Chapter 5

Generation and Maintenance of iPSCs From CD34+Cord Blood Cells on Artificial Cell Attachment Substrate

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Additional information is available at the end of the chapter

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1. Introduction

Cord blood (CB) cells are commonly used for the treatment of leukemia and inherited metabolic diseases. To date, more than 20,000 bone marrow transplants have been performed on children and adults with cord blood cells, and There are more than 450,000 HLA-defined CB collections stored frozen cryoperserved form in more than 50 units public CB banks and more than 2,000 CB transplants are being performed world-wide per year. CB cells are the youngest somatic cells and in theory have no post natal DNA damage such as caused by UV or chemical irritant exposure. Therefore, our previous study thought that use to the ability to cryopreserve CB HSC long-term in bank, which conferring a unique advantage to CB cell as a suitable material for generating induced pluripotent stem (iPSC) cells for future clinical use.[1]

iPSC should be generated with methods that do not require integration of exogenous DNA, thereby reducing the chance of tumorigenicity caused by random chromosomal insertion of exogenous genes. Several non-integrating reprogramming methods using EBNA based-plasmids vector [2, 3, 4, 5], piggy-back transposons [6, 7], human artificial chromosome vectors [8], small peptides [9, 10], mRNA [11] and proteins [12] have been reported. Among the vectors employed for these experiments, the Sendai virus (SeV) vector (that lacks a DNA phase) is recognized as a potent reagent for reprogramming of somatic cells [13-15]. However, complete elimination of the SeV construct carrying reprogramming factors is a key issue to assure three germ layer differentiation of individual cells. The presence of residual reprogramming factors in transfected cells could impede differentiation and contribute to formation of tumors after implantation. Therefore, the possible presence of the SeV construct should be checked at a

single cell level (not at a cell clump level) utilizing single cell cloning techniques in the naïve state [16-18]. Recently, feeder-free culture systems utilizing Laminin 511, LM-E8s or Matrigel have been reported for the maintenance of established iPSCs or ES cells [19-23]. The generation of iPSCs from fibroblasts on vitronectin-coated dishes and maintenance of iPSCs in chemically defined medium on vitronectin-coated dishes has been reported [23]. These studies were to characterize as substrates that support hESCs in a sustainable undifferentiated state under a xeno-free and chemical defined culture condition [20, 23]. On the other hands, multiple matrix proteins, such as laminin, vitronectin fibronectin and synthetic polymer surfaces support hESC/iPSC growth and maintenance. Most of these materials are too expensive for large-scale usage. Because, recombinants vitronectin is relatively easy to over-express and purify, we tested vitronectin in two feeder-free ES/iPS mediums. (mTeSR-1 and ReproFF2).

In this chapter, we describe the generation of iPSC clones from cord blood cells (CBCs) in feeder-free thought naïve conditions using temperature sensitive SeV vector. Additional, human naïve iPSC culturing methods using feeder-free systems and we introduce to low-cost and stable and easy maintenance culturing methods of hESC/iPSC.

2. Experimental procedures, materials and methods

2.1. Cord blood

$CD34^+$CBCscan be procured from Riken Bio Resource center (Riken BRC, Ibaraki, Japan) or other commercial suppliers. Alternatively $CD34^+$CBCscan be obtained from fresh cord blood using a mononuclear cells isolation kit (Lymphoprep TM, Cosmo Bio Co., Japan), and a human CD34 Micro Bead kit (Miltenyi Biotec, 130-046-702) or Auto Macs columns (Miltenyi Biotec, Germany) in accordance with the manufacturer's instruction. $CD34^+$CBCs were cultured in the density of 1.0 x 10^5 cells in two mL of hematopoietic culture medium [serum-free X-Vivo10 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), and 20 ng /mL Flt3/4 ligand (R&D System, Bostone, USA)] for one day prior to viral infection [23].

2.2. Preparation of coated dish for feeder-free generating iPS cells

PronectinF$^{plus®}$ coated-dish for reprogramming of CBCs is prepared as follows: One mg/mL stock solution PronectinF$^{plus®}$ (hereafter, Pronectin F, Sanyo Chemical Industries, Japan) was prepared by adding one mL of 37 ^oC deionized water to lyophilized Pronectin F. Ten ug/mL of Pronectin F working solution was prepared by diluting the stock solution with phosphate buffered saline (PBS). The culture dish (BD Life Science, Canada) was covered completely with Pronectin F and left overnight at room temperature. The coating solution was then removed by aspiration., and then dish was rinsed twice with PBS.

To make vitonectin-coated culture dish, the vitronectin-N (VTN-N) (Life Technology,USA) is used for a six-well plate. Dilute thawed VTN-N with 1xPBS (Life Technology,USA). in

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accordance with the manufacturer's instruction. Keep coated wells in culture medium at 37°C, 5% CO_2 during passaging procedure until cells are ready to be re-plated.

2.3. Sendai virus infection and reprogramming

Temperature-sensitive Sendai virus vector constructs inserting four reprogramming factors (SeV18+HS-*OCT3/4*/TS⊿F, SeV18+HS-*SOX2*/TS⊿F, SeV18+HS-*KLF4*/TS⊿F, SeV(*HNL)c-MYC*/TS15⊿F, SeV18+*GFP*/TS⊿F) were supplied by DNAVEC Corp. 1.0 x 10^4 $CD34^+$CBCs were transferred to one well of a 96-well plate in 180 μL of hematopoietic cell culture medium with 20 μL of viral supernatant containing 20 M.O.I. each of SeV constructs at 5% CO_2, 37 °C. The medium was changed to fresh medium in the following days (15-18 hours after infection). Infected cells were cultured another three days in hematopoietic culture medium in 96-well plates, after which 1 x 10^4 infected CBC were seeded on a Pronectin F-coated 6-well dish in primate ES cell medium ReproFF2 supplemented with 5 ng/mL bFGF (ReproCELL Inc, RCHEMD006B, JAPAN) to generate ES cell-like colonies under 20% O_2, 37 °C conditions. The amount of SeV constructs in the transfected cells was reduced by incubation cells at 5% CO_2, 38 °C for three days.

2.4. Cell culture in naïve state

After heat treatment, three hundred cells were resuspened in 100ml of naïve cell culture medium (see below). The cells were seeded in ten well of 96-well plate (100μl/well) pre-coated with Pronectin F. Approximately single cell in every three wells was seed in a 96-well plate. The presence of a single cell per individual well was verified by microscopic observation (phase contrast Olympus CKX31) in the same manner as single cell cloning. These cells were cultured at 37 °C in 5% O_2, 5% CO_2 condition in naïve cell culture medium to form dome-shape colonies. 50 mL of naïve ES/iPS cell culture medium was prepared by mixing 24 mL DMEM/F-12 medium (Invitrogen, 11320, Osaka), 24 mL Neurobasal medium (Invitrogen, 21103), 0.5 mL x100 nonessential amino acids (Invitrogen, 11140), 1 mL B27 supplement (Invitrogen; 17504044), and 0.5 mL N_2 supplement (Invitrogen; 17502048). The medium also contained final concentrations of 0.5 mg/mL BSA Fraction V (Sigma, A8412, Nebraska), penicillin-streptomycin (final x 1, Nacalai, Kyoto), 1 mM glutamine (Nacalai), 0.1 mM β-mercaptoethanol (Invitrogen 21985), 1 μM PD0325901 (Stemgent, 04-0006, Cambridge), 3 μM CHIR99021 (Stemgent, 04-0004), 10 μM Forskolin (Sigma, F6886) and 20 ng/mL of recombinant human LIF (Millipore; LIF1005, Billerica).

