# Chapter 2

# Derivation of Induced Pluripotent Stem Cells by Retroviral Gene Transduction in Mammalian Species

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## Abstract

Pluripotent stem cells can provide us with an enormous cell source for in vitro model systems for development. In 2006, new methodology was designed to generate pluripotent stem cells directly from somatic cells, and these cells were named induced pluripotent stem cells (iPSCs). This method consists of technically simple procedures: donor cell preparation, gene transduction, and isolation of embryonic stem cell-like colonies. The iPSC technology enables cell biologists not only to obtain pluripotent stem cells easily but also to study the reprogramming events themselves. Here, we describe the protocols to generate iPSCs from somatic origins by using conventional viral vectors. Specifically, we state the usage of three mammalian species: mouse, common marmoset, and human. As mouse iPSC donors, fibroblasts are easily prepared, while mesenchymal stem cells are expected to give rise to highly reprogrammed iPSCs efficiently. Common marmoset (*Callithrix jacchus*), a nonhuman primate, represents an alternative model to the usual laboratory animals. Finally, patient-specific human iPSCs give us an opportunity to examine the pathology and mechanisms of dysregulated genomic imprinting. The iPSC technology will serve as a valuable method for studying genomic imprinting, and conversely, the insights from these studies will offer valuable criteria to assess the potential of iPSCs.

**Key words:** Genomic imprinting, Induced pluripotent stem cells, Embryonic stem cells, Reprogramming, Pluripotency, Epigenetics, Germ cells, Cell culture, Common marmoset, Disease model

## 1. Introduction

Induced pluripotent stem cells (iPSCs) can be generated by transduction of various sets of defined factors into somatic cells (1, 2). Molecular and cellular properties of iPSCs are quite similar to those of embryonic stem cells (ESCs), and they have pluripotency in vivo and in vitro. Among pluripotent stem cells, there is a great advantage in utilizing iPSCs: facile derivation from individuals.

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Because of this, iPSC technology holds an enormous utility as a cell source for studying many genetic mutants, including those involved in human disease. In humans, dysregulation of imprinted genes is correlated with tumorigenesis or various disorders such as Beckwith–Wiedemann syndrome, Prader–Willi syndrome, and Angelman syndrome. Therefore, generation of patient-specific iPSCs could bring us better understanding of the pathology of genomic imprinting-related disorders (3). Moreover, iPSCs have become valuable cell sources for parthenogenesis, which is a unique model for studying genomic imprinting. It has been shown that parthenogenetic blastocysts and ESCs exhibit partial loss of imprinting (4). Recently, bimaternal parthenogenetic iPSCs were established from mouse neural stem cells (5). The iPSCs have lost the parthenogenetic imprinting pattern despite their origin, suggesting an attenuation of parthenogenetic imprinting through reprogramming process. Given that parthenogenetic cells generally exhibit growth defects, the iPSC technology would also provide ideal materials and methods to dissect this phenomenon as well.

Epigenetic regulation is intimately tied to the input and output of artificial reprogramming by somatic cell nuclear transfer (SCNT), cell fusion, and iPSC technology (6). For example, SCNT sometimes results in abnormal embryogenesis correlated with dysregulated genomic imprinting (7). Since iPSCs with somatic origin do not receive any germline-derived factors during reprogramming, it is important to examine the genomic imprinting pattern for quality assessment of iPSCs. Indeed, recent studies have revealed an important role of genomic imprinting in developmental potential of iPSCs. Gene expression profiling found aberrant silencing of the *Dlk1-Dio3* imprinted locus in mouse iPSCs, although overall gene expression was indistinguishable with that of ESCs (8, 9). The activation of the *Dlk1-Dio3* imprinted locus is positively correlated with a fully reprogrammed status, and notably, the developmental potency in partially reprogrammed iPSCs can be rescued by reactivation of this locus (8). Similarly, human iPSCs also exhibit aberrations in imprinted genes such as *H19* and *PEG3* in their allele-specific expression pattern, expression intensity, and DNA methylation status (10). Thus, the proper genomic imprint could serve as a vital marker to identify fully reprogrammed and clinically applicable iPSCs lines.

Obviously, the advantage of pluripotent stem cells in life sciences is their pluripotency and as an in vitro system to elucidate the mechanisms of development and differentiation. Since the pioneering work on germ cell production from ESCs in culture (11–13), it has been revealed that this potential is commonly observed among pluripotent stem cell lines (14–16). Furthermore, this propensity is valid for iPSCs as well; presumptive germ cells can be induced by in vitro differentiation of mouse and human iPSCs

(17–19). In these studies, genomic imprinting is a standard subject of analyses because germ cells undergo a dynamic alteration of genomic imprinting status in a developmental phase-specific manner. Hence, the imprinting status is one of the landmarks used to define iPSC-derived cells as "germ cells."

Here, we describe the protocols to obtain iPSCs from somatic cells derived from three mammalian species: mouse, common marmoset (*Callithrix jacchus*), and human. Because, to date, iPSCs have been successfully established in various animals using basically similar protocols (1, 20–25), the current methods could be applicable for other mammals of interest. We believe that this information will help accelerate elucidation of genomic imprinting with molecular and cellular biological approaches.

## 2. Materials

### *2.1. Mouse iPSCs from Fibroblasts*

#### *2.1.1. General Equipment*

1. Tissue culture plates and dishes: 100-mm, 6-, 24-, and 96-well (BD Falcon).
2. Conical tubes: 15- and 50-ml (BD Falcon).
3. Plastic disposable pipettes: 1-, 5-, 10-, and 25-ml (BD Falcon).
4. 0.22-μm Bottle-top filter (Techno Plastic Products, Trasadingen, Switzerland).
5. 0.22-μm Pore size filter (Millipore, Billerica, MA, USA).
6. 10-ml Disposable syringe (Terumo, Tokyo, Japan).
7. Cell-freezing container (Nalgene, Rochester, NY, USA).
8. Cryovial (Nunc, Waltham, MA, USA).

#### *2.1.2. Cell Culture*

1. PBS.
2. 0.25% (w/v) Trypsin/EDTA solution (Invitrogen).
3. Recovery Cell Culture Freezing Medium (Invitrogen).
4. mDMEM/10% FBS: DMEM containing 4.5 g/l glucose (Nacalai Tesque, Kyoto, Japan) supplemented with 10% (v/v) Fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 50 U/ml penicillin and 50 mg/ml streptomycin (Invitrogen). Filter with a 0.22-μm bottle-top filter and store at 4°C up to a week.
5. mESC medium: DMEM supplemented with 15% (v/v) FBS, 2 mM L-Glutamine (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen) (see Note 1), 50 U/ml penicillin and 50 mg/ml streptomycin, and 1,000 U/ml ESGRO (Millipore). Filter with a bottle-top filter and store at 4°C up to a week.

6. Gelatin-coated culture dishes: To prepare 10× stock solution, dissolve 1 g of gelatin powder (Sigma, St. Louis, MO, USA) in 100 ml of distilled water, autoclave, and store at 4°C for 2 months. To prepare 1× gelatin solution, warm the 10× gelatin stock to 37°C, add 50 ml of the stock to 450 ml of distilled water. Filter the solution with a bottle-top filter and store at 4°C up to 2 weeks. Add 0.1% (w/v) gelatin solution to cover the entire area of culture dishes. Incubate for at least 30 min at 37°C. Aspirate the solution immediately before plating cells.

*2.1.3. Preparation of Fibroblasts from Mouse Embryos and Adult Mouse Tail*

1. Sterilized forceps and scissors.

*2.1.4. Retrovirus Production*

1. pMXs vectors containing the cDNAs of *Oct4* (Plasmid 13366), *Sox2* (Plasmid 13367), *Klf4* (Plasmid 13370), *c-Myc* (Plasmid 13375), and *DsRed* (Plasmid 22724) (Addgene, Cambridge, MA, USA).
2. Plat-E packaging cells (Available from Dr. Toshio Kitamura at the University of Tokyo; kitamura@ims.u-tokyo.ac.jp).
3. Puromycin: Dissolve puromycin powder (Sigma) in distilled water at 10 mg/ml concentration, and filter it through a 0.22-μm filter (Millipore). Aliquot and store at −20°C.
4. Blastocidin S: Dissolve blastocidin S hydrochloride (Funakoshi, Tokyo, Japan) in distilled water at 10 mg/ml concentration, and filter it through a 0.22-μm filter. Aliquot and store at −20°C.
5. 0.05% Trypsin/EDTA: Mix 10 ml of 0.25% (w/v) Trypsin/EDTA solution (Invitrogen) and 40 ml of PBS. Store at −20°C.
6. Opti-MEM I Reduced-Serum Medium (Invitrogen).
7. FuGENE 6 transfection reagent (Promega, Madison, WI).
8. 0.45-μm cellulose acetate filter (Schleicher & Schuell, Keene, NH, USA).
9. Polybrene solution: To prepare the stock solution at 8 mg/ml concentration, dissolve 80 mg of polybrene (Nacalai Tesque) in 10 ml of distilled water and filter it through a 0.22-μm filter. Store at 4°C.

