective generation of iPS cells from CB cells. However, the molecule(s) responsible for SeV viral entry into the cell is elusive. Hemagglutinin-neuraminidase (HN), an envelope protein of Sev is reported to bind to sialomucin (28) and facilitate the cellular entry of virus. CD34 belongs to the sialomucin family. Although SeV is not able to infect $CD34^-$ cells from freshly isolated (non-cultured) CB cells, SeV is able to infect $CD34^-$ cells that have differentiated from $CD34^+$ CB cells after seven days of culture in hematopoietic cell culture media. With limited information, we cannot conclude that CD34 is the SeV entry molecule. Rather, it appears that a set of molecules other than CD34, expressed in $CD45^{low+}$ cells, might be responsible for it.

As a cell source for generating iPS cells, CB cells have certain advantages over other somatic cells. Unlike cultured cells or those obtained by biopsy from a variety of tissues at various ages, freshly isolated (non-cultured) $CD34^+$ CB cells are the youngest stem cell population available following birth. They also have distinct genetic and epigenetic profiles as hematopoietic stem cells and progenitors and lack genetic alternations like rearrangements or possible post-natal genomic damage caused by UV irradiation or chemical irritants. Furthermore, generating iPS cells from this fraction would facilitate our understanding of the reprogramming process, since the genetic profiles of the cell source and the reprogrammed cells are known. Another advantage of using cord blood cells would be the possibility of collaborating with the existing world-wide network of public cord blood banks. Extensive discussions concerning the conditions and ethical issues are necessary before such clinical applications are pursued. Nonetheless, the use of CB as a source for iPS cells is a realistic option for generating "bona fide" iPS cells for future clinical use.

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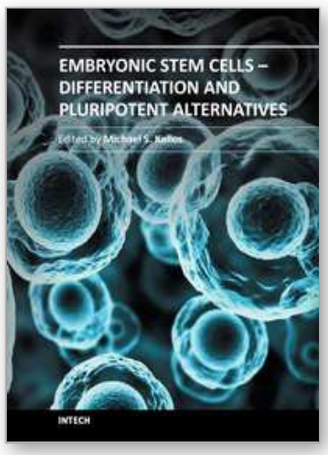

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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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