2.5. Gene chip analysis

Total RNAs from several established iPSCs lines (prime [1st, 2nd] and naïve), khES-1 (Riken BRC) and $CD34^+$CBCs (Riken BRC) were purified with an RNeasy Mini kit (QIAGEN), amplified Ovation Pico WTA System (Takara cat#3300–12), labeled with an Encore Biotin Module (Takara catalog number 4200–12) and then hybridized with a human Gene Chip (U133 plus 2.0 Array Affymetrix).

2.6. Karyotype analysis

After the iPS cells have reached the 80% of confluence, it must be harvested and fixed to make a cytogenetic suspension. iPS cells are growth arrested and accumulated in metaphase or prometaphase by inhibiting tubulin polymerization and thus preventing the formation of the mitotic spindle using colcemid (Sigma, #D7385). Following exposure to colcemid, iPS cells are treated with a hypotonic solution to enhance the dispersion of chromosomes and fixed with carnoy fixative (Methanol: Acetic Acid=3:1). Once fixed, the cytogenetic preparation can be stored in cell pellets, under fixative conditions and 20°C for several months. Fixed cells are spread on slides and air-dried, to be finally banded for the correct identification of chromosomes.

3. Results

3.1. Selection of coating materials for feeder-free generating iPS cells

Using gene chip approach, we investigated the levels of adhesion molecule expression on (i) $CD34^+$CBCs, (ii) the resulting iPSC cells and (iii) naïve iPSC on SNL (SNL76/7, ECACC) cultured in naïve cell medium. We identified several molecules that were expressed by $CD34^+$CBCs and by the resulting primed and naïve iPSCs cultured on feeder cell SNL (Table 1). These data prompted us to use their ligands to anchor CBCs to dishes for reprogramming in a feeder-free system. In this context, fibronectin or a relevant material, which has an-Arg-Gly-Asp-(RGD) motif that can bind to the integrin $\alpha 5/\beta 1$ dimmer expressed on CBCs, was selected as a candidate for a coating material for the generation of iPSCs.

Gene ID	Description	Cord blood cell (CBC)	iPS cell from $CD34^+$ on SNL cells	Naïve state iPSCs on SNL cells
		$CD34^+$ (n=3)	SeV-CB iPS (n=3)	Naïve state SeV-CB-iPS (n=3)
COL1A1	Collagen, type I, alpha 1	6.87 ± 3.89	21,022.23 ± 1,3691.11	18,154.75 ± 6,733.33
COL1A2	Collagen, type I, alpha 2	9.15 ± 7.69	4,170.04 ± 1,796.98	22,075.02 ± 9,436.53
COL9A3	Collagen, type IX, alpha 3	5.20 ± 2.01	1,793.79 ± 452.86	756.78 ± 130.64
COL18A1	Collagen, type XVIII alpha 1	132.90 ± 73.98	2,565.17 ± 877.84	1,558.63 ± 897.07
ITGA5	Integrin alpha 5	284.25 ± 38.21	366.91 ± 24.61	372.02 ± 22.96
ITGB1	Integrin, beta 1 (ITGB1)	9,034.55 ± 2,178.51	22,946.52 ± 7,287.56	25,447.85 ± 7,076.74
SDC2	Syndecan 2	132.13 ± 20.79	6,224.00 ± 813.19	1,095.14 ± 651.86
SDC4	Syndecan 4	334.2 ± 161.06	1,973.21 ± 565.5	11,594.85 ± 6,677.37
FN1	Fibronectin1	7.92 ± 1.14	9,922.15 ± 3,769.53	14,929.00 ± 6,824.63
TJP1	Tight junction protein 1	53.17 ± 23.59	8,351.35 ± 1,682.14	6,850.59 ± 801.16
TJP2	Tight junction protein 2	1,056.53 ±309.49	3,023.60 ± 59.05	4,144.96 ± 1,675.39

Mean and standard deviation of signal values for the expression of indicated genes from three independent experiments.

Table 1. Gene chip analysis of adhesion molecules on $CD34^+$cells, and primed and naive iPSCs cultured on SNL.

From the point of view of quality control and reagent tracking, synthetic peptides expressing the RGD motif are preferable to natural ligands. Thus, Pronectin F which mimics the peptide

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structure of fibronectin, was chosen and tested for reprogramming CBCs. Pronectin F was synthesized by fusing two amino acid motifs, RGD and $(GAGAGS)^9$ in tandem to produce a-RGD-$(GAGAGS)^9$-RGD-$(GAGAGS)^9$-RGD-$(GAGAGS)^9$-RGD-polypeptide. This polypeptide has thirteen RGD motifs and is folded at the RGD sequence. Thus, the RGD motif is effectively exposed at the limbs of the peptide bundle, facilitating its potent binding affinity to the integrin $\alpha 5/\beta 1$ dimer.

3.2. Generation of iPS cells on synthetic peptide (Pronectin F®)

Protocol for generating iPSC on feeder less condition is shown in Figure 1.

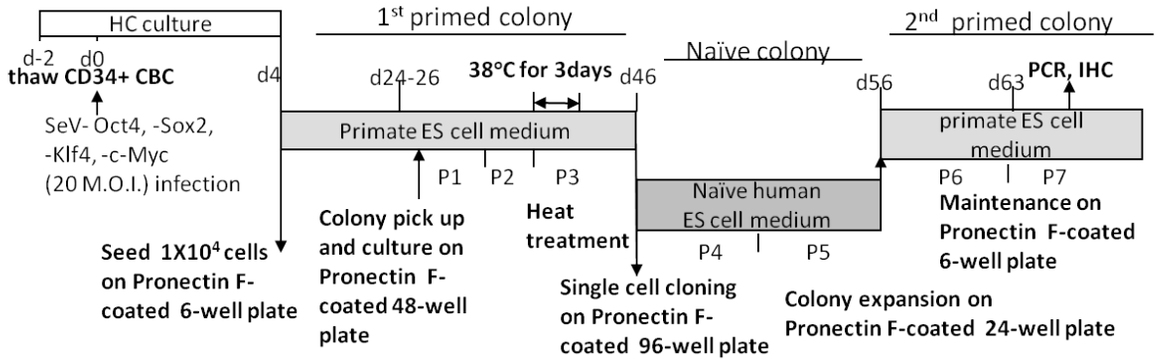

Figure 1. Protocol for generation of iPSCs from $CD34^+$CBCs on Pronectin F-coated dishes with temperature sensitive SeV vectors. P: passage.

Human ES cell-like colonies (first prime state) were picked up at day 24 and cultured on Pronectin F-coated dishes. The colonies were subjected to heat treatment (38°C, three days) at passage three (P3). Colonies emerged from single cells in Pronectin F-coated 96-well plates under naïve conditions at P4, dome-shaped colonies at P5 under naïve conditions, ES cell-like colonies (second primed) cultured under primed culture conditions at P6,P7.

The medium was changed every other day for transformed adherent cell stage (day 1-12). However, during day 13-17, primate ES medium was changed every day. The reprogramming process was monitored by checking the morphology of the transfected cells. CD34+cells infected with SeV constructs were cultured in serum-free hematopoietic cell culture, as shown in Figure 2 (day1). Some cells attached to Pronectin F-coated dishes by day four in Figure 2 (day 4). Cobble stone-like cell colonies emerged at day nine and cell clumps with round and small cells emerged inside the colonies at day 13 on Pronectin F-coated dishes (Figure 2, day 9, day 13). Cell clumps within cobble stone-like colonies grew (Figure 2, day 17) and finally human ES cell-like colonies emerged (Figure 2, day 24) on Pronectin F-coated dishes which were then picked up for serial passage. Fifteen to twenty-two dish-shape human ES cell-like colonies were picked out of 10,000 CD34+CBCs seeded on Pronectin F-coated dish in primate ES medium. Colonies were picked approximately three weeks after viral infection. Cells from individual colonies were transferred to a Pronectin F-coated 48-well plate to select passage-able ES cell-like colonies capable of passage.