*2.1.5. iPSCs' Derivation from Mouse Fibroblasts*

1. SNL medium: DMEM supplemented with 7% (v/v) FBS, 2 mM L-Glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. Filter with a bottle-top filter and store at 4°C up to a week.
2. SNL feeder cells: SNL cells (SNL 76/7; DS Pharma Biomedical, Osaka, Japan) are a derivative of STO cells, which express

neomycin-resistant gene and leukemia inhibitory factor (LIF). Cultivate the cells with SNL medium on gelatin-coated culture dishes. At 80–90% confluency in 100-mm culture dishes, add 0.3 ml of 0.4 mg/ml mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan) solution to the culture medium and incubate at 37°C for 2.5 h (see Note 2). After washing with 10 ml of PBS twice, trypsinize, and count the cell number. Resuspend with SNL medium and seed the cells on gelatin-coated culture dishes at $1 \times 10^6$ cells per 100-mm culture dish. Use within 2 weeks.

### 2.2. Mouse iPSCs from Mesenchymal Stem Cells

#### 2.2.1. General Equipment

1. Tissue culture plates and dishes: 100- and 60-mm dish (BD Falcon).
2. Conical tubes: 15- and 50-ml (BD Falcon).
3. Plastic disposable pipettes: 1-, 5-, 10-, and 25-ml.
4. 0.22-μm bottle-top filter (Techno Plastic Products).

#### 2.2.2. Animals

1. *Nanog*$^{\text{GFP-IRES-Puro}}$ mice (available from RIKEN BioResource Center, Tsukuba, Japan).

#### 2.2.3. Cell Culture

1. PBS.
2. 2× PBS containing 4% FBS: 10× PBS is diluted five times with sterile water and supplemented with 4% (v/v) FBS.
3. HBSS$^{+}$: HBSS (Nacalai Tesque) supplemented with 2% (v/v) FBS, 10 mM HEPES, and 50 U/ml penicillin and 50 mg/ml streptomycin (Invitrogen).
4. Mesenchymal stem cell (MSC) medium: MEM Alpha + GlutaMAX-I (GIBCO) supplemented with 10% (v/v) FBS, 10 mM HEPES, and 50 U/ml penicillin and 50 mg/ml streptomycin.
5. mESC medium: see Subheading 2.1.2, item 5.

#### 2.2.4. Preparation of Bone Marrow Cell Suspension

1. Enzymatic dissociation solution: 0.2% (w/v) collagenase (Wako, Osaka, Japan) in DMEM containing 1.0 g/l glucose supplemented with 10 mM HEPES and 50 U/ml penicillin and 50 mg/ml streptomycin.
2. Cell strainer: 70-μm pore size (BD Falcon).
3. Sterile water.

#### 2.2.5. Purification of MSCs

1. Fluorescently conjugated antibodies (eBioscience, San Diego, CA, USA): PE-conjugated CD45 (Clone: 30-F11, 12-0451), TER119 (Clone: TER-119, 12-5921), APC-conjugated PDGFRα (Clone: APA5, 17-1401), and FITC-conjugated Sca-1 (Ly6A/E, Clone: D7, 11-5981).

2. Fluorescently conjugated isotype controls (eBioscience): Rat IgG2b K Isotype Control PE (12-4031), Rat IgG2a K Isotype Control APC (17-4321), and Rat IgG2a K Isotype Control FITC (11-4321).
3. Propidium iodide solution (Sigma).
4. Triplelaser cell sorter such as MoFlo (Dako) and JSAN (Bay Bioscience).

*2.2.6. Retrovirus Production*

1. pMXs vectors: see Subheading 2.1.4, item 1.
2. Plat-E packaging cells (available from Dr. Toshio Kitamura at the University of Tokyo; kitamura@ims.u-tokyo.ac.jp).
3. mDMEM/10% FBS: see Subheading 2.1.2, item 4.
4. 0.05% Trypsin/EDTA: see Subheading 2.1.4, item 5.
5. FuGENE 6 transfection reagent (Promega).
6. 0.45-μm Cellulose acetate filter (Schleicher & Schuell).
7. Polybrene solution: see Subheading 2.1.4, item 9.

*2.2.7. Induction of iPSCs from PαS Cells*

1. SNL medium: see Subheading 2.1.5, item 1.
2. SNL feeder cells: see Subheading 2.1.5, item 2.
3. Puromycin: see Subheading 2.1.4, item 3.

### *2.3. Marmsoet iPSCs from Fetal Liver Cells*

*2.3.1. General Equipment Required Through Experiments*

1. Tissue culture plates and dishes: 100-mm (Greiner bio-one, Frickenhausen, Germany) and 96-well (Iwaki, Tokyo, Japan).
2. Gelatin-coated culture dishes: 100-mm and 12-well (Iwaki).
3. Conical tubes: 15- and 50-ml (BD Falcon).
4. Plastic disposable pipettes: 1-, 5-, 10- (BD Falcon, 357551), and 25-ml (BD Falcon).

*2.3.2. Cell Culture*

1. cjDMEM/10% FBS: Dulbecco's modified Eagle's medium (Wako) supplemented with 10% (v/v) FBS (Biowest) and 1% (v/v) antibiotic–antimycotic solution (Invitrogen).
2. cjESC medium: Knockout DMEM (Gibco) supplemented with 10% (v/v) Knockout Serum Replacement (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM MEM nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), and 1% (v/v) antibiotic–antimycotic solution (Gibco).
3. Trypsin solution for ESCs: 0.25% (v/v) Difco trypsin 250 (BD, Baltimore, MD, USA), 1 mM $CaCl_2$, and 20% (v/v) KSR.
4. Hank's buffered salt solution without calcium or magnesium (Gibco).

*2.3.3. Virus Production*

1. pMXs retroviral vectors carrying human *OCT4* (Addgene, Plasmid 17217), *SOX2* (Addgene, Plasmid 17218), *KLF4* (Addgene, Plasmid 17219), *C-MYC* (Addgene, Plasmid 17220), *NANOG* (kindly provided by Dr. Yamanaka), *LIN28* (kindly provided by Dr. Yamanaka), and *GFP* (kindly provided by Dr. Yamanaka) (see Note 3).
2. pVSV-G vector and GP-2 cells (Retroviral Gene Transfer and Expression; TaKaRa, Shiga, Japan).
3. Opti-MEM I Reduced-Serum Medium (Invitrogen).
4. FuGENE 6 transfection reagent.
5. 0.45-μm pore-size cellulose acetate filter (Sartorius, Goettingen, Germany).
6. Poly-L-lysine (Sigma).

*2.3.4. Preparation of Fetal Liver Cells*

1. Sterilized forceps and scissors.
2. Collagenase solution: Dissolve Collagenase type I in DMEM at 0.5% (w/v) concentration.

*2.3.5. Retroviral Infection of Marmoset Cells*

1. Polybrene (Nacalai Tesque).
2. Mitomycin C-treated or irradiated MEF feeder cell plates.

*2.3.6. Passage of iPSCs*

1. Cell strainer, 100-μm nylon (BD Falcon).
2. Mitomycin C-treated or irradiated MEF feeder cell plates.

*2.3.7. Storage of Established iPSCs*

1. Cell Banker 2 (ZENOAQ, Koriyama, Fukushima, Japan).
2. 2-ml plastic cryogenic vial (Iwaki).

### *2.4. Human iPSCs from Fibroblasts*

*2.4.1. General Equipment*

1. Tissue culture plates and dishes: 100-mm (FPI, Kobe, Japan), 6-, 24-, and 96-well (Nunc).
2. Conical tubes: 15- and 50-ml (Greiner).
3. Plastic disposable pipettes: 2-, 5-, 10-, 25-, and 50-ml (Greiner).
4. 0.22-μm bottle-top filter (Techno Plastic Products).
5. 0.22-μm pore size filter (Millipore).
6. 10-ml disposable syringe (Terumo).
7. Cryovial (Nunc).

*2.4.2. Cell Culture*

1. PBS.
2. 2.5% Trypsin.
3. 0.25% Trypsin/EDTA solution and 0.05% Trypsin/EDTA solution.
4. Water (Sigma).
5. Gelatin-coated culture dishes: see Subheading 2.1.2, item 6.
6. mDMEM/10% FBS: see Subheading 2.1.2, item 4.

*2.4.3. Preparation and Culture of Human Dermal Fibroblasts*

1. Dermapunch (Maruho, Osaka, Japan).
2. Sterilized forceps and scissors.
3. Cell Banker 2 (ZENOAQ).

*2.4.4. Lentivirus Production*

1. pLenti6/UbC vector containing mouse *Slc7a1* gene (Plasmid 17224, Addgene).
2. 293FT cells (Invitrogen).
3. CalPhos Mammalian Transfection kit (TaKaRa).
4. Virapower Lentiviral expression system (Invitrogen).
5. Solution A: Dilute 3 μg of Virepower packaging mix (pLP1, pLP2, and pLP/VSVG mixture) and 1 μg of pLenti6/UbC/mSlc7a1 in 12.4 μl of 2 M Calcium Solution, and add up to 100 μl with sterile water.
6. Solution B: Transfer 100 μl of 2× HBS into a 60-mm dish.
7. 0.45-μm pore size cellulose acetate filter.
8. Blastocidin S hydrochloride: see Subheading 2.1.4, item 4.

*2.4.5. Retrovirus Production*

1. pMXs retrovial vectors containing the cDNAs of human *OCT4* (Plasmid 17217), human *SOX2* (Plasmid 17218), human *KLF4* (Plasmid 17219), and human *C-MYC* (Plasmid 17220) (Cell biolabs, Inc., San Diego, CA, USA; http://www.cellbiolabs.com/).
2. Plat-E packaging cells (available from Dr. Toshio Kitamura at the University of Tokyo; kitamura@ims.u-tokyo.ac.jp).
3. OPTI-MEM I.
4. FuGENE 6 transfection reagent.
5. Polybrene solution: see Subheading 2.1.4, item 9.
6. Puromycin: see Subheading 2.1.4, item 3.
7. Blastocidin S hydrochloride: see Subheading 2.1.4, item 4.

*2.4.6. Induction of iPSCs from Human Fibroblasts*

1. SNL medium: see Subheading 2.1.5, item 1.
2. SNL cells: see Subheading 2.1.5, item 2.
3. 0.4 mg/ml mitomycin C: Dissolve 10 mg of mitomycin C in 25 ml of water. Filter through a 0.22-μm pore size filter, aliquot, and store at –20°C.
4. SNL feeder cells: Incubate SNL cells at 80–90% confluency with 12 μg/ml mitomycin C for 2 h and 15 min in 37°C, 5% $CO_2$ incubator. Wash the cells with 4.5 ml of PBS twice, trypsinize, and count the cell number. Plate the cells at $2.6\times10^4$ per $cm^2$ onto gelatin-coated culture dishes; $1.5\times10^6$ cells/dish (100-mm culture dish), $2.5\times10^5$ cells/well (6-well culture plate), and $5.2\times10^4$ cells/well (24-well plate) (see Note 4).

5. hESC medium: DMEM/F12 containing 20% KSR, 2 mM L-glutamine, 0.1 mM non-essential amino acids (Sigma), 0.1 mM 2-mercaptoethanol (Sigma), and 50 U and 50 mg/ml penicillin and streptomycin. Filter through a 0.22-μm bottle-top filter and store at 4°C up to 2 weeks.
6. 50 μg/ml FGF-2: Dissolve 1 mg of FGF-2 (PeproTech, Rocky Hill, NJ, USA) in 20 ml of hESC medium. Aliquot and store at –20°C.

*2.4.7. Picking, Expanding, Freezing, and Thawing Human iPSCs*

1. 10 mg/ml Collagenase IV: Dissolve 1 g of collagenase IV (Invitrogen) in 100 ml of water, and filter through a 0.22-μm pore size filter. Aliquot and store at –20°C.
2. 0.1 M $CaCl_2$: Dissolve 555 mg of $CaCl_2$ in 50 ml of water, and filter through a 0.22-μm pore size filter. Store at 4°C.
3. CTK solution: Mix 10 ml of 2.5% Trypsin, 10 ml of 10 mg/ml collagenase IV, 1 ml of 100 mM $CaCl_2$, and 20 ml of KSR with 59 ml of PBS (26). Aliquot and store at –20°C. Avoid repeated freezing and thawing. Store at 4°C up to 1 week.
4. Cell scraper (Iwaki).
5. 10 mM Y-27632: Dissolve 5 mg of Y-27632 (Wako) in 1.48 ml of sterile water. Aliquot and store at –20°C.
6. DAP213 solution: Mix 1.42 ml of DMSO (Sigma), 0.59 g of acetamide (Sigma), and 2.2 ml of propylene glycol (Sigma) with 6 ml of hESC medium. Filter through a 0.22-μm pore size filter and store at –80°C.

## 3. Methods

### *3.1. Mouse iPSCs from Fibroblasts*

In most experiments of iPSC generation, reprogrammed cells have been selected based on the expression of fluorescence protein or drug-resistance genes driven by the promoter of pluripotency-related genes such as *Nanog* and *Oct4*. Although this helps to select highly reprogrammed cells, it is not always necessary to take advantage of the system. For a wider usage of the iPSC technique, in this part, we described the mouse iPSCs' generation from embryonic and adult fibroblasts without reporter-dependent selection.

*3.1.1. Preparation of Fibroblasts from Mouse Embryos*

1. Euthanize female mice on the day 13.5 of pregnancy by cervical dislocation (see Note 5). Wipe with 70% ethanol, and isolate uteri using sterilized forceps and scissors into 100-mm culture dishes containing PBS. Separate the embryos from their placenta and wash them with PBS twice. Remove the embryo's head, visceral tissues, and gonads.

2. Transfer the remaining bodies to a new 100-mm culture dish containing PBS and mince them into small pieces. Transfer into a 50-ml conical tube containing 0.25% Trypsin/EDTA solution (3 ml per embryo) and incubate at 37°C for 20 min. Then, add an additional 0.25% Trypsin/EDTA solution (3 ml per embryo) and incubate at 37°C for 20 min. Invert the tube gently several times and add an equal amount of mDMEM/10% FBS (6 ml per embryo). Pipette up and down to dissociate the tissues.
3. Centrifuge at 200 g for 5 min, discard the supernatant, and resuspend the pellet in mDMEM/10% FBS. Count the cell number and plate $1 \times 10^7$ cells per 100-mm gelatin-coated culture dish containing 10 ml of mDMEM/10% FBS. Incubate at 37°C with 5% $CO_2$ overnight (passage 1), and the next day replace the medium to remove floating cells.
4. When the cells grow to confluency, split or freeze them at 1:4 dilution. Remove the medium, wash once with PBS, and trypsinize with 1 ml of 0.25% Trypsin/EDTA at 37°C for 5 min. Then, add 9 ml of mDMEM/10% FBS and resuspend by pipetting. For passage, split the cells to new 100-mm gelatin-coated culture dishes at 1:4 dilution (passage 2) (see Note 6).
5. To prepare the freeze stocks, transfer the cell suspension to 15-ml conical tubes and centrifuge at 200 g for 5 min. Discard the supernatant and resuspend the cells with Recovery Cell Culture Freezing Medium. Aliquot 1 ml of the cell suspension per freezing vial. Keep the vials in a cell-freezing container at –80°C overnight and then transfer them into a liquid nitrogen tank.

*3.1.2. Preparation of Fibroblasts from Adult Mouse Tail*

1. Cut the tail from an adult mouse and wash with PBS (see Note 5). Incise using sterilized scissors, peel superficial dermis by hand, and mince the remaining tail into 1-cm pieces with scissors. Place two pieces per well of 6-well gelatin-coated plates, add 2 ml of mDMEM/10% FBS, and incubate at 37°C with 5% $CO_2$ for 5 days.
2. Remove the tissues of tails and replace the medium with 2 ml of fresh mDMEM/10% FBS. When they reach confluency, aspirate the medium, wash twice with 2 ml of PBS, add 0.3 ml of 0.25% Trypsin/EDTA, and incubate at 37°C for 10 min. Add 2 ml of mDMEM/10% FBS, suspend the cells, and transfer to a 15-ml conical tube. Centrifuge the cells at 200 g for 5 min.
3. Discard the supernatant, resuspend the cells with 10 ml of mDMEM/10% FBS, and plate to a 100-mm gelatin-coated culture dish (passage 2). When the cells become confluent, trypsinize with 1 ml of 0.25% Trypsin/EDTA at 37°C for 5 min, and resuspend with 9 ml of mDMEM/10% FBS.

Passage to new 100-mm gelatin-coated culture dishes at 1:4 dilution (passage 3). These cells usually become confluent within 3–4 days (see Note 6).