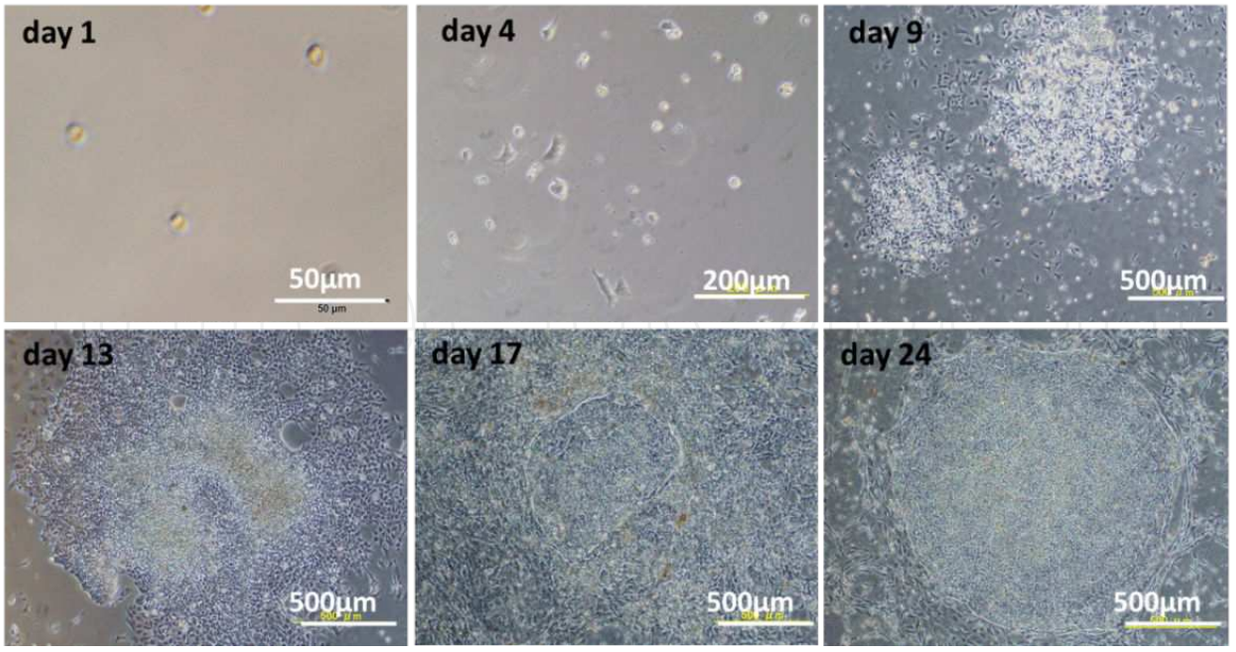

Figure 2. Phase contrast light microscopic observation of cells during reprogramming. Images captured on a Pronectin F-coated dish on days 1, 4, 9, 13, 17 and 24. day 1; Infected CD34^{+}CBCs with Sendai virus seed on Pronectin F$^{®}$-coated dish. day 4; Infected CD34^{+}CBCs were attachment and little spread on Pronectin F-coated dish. day 9: Infected CD34^{+}CBCs expansion on Pronectin F-coated dish. day 13; Infected CD34^{+}CBCs expansion with colony-like state. day 17; generation of small ES-like colony around spreading apart of infected CD34^{+}CBCs. day 24; Human ES cell-like colonies emerged on Pronectin F-coated dishes.

Human ES cell-like colonies (first primed state) were picked up at day 24 and cultured on Pronectin F-coated dishes. The colonies were subjected to heat treatment (38 ^{o}C, three days) at passage three (P3) to reduce the SeV constructs (Figure 3).

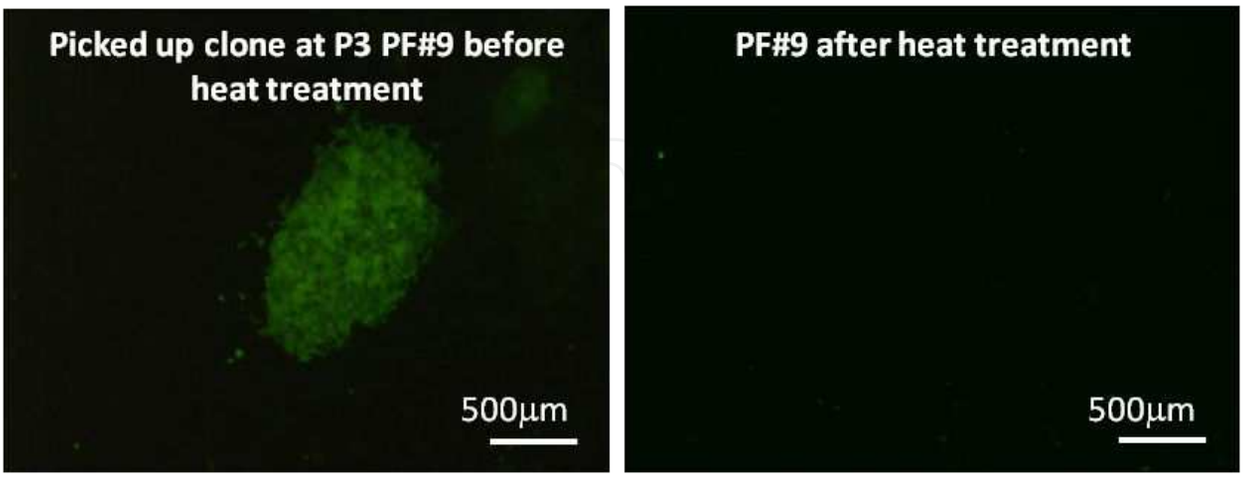

Figure 3. Expression of SeV in reprogrammed cell clone before and after heat treatment.

Expression of SeV in ES cell-like colonies before heat treatment at passage three (SeV at P3) and after heat treatment and single cell cloning at passage.

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Reprogrammed cell clone before single cell cloning in the naïve state was named **PF** (**P**ronectin F –coated **F**eeder-less clones). The level of SeV protein expression was determined by immunostaining with SeV HN antibody (polyclonal-rabbit, gift to DNAVEC Corp., Ibaraki).

Then, single cells from dish-shaped (first primed) primate ES cell-like colonies at passage three were seeded on a Pronectin F-coated 96 well plate at approximately one cell per three wells and cultured in naïve medium under hypoxic conditions (5% O_2, 5% CO_2 at 37° C). After five or six days, dome-shaped mouse ES cell like-colonies were collected and expanded on Pronectin F-coated dishes. Next, cell clumps were transferred to primate ES medium under 20% O_2 again to culture them in the primed state in Figure 4.