*3.1.3. Retrovirus Production*

1. Thaw a vial of Plat-E cells in 37°C water bath. Resuspend the cells with 10 ml of mDMEM/10% FBS and transfer to a 100-mm gelatin-coated culture dish. Incubate the cells in 37°C, 5% $CO_2$ incubator. From the next day onwards, cultivate the cells in 10 ml of mDMEM/10% FBS supplemented with 1 μg/ml puromycin and 10 μg/ml blastocidin S. Split the cells at 1:5 dilution when they reach confluency.
2. Twenty-four hours before transfection, aspirate the medium, gently wash with PBS once, and add 1 ml of 0.05% Trypsin/EDTA. After incubation at room temperature for 5 min, suspend with 10 ml of mDMEM/10% FBS, and transfer to a 50-ml conical tube. Count the cell number and plate the cells in mDMEM/10% FBS at $3.6 \times 10^6$ cells per 100-mm culture dish, $1.5 \times 10^6$ cells per 60-mm culture dish, or $6 \times 10^5$ cells per well of a 6-well culture plate. For the four iPSC factors to be transduced, prepare five culture dishes to transfect the five plasmids pMXs-Oct4, Sox2, Klf4, c-Myc, and DsRed separately.
3. Transfer 0.3 ml of Opti-MEM I Reduced-Serum Medium to 1.5-ml plastic tubes. Add 27 μl of FuGENE 6 transfection reagent, mix gently by tapping, and incubate at room temperature for 5 min. Then, add 9 μg of pMXs plasmid DNA, mix gently by finger tapping, and incubate at room temperature for 15 min (see Note 7).
4. Add the DNA/FuGENE 6 mixture to the Plat-E cell culture dishes dropwise and incubate at 37°C, 5% $CO_2$ overnight. Replace the medium with 10 ml of fresh mDMEM/10% FBS and further incubate overnight.
5. Collect the supernatants from the Plat-E cell culture dishes and filter them through a 0.45-μm cellulose acetate filter (Fig. 1). Combine an equal volume of the virus supernatants containing each factor. For the transduction of four iPSC factors, mix the supernatants of *Oct4*, *Sox2*, *Klf4*, *c-Myc*, and *DsRed* at 1:1:1:1:4 ratio. For three iPSC factors without *c-Myc*, mix the supernatants of *Oct4*, *Sox2*, *Klf4*, and *DsRed* at 1:1:1:3. Add polybrene solution to the virus supernatant mixture at the final concentration of 4 μg/ml and mix gently. Use immediately for transduction (see Note 8).

*3.1.4. iPSCs' Derivation from Mouse Fibroblasts*

1. Twenty-four hours before retroviral gene transduction, trypsinize MEF or TTF within passage 3, and plate $8 \times 10^5$ cells per 100-mm gelatin-coated culture dishes. Prepare one extra culture dish for transduction of *DsRed* in addition to those for the iPSC factors. The next day, aspirate the medium and add

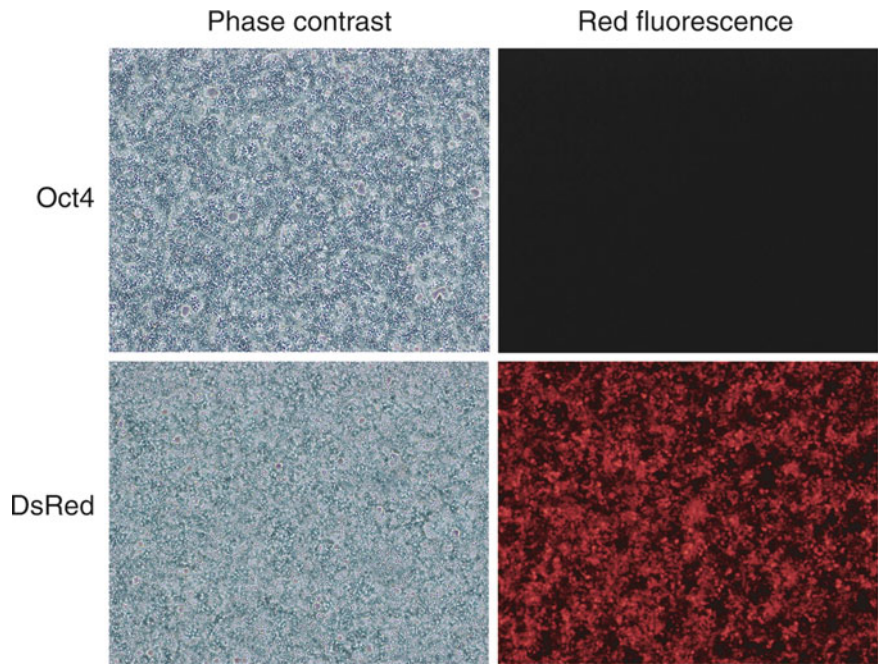


Fig. 1. Plat-E packaging cells after transfection of pMXs retrovirus plasmids. Phase and fluorescence images of Plat-E cells just before collection of virus supernatants (*Oct4* and *DsRed*). The Plat-E cells with pMXs-DsRed transfection show high Red fluorescence when the virus is properly produced.

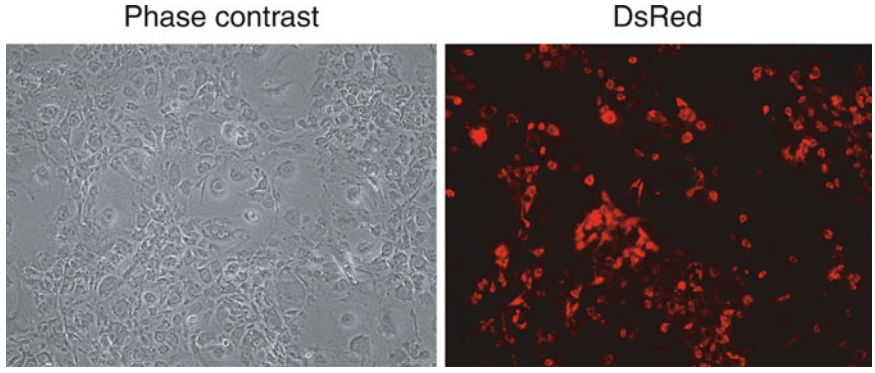


Fig. 2. Fibroblasts with successful gene transduction. Retroviral gene transduction can be monitored by red fluorescence in mouse fibroblasts infected with the pMXs-DsRed retrovirus. The image was photographed after replating onto SNL feeder cells.

the retrovirus supernatant mixture prepared at step 5 of Subheading 3.1.3. Incubate the cells at 37°C, 5% $CO_2$ overnight and replace the medium with 10 ml of fresh mDMEM/10% FBS. Two days later, exchange the medium with 10 ml of fresh mDMEM/10% FBS again (see Note 9) (Fig. 2).

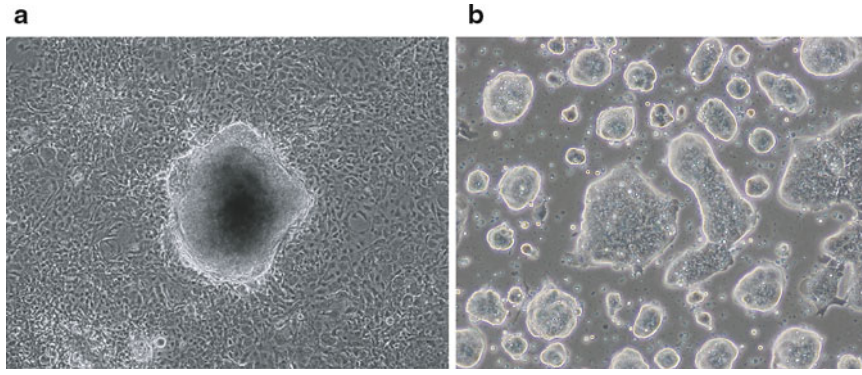


Fig. 3. Derivation of mouse fibroblast-derived iPSCs. (**a**) Morphology of mouse iPSC colony derived from fibroblasts (with three iPSC factors: *Oct4*, *Sox2*, *Klf4*) just before picking. (**b**) Expansion culture of fibroblast-derived mouse iPSCs. The image was photographed at passage 8 on gelatin-coated culture dish.

2. On the day 8 after transduction, trypsinize the cells with 0.25% Trypsin/EDTA. Resuspend with 10 ml of mDMEM/10% FBS and count the cell number. Replate them at a density of $0.5–5 \times 10^4$ cells (with four iPSC factors) or $3.5 \times 10^5$ cells (with three iPSC factors) per 100-mm culture dishes with SNL feeder cells (see Note 10). Incubate the cells at 37°C, 5% $CO_2$ overnight and replace the medium with 10 ml of mESC medium the next day.
3. Exchange the medium with 10 ml of fresh mESC medium every other day until the emerging iPSC colonies grow large enough to be picked (Fig. 3a).

*3.1.5. Picking and Expanding iPSC Colonies*

1. Aliquot 20 µl of 0.25% Trypsin/EDTA per well of a 96-well culture plate. Then, aspirate the mESC medium and wash the cells with 10 ml of PBS once. Add 5 ml of PBS, pick colonies with ESC-like morphology using a 10-µl pipette, and transfer each colony to one well of the 96-well culture plate. When picking iPSC colonies, choose the colonies without DsRed fluorescence to select highly reprogrammed cells (see Note 11). Incubate at 37°C for 15 min.
2. Add 180 µl of mESM medium to each well and pipette up and down to dissociate the colony into single cells. Transfer each cell suspension into one well of a 24-well plate with SNL feeder cells and add 300 µl of mESC medium. Incubate at 37°C, 5% $CO_2$ until the cells grow to 50–60% confluency.
3. To expand the iPSCs, aspirate the medium, wash with PBS once, and add 0.1 ml of 0.25% Trypsin/EDTA per well of a 24-well culture plate. Incubate at 37°C for 10 min and add 0.4 ml of mESC medium. Carefully pipette up and down to obtain a single-cell suspension and transfer to a well of 6-well culture plates. Add 1.5 ml of mESC medium and cultivate at 37°C, 5% $CO_2$ until the cells reach 80–90% confluency (Fig. 3b).

*3.1.6. Freezing and Thawing iPSCs*

1. Aspirate the medium, wash with PBS once, and add 0.3 ml of 0.25% Trypsin/EDTA per well of 6-well culture plates. Incubate at 37°C for 10 min. Add 2 ml of mESC medium and carefully pipette up and down to obtain single-cell suspension. Transfer the cell suspension to a 15-ml conical tube.
2. Centrifuge the tube at 200 g for 5 min. Discard the supernatant and resuspend the cells with Recovery Cell Culture Freezing Medium at $1–2 \times 10^6$ cells/ml. Aliquot 1 ml of the cell suspension per freezing vial. Keep the vials in a cell-freezing container at −80°C overnight and then transfer them into a liquid nitrogen tank the next day.
3. To thaw the iPSC freeze stocks, warm the vials in 37°C water bath until half of the ice crystals disappear. Transfer the cell suspension into a 15-ml conical tube containing 9 ml of mDMEM/10% FBS. Centrifuge at 200 g for 5 min and gently resuspend the cells with 2 ml of mESC medium. Plate the cells to a well of a 6-well culture plate with SNL feeder cells.

### *3.2. Mouse iPSCs from Mesenchymal Stem Cells*

MSCs are defined as plastic-adherent, fibroblast-like cells which undergo sustained in vitro growth and can give rise to multiple mesenchymal lineages (bone, adipose and cartilage tissue, etc.). We previously established a method for isolating highly enriched MSCs from adult murine bone marrow based on their expression of PDGFRα and Sca-1 (27). The iPSCs generated from purified MSCs (PαS) by *Oct4*, *Sox2*, and *Klf4* seem to be the closest equivalent to ESCs by global gene profile and germline transmission, compared with those from PDGFRα$^-$/Sca-1$^-$ osteoprogenitors and tail-tip fibroblasts (28). These results suggest that tissue stem cells could be a promising cell source for producing high-quality iPSCs.

*3.2.1. Preparation of Bone Marrow Cell Suspension*

1. Dissect femurs and tibias from adult mice (3–20 mice) and remove residual tissues from the bones. Wash with PBS three times.
2. Put the bones on a mortar and crush them with a pestle (see Note 12). Wash the crushed bones several times with HBSS$^+$ to remove the hematopoietic cells.
3. Incubate the bone fragments in 20 ml of enzymatic dissociation solution in 50-ml conical tube for 1 h at 37°C with shaking (110 rpm/min). Filter the suspension through a cell strainer (70-μm pore size), and collect the cells by centrifugation at 280 g for 7 min at 4°C.
4. Discard the supernatant. Resuspend the pellet with 1 ml of sterile water for 5–10 s to burst red blood cells, and add 1 ml of 2× PBS containing 4% FBS. At this step, cell debris can be seen. Then, resuspend the cells in 10 ml of HBSS$^+$. To remove

the debris, filter the cell suspension through a cell strainer (70-μm pore size).

5. Collect the cells by centrifugation at 280 g for 7 min at 4°C. Discard the supernatant, and resuspend the cells in 1 ml of $HBSS^+$.

*3.2.2. Purification of MSCs from Bone Marrow Cell Suspension*

1. To compensate the fluorescence interference, transfer approximately $1 \times 10^5$ cells into five 1.5-ml plastic tubes as "control tubes." Add a single fluorescence-conjugated antibody (PE, APC, or FITC) to the "control tubes" one by one. The final concentration of antibodies is 0.8 (anti-CD45), 1 (anti-TER119), 1.4 (anti-PDGFRα), and 2.5 μg/ml (anti-Sca-1). Prepare one extra tube for an unstained (negative) control. Avoid light exposure and incubate the tubes at 4°C for 30 min.
2. Add all fluorescence-conjugated antibodies to the remaining cell suspension in 50-ml conical tube as "sample tube." Avoid light exposure and incubate the tube at 4°C for 30 min.
3. Add 500 μl and 9 ml of $HBSS^+$ to the "control tubes" and "sample tube," respectively. Centrifuge the "control tubes" at 800 g for 3 min at 4°C. For the "sample tube," centrifuge at 280 g for 7 min at 4°C. Discard the supernatant, and add 300–500 μl and 5–9 ml of $HBSS^+$ containing 2 μg/ml PI to the "control tubes" and the "sample tube," respectively.
4. For sorting cells, use a triplelaser cell sorter such as MoFlo (Dako) or JSAN (Bay Bioscience), following to the instrument calibration and standardization by the protocols established in your laboratory. Compensate each laser using the "control tubes," measure PI fluorescence, and define a live cell gate excluding PI-positive cells. Define additional gates to isolate the cells positive for PDGFRα and Sca-1 but negative for CD45 and TER119, according to the isotype control fluorescence intensity (see Note 13). Routinely, 0.1–2% of bone marrow cells are $CD45^-/TER119^-$, and 10–20% of $CD45^-/TER119^-$ cells are $PDGFR\alpha^+/Sca\text{-}1^+$ (Fig. 4).
5. Collect the sorted $PDGFR\alpha^+/Sca\text{-}1^+/CD45^-/TER119^-$ cells by centrifugation at 280 g for 7 min at 4°C. Discard the supernatant and resuspend the cells with 1 ml of MSC medium. Repeat this washing step again.
6. Plate the sorted cells onto a 100-mm tissue culture dish containing 10 ml of MSC medium. Exchange the medium every 3 days until the cells reach confluency (Fig. 5a).

*3.2.3. Retrovirus Production*

1. Seed Plat-E cells at $8 \times 10^6$ cells per 100-mm dish.
2. On the next day, introduce 9 μg of pMX-based retroviral vectors for *DsRed*, *Oct4*, *Sox2*, *Klf4*, and *c-Myc* individually into

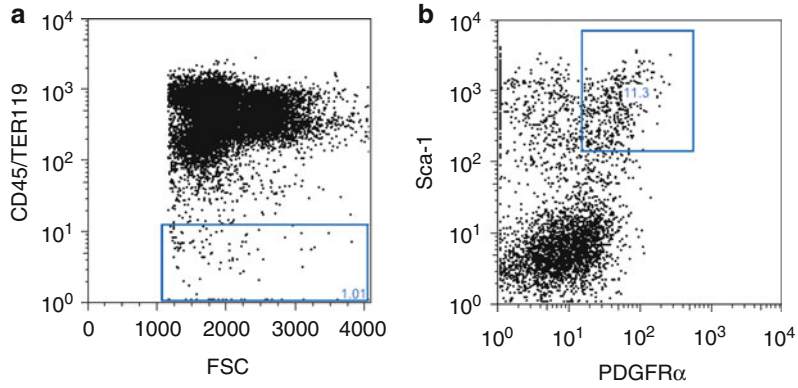


Fig. 4. Isolation of mouse PαS MSCs from adult bone marrow. (**a**) Cell sorter profile of CD45⁻/TER119⁻ non-blood cells in whole bone marrow cells. In this experiment, 1.01% of cells are CD45⁻/TER119⁻. (**b**) Cell sorter profile of PDGFRα⁺/Sca-1⁺ MSCs in CD45⁻/TER119⁻ cells. PDGFRα⁺/Sca-1⁺ cells were separated after gating on CD45⁻ and TER119⁻. In this experiment, 11.3% of cells are PDGFRα⁺/Sca-1⁺. In toto, 0.01–0.4% of cells are usually isolated as PαS MSCs from bone marrow.