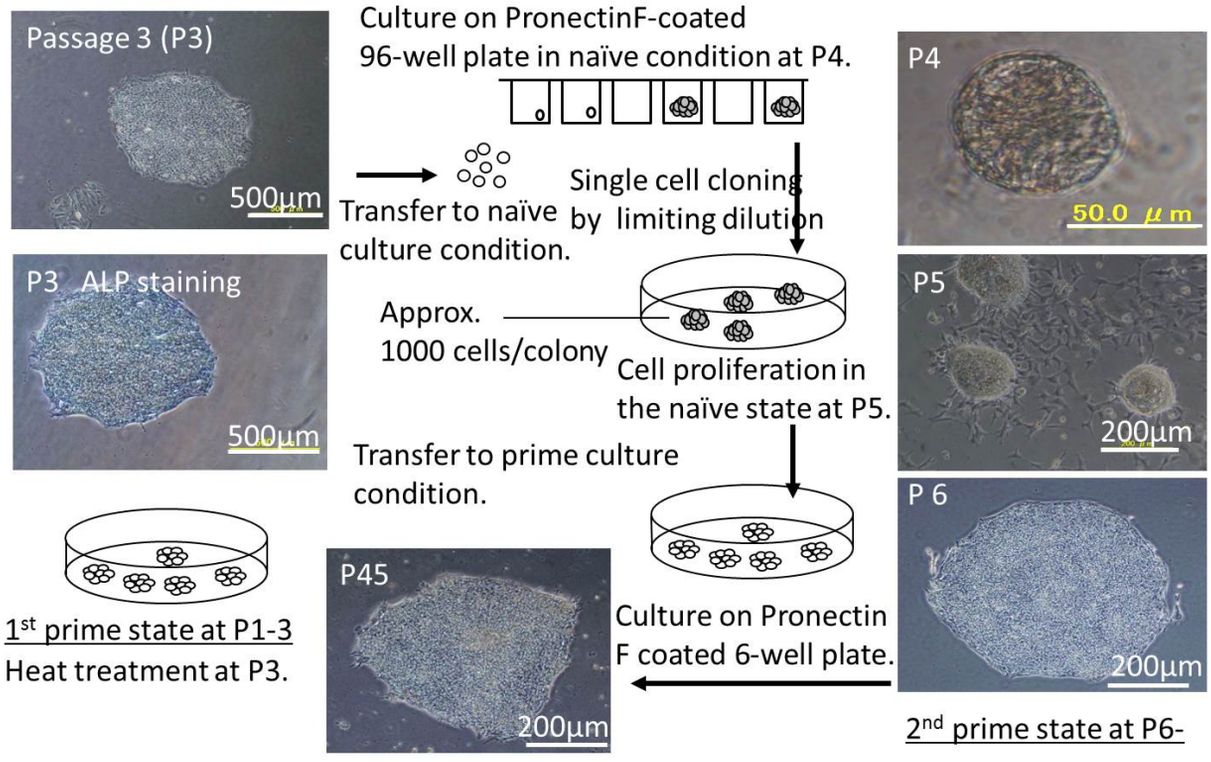

Figure 4. Generation of reprogrammed cell clone from a single cell via the naïve state.

Light microscopic image and ALP staining at P3 are shown in upper and lower panels, respectively. Colonies emerged from single cells in Pronectin F-coated 96-well plates under naïve condition at P4, dome-shaped colonies at P5 under naïve condition, ES cell-like colonies (second primed) cultured under primed culture condition at P6 or long-term passaged clone at P45 are shown. And, the colonies were subjected to heat treatment (38°C, three days). Colonies emerged from single cells in Pronectin F-coated 96-well plates under naïve conditions

at P4, dome-shaped colonies at P5 under naïve conditions, ES cell-like colonies (second primed).

Long-term passaged clone (PFX#9) at P45 is shown. After single cell cloning in the naïve state, picked up cell clones were named as **PFX** (**P**ronection F-coated **F**eeder-free iPSC derived from female (**X**X) cord blood cell. We used female cord blood cells (XX) to check the status of being in the naïve stage manifested by reactivation of X-chomosome inhibition. Culturing cells in the naïve state was useful for a single cell cloning in limited dilution, but we fail to support cell culture in the naïve stage robustly for more than five passages. Therefore cells were kept culturing in the primed condition (20% O_2, the ES cell medium containing bFGF) after single cell cloning in the naïve state for further appraisal and passages.

Whether dome–shape cells cultured in the naïve condition (Figure 4, P5) was indeed in the naïve state or not a reactivation of X-chromosome inhibition was determined by gene chip analysis (Table 2.) and RT-PCR (Figure 5) with each states (prime [1st, 2nd] and naïve).

probe no.	Gene description	khES-1*(XY)* 1st *primed*	13PFX #1*(XX)* 1st primed	PFX#2 *(XX)* naïve	PFX#7 *(XX)* naïve	PFX#9 *(XX)* naïve	PFX#2 *(XX)* 2nd primed	PFX#7 *(XX)* 2nd primed	PFX#9 *(XX)* 2nd primed
214218_s_at	*Xist*	23.19	2870.9	40.24	39.2	47.7	1,610.52	2,615.94	1,278.82
221728_x_at	*Xist*	46.12	6727.3	5.53	71.2	75.7	2,949.19	5,036.44	2,275.75
224588_at	*Xist*	20.47	10579.6	8.09	17.4	48.2	2,891.72	6,009.63	2,604.95
227671_at	*Xist*	65.22	5964.9	3.24	76.5	79.3	2,761.26	5,532.28	2,217.70

Table 2. *Xist* gene expression analysis by gene chip for X-chromosome activite / inactivite states using four different probes.

Naïve PFXs were cultured in the naïve state and 2nd primed PFs were cultured in the naïve state. PF #13 1st prime and khES-1 1st primed were cultured in the primed state (without being in the naïve state). PF #13 and PFXs are female (XX) in origin, while human ES cell line khES01 is male in (XY) origin.

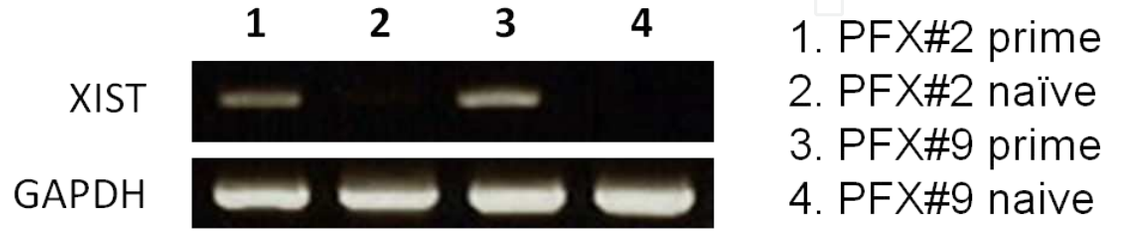

Figure 5. Expression of *Xist* genes in naïve and prime state iPS cell determined by RT-PCR.

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RT-PCR determination of naïve state in iPSC colony, second primed colonies 1; PFX#2 or 3; PFX#9, naïve state colonies before each second prime state colonies (2; PFX#2 naïve, 4; PFX#9 naïve). Values were normalized using the housekeeping gene *GAPDH*.

3.3. Maintenance and characterization of reprogrammed cells

3.3.1. Maintenance of reprogrammed cells established in feeder free condition

Once established on a Pronectin-coated dish, reprogrammed cell colonies can be maintained either in a Pronectin F-, Laminin-or Matrigels-coated dish for serial passage. 100-200 cell clumps (50-100 μm diameters) were seeded on 100mm dish or in six wells of a 6-well plate and cultured until colonies reach 70-80% confluence. The split ratio was routinely 1:3. This is a protocol for passage via cell clump, not via single cell suspension.

It is possible to conduct cell passaging via single cell suspension in serum-free media (mTeSR1, TeSR2 and ReproFF) in the primed condition with the use of Rock inhibitor (Y-27632, Stemgent, #2514).

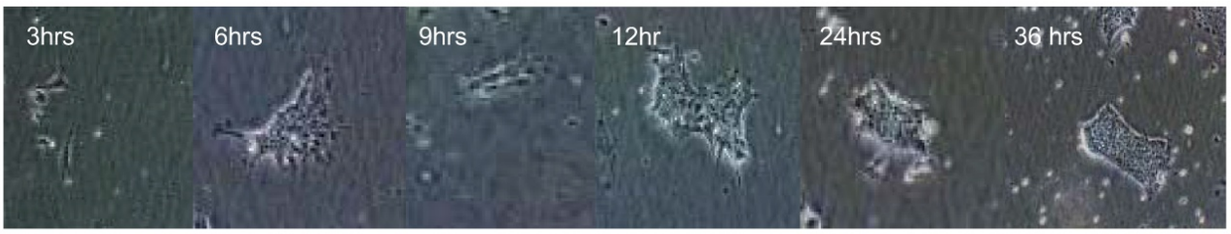

Figure 6. Photograph of forming cell colony from single iPS cell suspension on Matrigel.