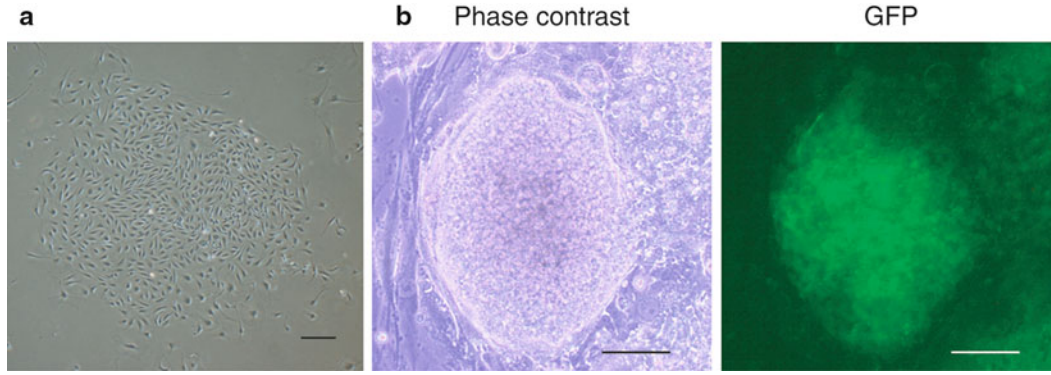


Fig. 5. Derivation of mouse PαS-derived iPSCs. (**a**) Morphology of PαS MSCs purified from adult bone marrow. (**b**) Phase and fluorescence images of PαS-derived iPSC colonies. When using transgenic mice with *Nanog*$^{GFP\text{-}IRES\text{-}Puro}$, fully reprogrammed iPSCs are visualized by GFP fluorescence driven by the promoter of pluripotency marker gene *Nanog*.

separate dishes of Plat-E cells using 27 μl of FuGENE 6 transfection reagent.

3. After 24 h, replace the medium with 10 ml of mDMEM/10% FBS and incubate overnight. Collect the virus-containing supernatants from the Plat-E cultures and filter them through 0.45-μm cellulose acetate filters.
4. Make a mixture of equal volumes of supernatants containing four or three (without c-Myc) iPSC factors and DsRed retroviruses. DsRed is used as a marker for infection efficiency and transgene silencing. Add polybrene solution into the filtered virus-containing supernatants at the final concentration of 4 μg/ml. Use immediately for transduction.

*3.2.4. Induction of iPSCs from PαS Cells*

1. Twenty-four hours before retroviral gene transduction, trypsinize PαS cells to seed at $1 \times 10^4$ cells per 60-mm culture dish covered with SNL feeder cells.
2. Remove the medium from PαS cell culture dishes and add the virus/polybrene-containing medium. Incubate the cells for 24 h and replace the medium with 5 ml of MSC medium.
3. Two days after infection, exchange the medium with 5 ml of mESC medium. The next day, plate $1 \times 10^4$ DsRed$^+$-infected cells per 100-mm culture dish covered with SNL feeder cells.
4. To select highly reprogrammed cells by *Nanog*$^{\text{GFP-IRES-Puro}}$, add puromycin to the culture medium at the final concentration of 1.5 μg/ml after 3 weeks (four iPSC factors) or 4 weeks (three iPSC factors: *OCT4*, *SOX2*, and *KLF4*) since the infection. Change the medium every day until iPSC colonies grow large enough to be picked (Fig. 5b).

### *3.3. Marmoset iPSCs from Fetal Liver Cells*

In this chapter, we describe a protocol to establish common marmoset iPSCs from fetal liver cells via retrovirus-mediated introduction of six human transcription factors, i.e., *OCT4*, *SOX2*, *KLF4*, *C-MYC*, *NANOG*, and *LIN28* (24). We found that *LIN28*, in addition to Yamanaka's four transcription factors, improved the efficiency of iPSCs' establishment in marmosets. The availability of marmosets, and their ease of breeding, may provide an alternative to the use of traditional Old World nonhuman primates. In the future, common marmosets and their iPSCs could provide a powerful preclinical model for the study of regenerative medicine and possibly increase interest in the field.

*3.3.1. Virus Production*

Retroviruses carrying the transcription factors were produced using the Retroviral Gene Transfer and Expression System according to the manufacturer's instructions.

1. Seed GP-2 cells at $3 \times 10^6$ cells per 100-mm poly-L-lysine-coated dish 1 day prior to transfection (see Note 14).
2. Mix 27 μl of FuGENE 6 transfection reagent with 0.3 ml of OPTI-MEM I in a 1.5-ml tube, and incubate at room temperature for 5 min.
3. Combine 6 μg of each pMX vector (carrying human *OCT4*, *SOX2*, *KLF4*, *C-MYC*, *NANOG*, *LIN28*, and *GFP*) and 6 μg of pVSV-G vector with the FuGENE 6 and OPTI-MEM I mixture. Mix gently, and incubate at room temperature for 15 min.
4. Add the DNA/FuGENE 6 complex to the GP-2 cell dish culture in 10 ml of OPTI-MEM I, and incubate at 37°C, 5% $CO_2$ overnight. The next day, replace the medium containing the DNA/FuGENE 6 complex with 10 ml of cjD-MEM/10% FBS.

5. At 48 and 72 h post transfection, collect the medium as a virus-containing supernatant, and filter with a 0.45-μm pore-size cellulose acetate filter. Aliquot and store the virus-containing supernatant at −80°C (see Note 15).

*3.3.2. Preparation of Fetal Liver Cells*

Common marmoset fetal liver cells were isolated from a miscarried female fetus.

1. Remove the fetus liver and mince (with sterilized scissors) on a 100-mm cell culture dish after washing twice with HBSS. Add 5 ml of collagenase solution, and transfer the cell suspension to a 50-ml centrifuge tube. Incubate at 37°C for 30 min with shaking.
2. Add 30 ml of cjDMEM/10% FBS, centrifuge at 190 g for 5 min, and discard the supernatant. Resuspend the cells with 10 ml of cjDMEM/10% FBS, plate onto 100-mm cell culture dishes, and culture at 37°C with 5% $CO_2$. Change the medium every other day.

*3.3.3. Retroviral Infection of Marmoset Cells*

1. Seed the liver cells at $1 \times 10^6$ cells per 100-mm cell culture dish 1 day prior to infection. The cells will reach 70–80% confluency the following day.
2. Mix equal volumes of each virus-containing supernatant with *OCT4*, *SOX2*, *KLF4*, *C-MYC*, *NANOG*, *LIN28*, and *GFP*. The final volume of the mixture is 8–12 ml. Add polybrene into the virus-containing mixture to the final concentration of 4 μg/ml.
3. Replace the culture medium with the virus-containing mixture, and incubate the cells for a minimum of 4 h (maximum overnight) at 37°C, 5% $CO_2$.
4. After infection, replace the virus-containing mixture with cjDMEM/10% FBS (Fig. 6a) (see Note 16). Replace the medium every other day.

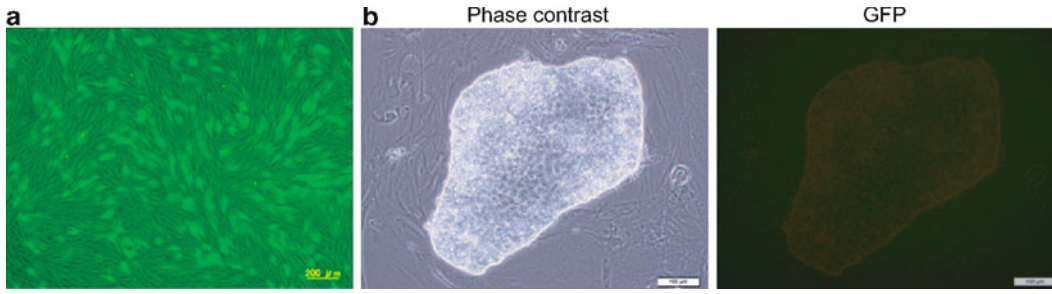


Fig. 6. Derivation of marmoset iPSCs. (**a**) GFP fluorescence 2 days after viral infection. Approximately 33% of the visible cells fluoresced. (**b**) Phase and fluorescence images of marmoset iPSCs emerged 3–5 weeks post infection with the six iPSC factors. All iPSCs exhibited flat, packed, tight colony morphology, and a high nucleus-to-cytoplasm ratio. Fully reprogrammed iPSCs are GFP negative under UV light because of the transgene silencing.

5. Seven days after transfection, harvest the cells by trypsinization and plate onto MEF feeder cells at $1–2 \times 10^5$ cells per 100-mm gelatin-coated dish. Replace the cjDMEM/10% FBS with cjESC medium. Change the medium every other day.

#### 3.3.4. Picking Colonies

Three to 5 weeks after introducing the six transcription factors, several colonies resembling ESCs will emerge (Fig. 6b) (see Note 17).

1. Aliquot 20 μl of cjESC medium (per well) into a 96-well plate. Pick each colony in the 96-well plate using a 20-μl pipette, and dissociate the colony to small clumps by repeated pipetting.
2. Transfer the cell suspension onto MEF feeder cells in gelatin-coated 12-well plates and culture the cells in cjESC medium. Change the medium every other day.

#### 3.3.5. Passage of iPSCs

Seven to 10 days after picking the colonies, the iPSC colonies develop to approximately 100–200 μm in a diameter.