As shown in Figure 6, it is notable that single cells migrate towards one another three to thirty-six hrs after passage to form cell clumps. This is a single cell passage, not a single cell cloning process. We failed to generate colonies from single cell in the primed state. That is a rationale for using naïve culture for single cell subcloning purpose. It is convenient to use singe cell suspension for passage. However, morphology of cell colony via single cell passage in longer period (P20 or over) is not uniform and is no longer round. We have not accumulated enough data how relevant this even is, but from a daily practical point of view, we perform cell passaging via cell clumps.

3.3.2. Characterization of reprogrammed cells by Reverse transcriptase polymerase chain reaction (RT-PCR)

The expression of pluripotecy related genes were determined by RT-PCR. Total RNA was purified with an RNeasy Plus Micro kit (QIAGEN 74034), according to the manufacturer's instructions, and One μg of total RNA was used for reverse transcription reactions with PrimeScript RT reagent kit (TAKARA, Japan). Result is shown in Figure 7. Primer sequences used for PCR are shown in Table 3.

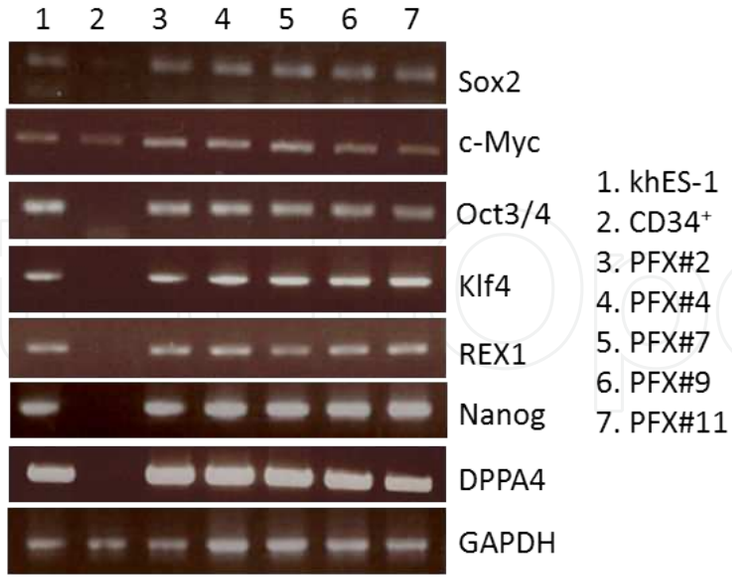

Figure 7. Expression of endogenous pluripotency related genes in reprogrammed cell determined by RT-PCR.

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		
hTERT-F3292	CCT GCT CAA GCT GAC TCG ACA CCG TG	undifferentiated ES cell	445
hTERT-R3737	GGA AAA GCT GGC CCT GGG GTG GAG C		
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		
SeV vector-F	GGATCACTAGGTGATATCGAGC	SeV vectors	193
SeV vector-R	CATATGGACAAGTCCAAGACTTC		
hGAPDH F	AAC AGC CTC AAG ATC ATC AGC	control	337
hGAPDH R	TTG GCA GGT TTT TCT AGA CGG		

Table 3. List of genes and the primers used for RT-PCR.

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3.3.3. Evaluation for remaining SeV construct

The remaining SeV construct after heat treatment and single cell cloning was determined by qRT-PCR and shown in Table 4.

Cell	CD34+ infected	PF#7 before HT	PF#9 before HT	CD34+	201B7	PFX#7 (P8)	PFX#9 (P8)
SeV	193736	19719	26850	6511	3997	5414	1135

Table 4. Quantitative RT-PCR determination of residual SeV viral genomes.

Quantitative RT-PCR determination of residual SeV viral genomes in CD34+CBCs three days after SeV infection (CD34 infected), first primed colony iPS#7 or iPS#9 before heat treatment at P2 (PF#7 before HT, PF#9 before HT), non-infected CD34+CBCs (CD34) or iPSC clone generated by retrovirus (201B7), established clones at P9 (PFX#7) or (PFX#9). Values were normalized using the housekeeping gene *GAPDH*.

The residual SeV viral genome was determined by qRT-PCR analysis for selection of non-integration and non-virus of established iPSC lines.

3.3.4. Characterization of reprogrammed cells by Immunohistological staining

ES cell like-colonies were stained with the Leukocyte Alkaline Phosphatase kit (VECTOR, Burlingame, CA, SK-5300) in accordance with the manufacturer's instructions. For immunochemical staining, these cells were fixed with 4% paraformaldehyde followed by staining with antibodies against Oct3/4 (1:100 sc-5279; Santa Cruz, Biotechnology USA), Nanog (1:500, RCAB0003P; Reprocell, Tokyo, Japan), SSEA-3 (1:200 MAB4303; Millipore), SSEA-4 (1:200, MAB4304, Millipore). Photomicrographs were taken with a fluorescent microscope (Olympus BX51, IX71, Tokyo) and a light microscope (Olympus CKX31).

ES cell-like clone PFX#9 at P8 was stained with antibodies against Nanog, Oct3/4, SSEA-3, or SSEA-4 as indicated. Alexa 594-and Alexa 488-conjugated secondary antibodies (red and green, respectively) were used to visualize the staining.

3.3.5. Characterization of reprogrammed cells by gene chip analysis and karyotyping

Total RNAs from several established iPSCs lines, ESCs lines (Riken BRC) and CD34+CBCs (Riken BRC) were purified with an RNeasy Plus Mini kit (QIAGEN 74136), amplified Ovation Pico WTA System (Takara cat#3300-12), labeled with an Encore Biotin Module (Takara catalog number 4200-12) and then hybridized with a human Gene Chip (U133 plus 2.0 Array Affymetrix) according to the manufacturer's instructions (Figure 9). Karyotyping G-band method of iPSCs is shown in Figure 10. The amount of metaphases obtained is sometimes inadequate for chromosome analysis, thus it is always necessary to keep growing the PFX#9 iPS cells. As shown in Figure 10, PFX#9 iPS cell on VTN-N was normal karyotypic cell.

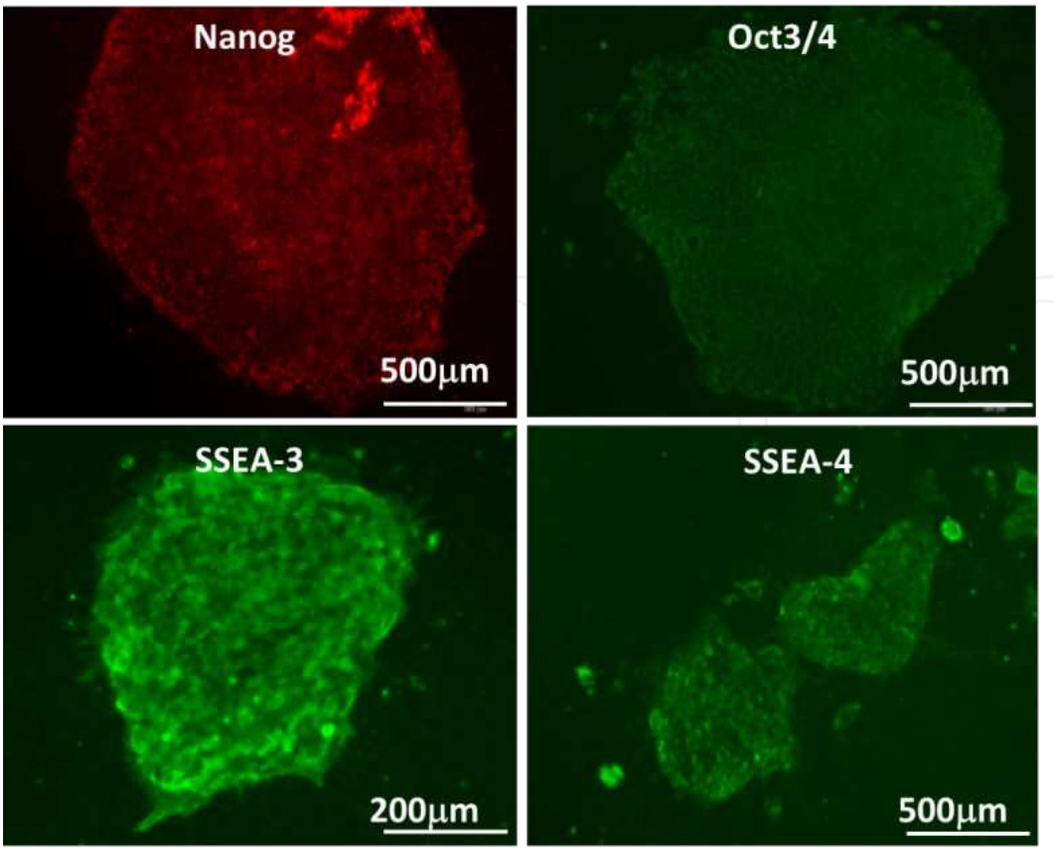

Figure 8. Characterization of established iPSC PFX#9 clones. Expression of pluripotency-related molecules in reprogrammed cell clones.

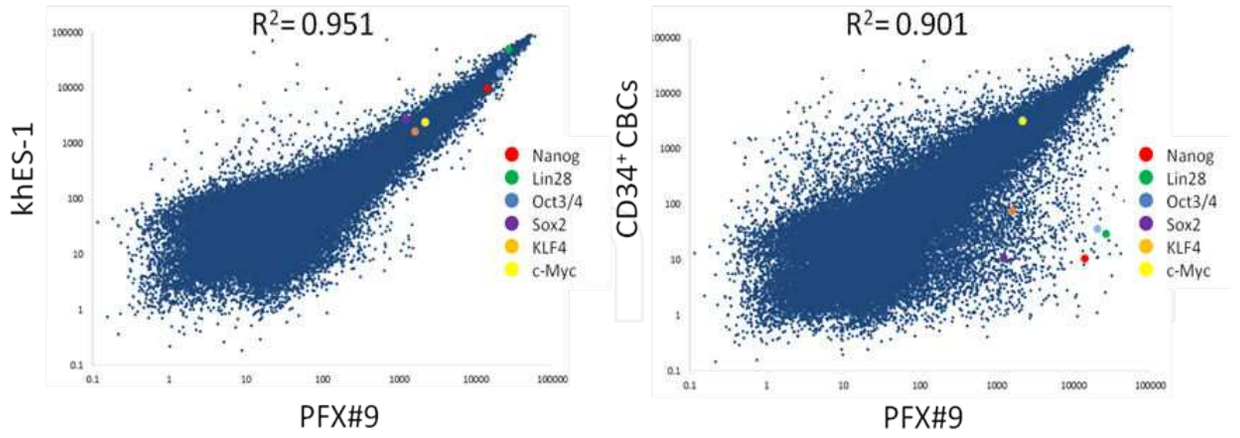

Figure 9. Gene expression comparison between the mean (mean) expression of clustered pluripotent stem cell. [PFX#9(iPSC from CBC with Yamanaka 4factors-heat treat Sendai virus without feeder) and HSC of Cord Blood ($CD34^+$CBC)] and gene expression of PFX#9, or that of khES-1, (iPSC from CBC with Yamanaka 4 factors-Sendai Virus on feeder) ((left panel)]. R^2: dicision coefficient

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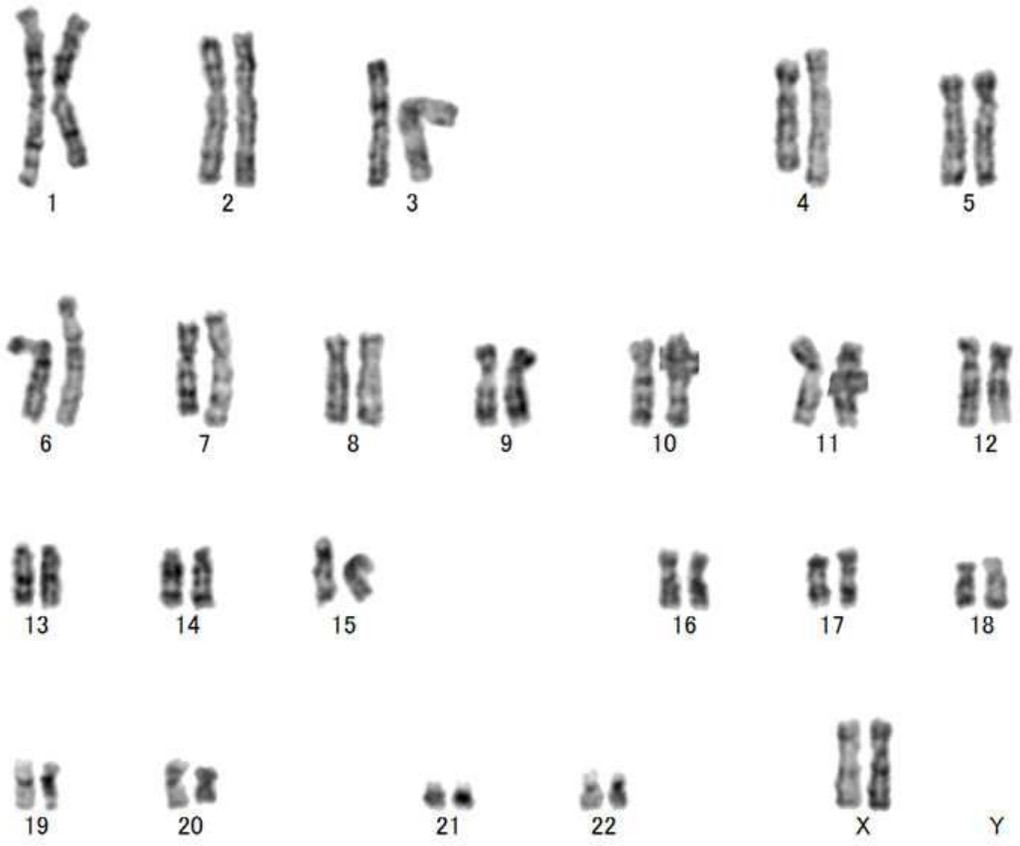

Figure 10. G-band karyotype analyses. PFX#9 (P45)

3.3.6. In vitro differentiation potentials of reprogrammed cells

The three germ layers differentiation potential of reprogrammed cells was tested via embryo body (EB) formation. Established ES cell-like clones were transferred to six-well, ultralow attachment plates (Corning) and cultured in DMEM/F12 containing 20% knockout serum replacement (KSR, Invitrogen) 2 mM L-glutamine, 1% NEAA, 0.1 mM 2-ME and 0.5% penicillin and streptomycin or ReproFF medium without bFGF to form EB. The medium was changed every other day. The resulting EBs were transferred to gelatin-coated plates for 16 days. Differentiation to ectodermal, mesodermal, or endodermal tissue was confirmed by detection of molecules related to three germ layers lineage differentiation such as α-feto-protein (endoderm), βIII-tubulin (ectoderm), GFAP (ectoderm), or Vimentin (mesoderm) with antibody against α-feto-protein (1:100 dilution MAB1368; R&D Systems), βIII-tublin (1:200 T4026; Sigma), GFAP(1:50 sc-6170 santa cruz biotechnology) or Vimentin (1:100 sc-5565; Santa Cruz Biotechnology) respectively. Antibodies were visualized with Alexa Fluor 488 goat anti-mouse (1:1,000; Invitrogen), Alexa Fluor 594 rabbit anti-mouse (1:1,000; Invitrogen), and Alexa Fluor 594 goat anti-rabbit (1:1,000; Invitrogen). Nuclei were stained with DAPI (1:1,000; Sigma) as shown in Figure 11.