1. Aspirate the culture medium and wash the cells twice with HBSS. Add 0.2 ml of Trypsin solution for the ESCs per well of the 12-well plate, and incubate at 37°C for 5 min.
2. Add 1 ml of cjESC medium and remove colonies from the feeder cells by repeated pipetting. Transfer the cell suspension to a 15-ml centrifuge tube, centrifuge at 190 g for 5 min, and discard the supernatant (see Note 18).
3. Dissociate the colonies by repeated pipetting to small clumps of 20–30 cells. Replate on new MEF feeder cells in a 100-mm gelatin-coated dish (see Note 19).

#### 3.3.6. Storage of Established iPSCs

1. Aspirate culture medium and wash the cells twice with HBSS. Add 2 ml of Trypsin solution for the ESCs to a 100-mm cell culture dish, and incubate at 37°C for 5 min.
2. Add 10 ml of cjESC medium and remove colonies from the feeder cells by repeated pipetting. Transfer the cell suspension to a 15-ml centrifuge tube, centrifuge at 190 g for 5 min, and discard the supernatant.
3. Add 3 ml of Cell Banker 2 and aliquot into 2-ml plastic cryogenic vials (see Note 20). Store the vials at –80°C.

### 3.4. Human iPSCs from Fibroblasts

In our laboratory, we have generated human iPSCs by retroviral transduction of four reprogramming factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*), which was initially introduced by Shinya Yamanaka in 2007 (29). A unique step of Yamanaka method is to introduce mouse solute carrier family 7 member 1 (Slc7a1) gene, which encodes an ecotropic retrovirus receptor, into human cells. Although there are currently several strategies to deliver reprogramming factors

(e.g., various virus vectors, nonviral DNAs, and miRNA), we usually utilize retrovirus vectors because of convenience and high efficiency. Here, we described our protocol, especially focusing on the problems that we encountered.

*3.4.1. Preparation of Culture Human Dermal Fibroblasts*

1. Obtain primary human fibroblasts from skin biopsy using a 5-mm dermapunch (see Note 21). Place the biopsy specimen immediately in mDMEM/10% FBS on ice, and transport it to the laboratory.
2. Transfer the biopsy sample to a 60-mm culture dish, and eliminate the outer layer of the skin. Cut the inner skin into 1 mm pieces using sterilized forceps and scissors. Place the four pieces per 60-mm culture dish. Routinely, three to four dishes with skin pieces can be prepared from a biopsy specimen.
3. When the pieces adhere to the culture dish, add 5 ml of mDMEM/10% FBS into the dish. If some pieces do not adhere, aspirate the medium and try this procedure again.
4. Incubate the cells in 37°C, 5% $CO_2$ incubator and leave them still for a week. When outgrowth of fibroblasts appears, exchange the medium twice a week.
5. When the cells grow to 30–50% confluency, split them at 1:3 dilution. Aspirate the medium, wash twice with PBS, and trypsinize with 0.5 ml of 0.05% Trypsin/EDTA at 37°C for 7 min. Add 3 ml of mDMEM/10% FBS and resuspend by pipetting. Split the cells to new 60-mm culture dishes at 1:3 dilution (see Note 22).
6. Prepare the freeze stocks when the cells grow to 80% confluency. Trypsinize with 1 ml of 0.05% Trypsin/EDTA at 37°C for 7 min. Add 6 ml of mDMEM/10% FBS and resuspend by pipetting. Transfer the cell suspension to a 15-ml conical tube, centrifuge at 160 g for 5 min, and discard the supernatant.
7. Resuspend the cells with Cell Banker 2 at $1 \times 10^6$ cells/ml approximately. Aliquot 1 ml of the cell suspension per freezing vial. Keep the vials in a freezing container at –80°C overnight, and transfer them to the gas phase in a liquid nitrogen tank.
8. To thaw the cell stocks, warm the vials in 37°C water bath until most (but not all) cells are thawed. Transfer the cells into a 15-ml conical tube containing 9 ml of mDMEM/10% FBS. Centrifuge at 160 g for 5 min, discard the supernatant, and resuspend the cells with 10 ml of mDMEM/10% FBS. Plate the cells into a 100-mm culture dish. Change the medium every other day.

*3.4.2. Lentiviral Production*

1. Thaw a vial of 293FT cells in 37°C water bath. Transfer the cells to a 15-ml conical tube containing 9 ml of mDMEM/10% FBS. Centrifuge at 160 g for 5 min and discard the supernatant.

Resuspend the cells with 10 ml of mDMEM/10% FBS, and transfer to a 100-mm culture dish. Incubate the cells in 37°C, 5% $CO_2$ incubator until the cells grow to 80–90% confluency. Exchange the medium every other day.

2. When the cells grow to 70–80% confluency, trypsinize with 1 ml of 0.25% Trypsin/EDTA at 37°C for 3 min and resuspend with mDMEM/10% FBS. Split the cells at 1:3 to 1:5 dilution.
3. The day before transfection, plate the cells at $1 \times 10^6$ cells per 60-mm culture dish. The next day, prepare Solution A and B, and drop Solution A onto Solution B dropwise. Incubate the solution mixture at room temperature for 20 min.
4. Add the solution mixture to 293FT cell culture dish, and incubate at 37°C, 5% $CO_2$ overnight (see Note 23). Then, replace the medium with 5 ml of mDMEM/10% FBS.
5. Twenty-four hours after the medium change, collect the supernatant from the 293FT cell culture and filter it through a 0.45-μm cellulose acetate filter. Store at –80°C.

*3.4.3. Lentiviral Transduction of Fibroblasts*

1. Twenty-four hours before transduction, plate the fibroblasts at $3.2 \times 10^5$ cells per 60-mm culture dish. Incubate at 37°C, 5% $CO_2$ overnight.
2. Replace the medium with 5 ml of the lentivirus supernatant supplemented with 4 μg/ml polybrene. Incubate at 37°C, 5% $CO_2$ overnight (see Note 24).
3. Twenty-four hours after transduction, aspirate the virus-containing medium and add 5 ml of fresh mDMEM/10% FBS.
4. When the cells grow to 70–80% confluency, passage the cells to two new 100-mm culture dishes (see Note 25).

*3.4.4. Retrovirus Production*

1. The day before transfection, seed Plat-E cells at $3.6 \times 10^6$ cells per 100-mm culture dish, and incubate at 37°C, 5% $CO_2$ overnight (see Note 26).
2. On the next day, mix 27 μl of FuGENE 6 transfection reagent with 0.3 ml of OPTI-MEM I in a 1.5 ml tube (see Note 26). Incubate at room temperature for 5 min.
3. Add 9 μg of pMXs vectors (encoding *OCT4*, *SOX2*, *KLF4*, *C-MYC*, and *GFP*) one by one into the FuGENE 6/OPTI-MEM I mixture. Mix gently and incubate at room temperature for 15 min.
4. Add the DNA/FuGENE 6 complex dropwise into the Plat-E cell culture dishes, and incubate at 37°C, 5% $CO_2$ overnight (see Note 27). After 24 h, replace the medium with 10 ml of mDMEM/10% FBS and incubate further overnight.

5. On the next day, collect the supernatant from each Plat-E cell culture, and filter through a 0.45-μm pore size cellulose acetate filter. Add polybrene solution into the filtered virus-containing medium at the final concentration of 4 μg/ml.
6. Make a mixture of equal volume of the supernatants containing each retrovirus (see Note 28).

*3.4.5. Induction of iPSCs from Human Fibroblasts*

1. The day before transduction, plate the fibroblasts expressing mouse *Slc7a1* which encodes an ecotropic retrovirus receptor at $3.2 \times 10^5$ cells per 60-mm culture dish (Fig. 7a) (see Note 29). Incubate at 37°C, 5% $CO_2$ overnight.
2. Aspirate the medium and add 5 ml of retrovirus mixture prepared at step 6 of Subheading 3.4.4. Incubate the cells at 37°C, 5% $CO_2$ overnight, and replace the medium with mDMEM/10% FBS. Exchange the medium every other day (Fig. 7b).
3. On the day 11 after infection, trypsinize the cells with 0.5 ml of 0.05% Trypsin/EDTA at 37°C for 5 min. Resuspend with 4 ml of mDMEM/10% FBS, and count the cell number.
4. Seed $5 \times 10^4$ or $5 \times 10^5$ cells onto 100-mm culture dishes covered with SNL feeder cells containing 10 ml of mDMEM/10% FBS. Incubate at 37°C, 5% $CO_2$ overnight.
5. The next day, replace the medium with 10 ml of hESC medium. Culture them in 37°C, 3% $CO_2$ incubator. Exchange the medium to 10 ml of hESC medium supplemented with 4 ng/ml FGF-2 every other day, until the iPSC colonies become large enough to be picked (see Note 30). Routinely, iPSC colonies are observed 2–3 weeks after the retroviral infection (Fig. 8) (see Note 31).