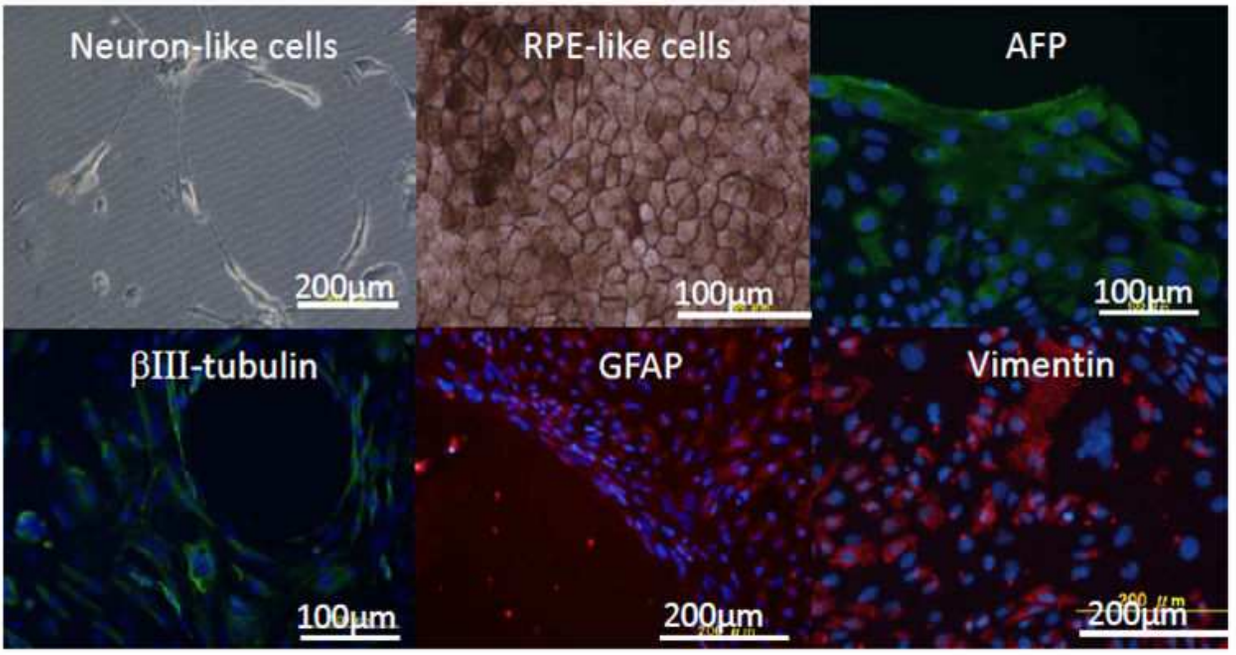

Figure 11. *In vivo* differentiation potential of established clones. Phase contrast images of neuron-like (top left) and retinal pigmented epithelium (RPE) differentiation (top middle) of established clone PFX#7. Cells were fixed and stained with antibodies against AFP, βIII-tubulin, GFAP and vimentin to identify specific cell lineages [18].

3.3.7. In vivo differentiation potential of reprogrammed cells by Teratoma formation assay

Reprogrammed cell lines should demonstrate differentiation potential reflecting three germ layers, *in vivo* as well as *in vitro*. To this end, one million iPSCs were injected beneath the testicular capsule of NOD-SCID mice (SLC Japan) to determine the ability of the transplanted cells to form teratomas containing cells of all three germ layers. Tumor formation was observed approximately four weeks after cell transplantation. Tumor tissues were fixed with 4% formalin, sectioned, and stained with hematoxylin and eosin (Figure 12).

3.3.8. Preservation of Feeder-free iPS cells

Human ES/iPS clones generated and maintained in a feeder-free system could be frozen in cell clumps using DMSO-free, chemically defined and serum-free freezing medium, CryoStem™ Freezing Medium (Stemgent), and could be cultured again on a Pronectin F-coated dish after thawing. Approximately 10-20% of the colony number scored before cryopreservation in CryoStem™ emerged after thawing.

3.3.9. Long-term, Low-cost and Stable maintenance of undifferentiated human induced pluripotent stem cells in feeder-free condition

Vitronectin provides a completely defined culture system for the maintenance of hiPSC under feeder-free conditions such as ReproFF2 medium (Figure 13, Figure 14, Table 5). This system

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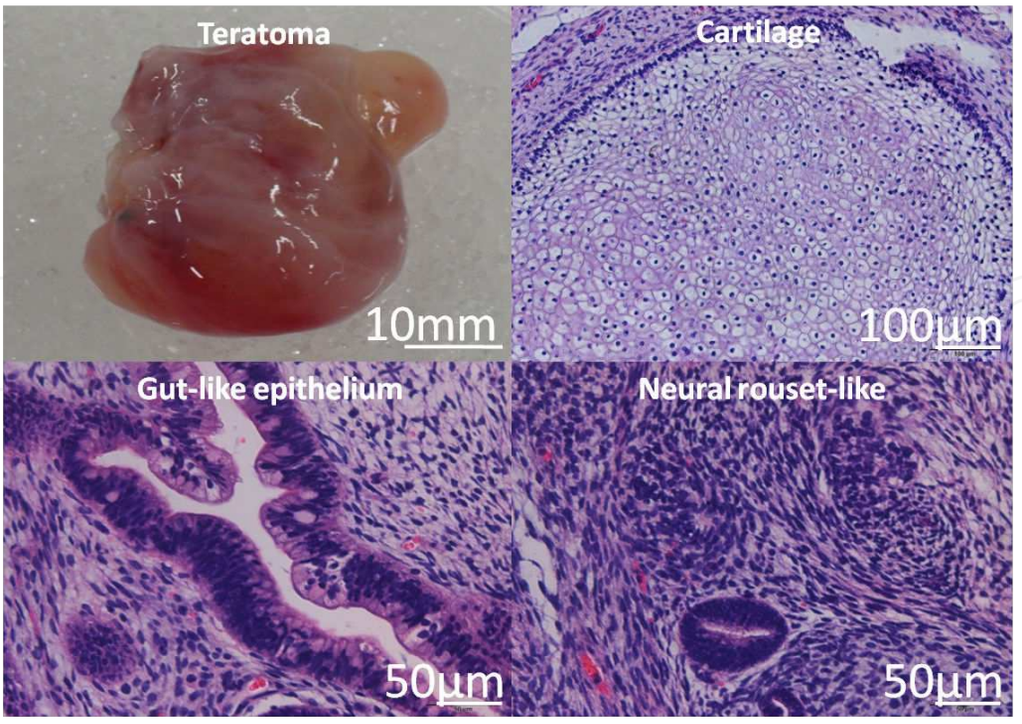

Figure 12. Teratoma with cystic structure. It was derived from iPSCs (PFX #9) implanted in the testicular capsule of a NOD-SCID mouse. It was stained with hematoxylin and eosin for histological observation.

allows complete control over the culture environment, resulting in more consistent cell populations and reproducible results in clinical applications.

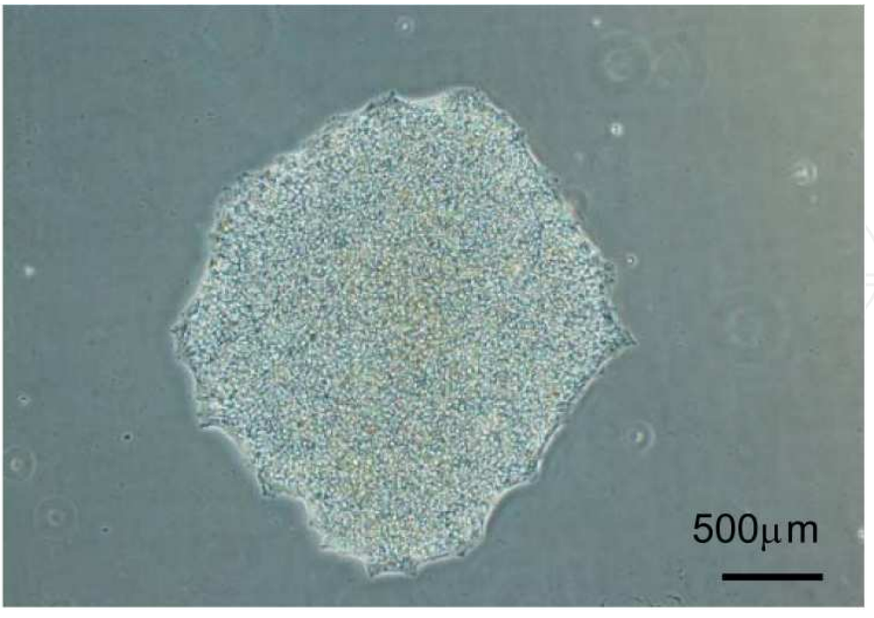

Figure 13. Maintenance of iPS cells (PFX#9) on recombinant vitronectin (VTN-N, Life Technology) in ReproFF2 medium.

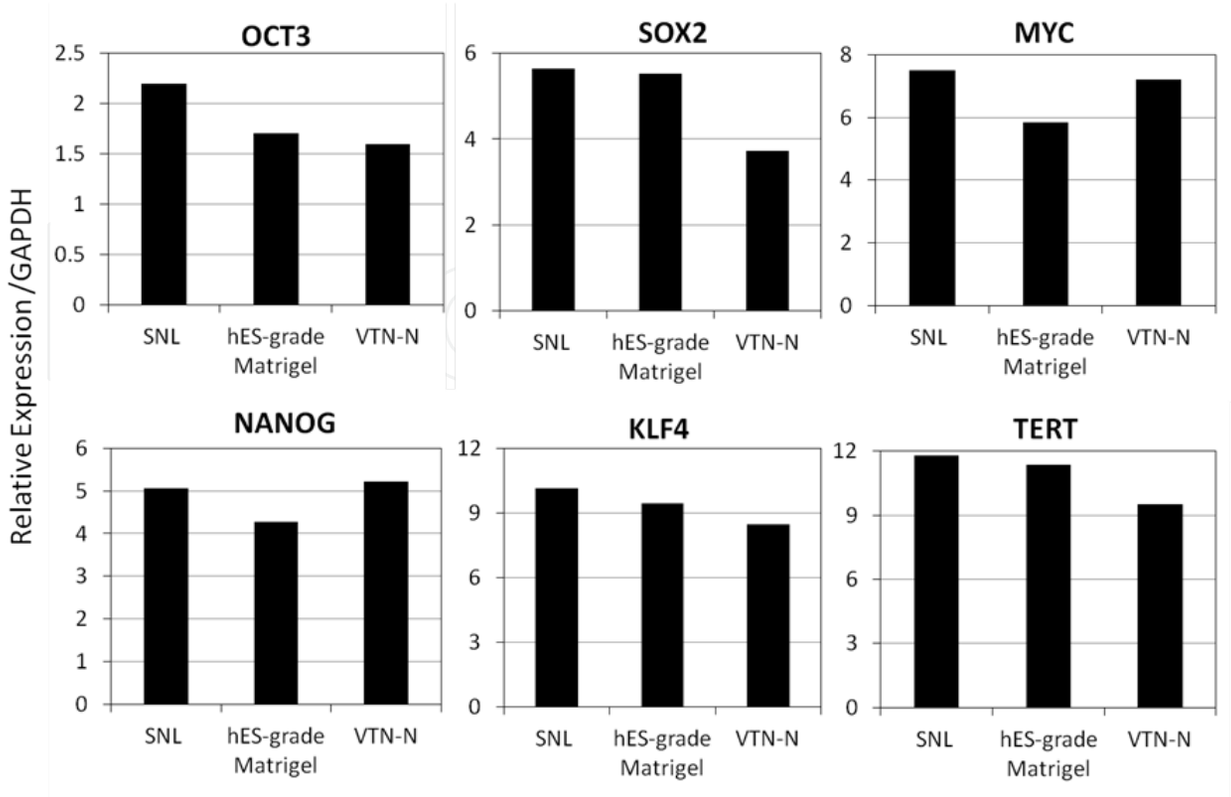

Figure 14. Expression of endogenous pluripotency related genes in iPSC (PFX#9) on VTN-N determined by qRT-PCR.

Following, the PFX#9 cells cultured with VTN-N was the gene expression of pluripotency markers comparable iPS cells cultured on Matrigel or on SNL in Figure 12. It was found that only a recombinant vitronectin (VTN-N) can be maintained in culture for long-term feeder free conditions.

Primers		Size(bp)	
hOCT3/4-F	GAA ACC CAC ACT GCA GCA GA	undifferentiated ES cell	103
hOCT3/4-R	TCG CTT GCC CTT CTG GCG		
hSOX2-F	GGG AAA TGG GAG GGG TGC AAA AGA GG	undifferentiated ES cell	151
hSOX2-R	TTG CGT GAG TGT GGA TGG GAT TGG TG		
hMYC-F	CGT CTC CAC ACA TCA GCA CAA	undifferentiated ES cell	68
hMYC-R	TCT TGG CAG CAG GAT AGT CCT T		
hKLF4-F	CGC TCC ATT ACC AAG AGC TCA T	undifferentiated ES cell	77
hKLF4-R	CGA TCG TCT TCC CCT CTT TG		
hTERT-F	CGT ACA GGT TTC ACG CAT GTG	undifferentiated ES cell	82
hTERT-R	ATG ACG CGC AGG AAA AAT GT		

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Primers		Size(bp)	
REX1-F	TGC AGG CGG AAA TAG AAC CT	undifferentiated ES cell	64
REX1-R	TCA TAG CAC ACA TAG CCA TCA CAT		
NANOG-F	CTC AGC TAC AAA CAG GTG AAG AC	undifferentiated ES cell	153
NANOG-R	TCC CTG GTG GTA GGA AGA GTA AA		
hGAPDH-F	CCA CTC CTC CAC CTT TGA CG	control	114
hGAPDH-R	ATG AGG TCC ACC ACC CTG TT		

Table 5. List of primers used for quantitative real-time PCR (qRT-PCR)

4. Conclusion

In this chapter we have shown the method for generating iPSC from non-cultured $CD34^+$cord blood cells using feeder-free conditions. The established cell clones were characterized at a single cell level. This robust iPSC generation method will solve some of the safety concerns related to tumorigenicity ariseing from chromosomal integration of exogenous genes and/or infection hazards associated with the use of by xenogeneic biological products in the culture system. These methods will contribute to future application of iPSCs-derived cell therapy.

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