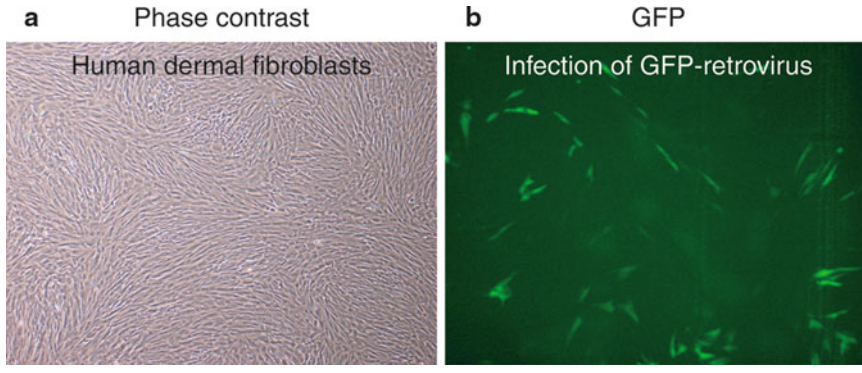


Fig. 7. Retroviral transduction of human dermal fibroblasts. (**a**) Human fibroblasts before the retroviral infection. Replate the cells at $3.2 \times 10^5$ cells on 60-mm culture dishes the day before infection. (**b**) Human fibroblasts 7 days after infection. The infection efficiency can be evaluated by transduction of GFP-retrovirus.

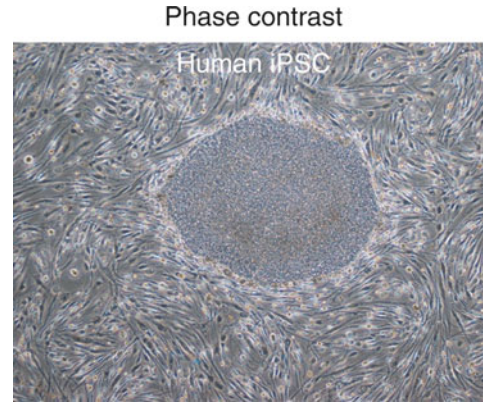


Fig. 8. Human iPSCs derived from fibroblasts. At this experiment, $5 \times 10^4$ cells of fibroblasts were replated onto SNL feeder cells in a 100-mm culture dish. ESC-like iPSC colonies emerge by day 30 after retroviral infection.

*3.4.6. Picking and Expanding Human iPSCs*

1. Aliquot 100 μl of hESC medium with FGF-2 per well of a 96-well culture plate. Pick iPSC colonies from the culture dish under the stereomicroscope using a 20-μl pipette, and transfer each colony to each well of the 96-well culture plate (see Note 32). Pipette up and down to dissociate the colonies to cell clumps composed of 20–30 cells (see Note 33).
2. Add 400 μl of hESC medium with FGF-2 per well, and transfer the cell suspensions into a 24-well plate with SNL feeder cells. Culture them in 37°C, 3% $CO_2$ incubator until the cells grow to 80–90% confluency.
3. To passage the iPSCs, aspirate the medium, wash with 0.5 ml of PBS, and add 0.1 ml of CTK solution. Aspirate an excess of CTK immediately, and incubate at 37°C for 5 min.
4. Add 0.5 ml of hESC medium with FGF-2 and transfer the cells into a 1.5-ml plastic tube without pipetting. Centrifuge at 160 g for 5 min at room temperature and discard the supernatant. Add 1 ml of hESC medium with FGF-2 and pipette carefully to obtain cell clumps composed of 20–30 cells.
5. Transfer the cell suspension to a well of 6-well culture plates with SNL feeder cells. Add 1 ml of hESC medium with FGF-2, and incubate in 37°C, 3% $CO_2$ incubator until cells grow to 80–90% confluency. Exchange the medium every day.
6. For further passages, aspirate the medium, wash with 2 ml/well of PBS, and add 0.5 ml of CTK solution. Incubate at 37°C for 2–5 min. Then, aspirate CTK solution and wash with 2 ml of PBS twice.
7. Add 2 ml of hESC medium with FGF-2 and detach iPSCs by using a cell scraper. Dissociate the iPSC colonies to cell clumps composed of 20–30 cells by pipetting. Add 8 ml of hESC

medium with FGF-2, and plate the cells into a 100-mm culture dish with SNL feeder cells. Culture in 37°C, 3% $CO_2$ incubator until the cells grow to 80–90% confluency again (see Note 34).

*3.4.7. Freezing and Thawing Human iPSCs*

To make the iPSC freeze stocks, prepare the cells which grow to 80–90% confluency. It is recommended to store the iPSCs at early passages. We usually use Y-27632, a specific inhibitor for p160-Rho-associated coiled-coil kinase (ROCK), to enhance a viability of the frozen cells (30).

1. Aspirate the medium and wash the cells with 6 ml of PBS. Add 1 ml of CTK solution and aspirate the excess immediately. Then, incubate at 37°C for 5 min.
2. Add 6 ml of hESC medium. Detach the iPSC colonies from dish by using a cell scraper, and transfer the cell suspension to two 15-ml conical tubes per 100-mm culture dish.
3. Centrifuge the cells at 160 g for 5 min. Remove the supernatant and resuspend the pellet with 0.2 ml of DAP213 solution by pipetting (see Note 33). Transfer the cell suspension to freezing vials. Put the vials quickly into liquid nitrogen (see Note 35).
4. To thaw the freeze stocks, warm 10 ml of hESC medium in 37°C water bath. Add 0.8 ml of pre-warmed hESC medium into each frozen viral, and thaw quickly by pipetting two to three times.
5. Transfer the cell suspension to the 15-ml conical tube containing hESC medium. Centrifuge at 160 g for 5 min at room temperature.
6. Aspirate the supernatant and add 4 ml of hESC medium supplemented with 4 μl of 10 mM Y-27632 and FGF-2. Plate the cells into 100-mm culture dishes with SNL feeder cells. Culture them in 37°C, 3% $CO_2$ incubator until the cells grow to 80–90% confluency. Do not move the dish for the initial 48 h, and then exchange the medium every day.

## 4. Notes

1. 2-Mercaptoethanol is toxic. Avoid inhalation, ingestion, or contact with skin and eye. Use protective gloves and safety glasses when handling.
2. Mitomycin C is toxic. Avoid inhalation, ingestion, or contact with skin and eye. Use protective gloves and safety glasses when handling.

3. Retroviral pMX vectors for human *OCT4*, *SOX2*, *KLF4*, *C-MYC*, *NANOG*, and *LIN28* were kindly provided by Dr. Yamanaka (29). These vectors except *NANOG* and *LIN28* are available from Addgene (http://www.addgene.org/Shinya Yamanaka).
4. Use SNL feeder cells within 3 days. Otherwise, the feeder cells might detach from the culture dish during iPSCs' induction culture. It is possible to utilize frozen stocks of SNL feeder cells kept at −80°C.
5. Experiments involving use of animals must be approved by the international and institutional regulations. Technically, all the procedures should be conducted aseptically.
6. We recommend using early passage fibroblasts (passage 3) as donors for iPSC derivation because the prolonged culture causes replicative senescence, which results in low efficiency of the iPSC derivation.
7. Prepare one tube of DNA/FuGENE 6 mixture for each pMXs plasmid. At this time, it is essential to prepare the proper control to confirm successful gene transduction. We normally utilize pMXs retroviral vector of red fluorescence protein DsRed to evaluate the transduction efficiency. Thus, in the case of four iPSC factors' transduction, a total five tubes of DNA/FuGENE 6 mixture are necessary for the production of *Oct4*, *Sox2*, *Klf4*, *c-Myc*, and *DsRed* viruses.
8. The viral medium should be used soon after collection. Storing of viral supernatant causes a significant reduction of transfection efficiency and consequently a lower number of iPSC colonies. Because the retrovirus infects Plat-E packaging cells themselves, the successful viral production can be conventionally evaluated by DsRed fluorescence in Plat-E cells transfected with pMXs-DsRed (Fig. 1).
9. Check the transduction efficiency by monitoring DsRed fluorescence. High efficiency of gene transduction is essential for successful iPSC derivation. We routinely observe >80% transfection efficiency.
10. The number of replated cells dramatically affects the frequency of the iPSC derivation. For the transduction of four iPSC factors, replating too many cells results in overgrowth of transformed cells and difficulty in isolation of highly reprogrammed iPSCs. On the other hand, without *c-Myc*, the iPSCs' frequency becomes much lower so that all the cells can be replated. Considering the number of iPSC colonies fluctuates among experiments, it is better to validate the optimal cell number by replating with a dilution series.

11. Highly reprogrammed iPSCs can be distinguished by a silencing of retroviral transgenes. By retroviral transduction of the iPSC factors in combination with *DsRed*, we can visually monitor the transgene silencing based on the fluorescence. The iPSC colonies without DsRed fluorescence are most likely to be highly reprogrammed iPSCs.
12. Crush the bones in pieces but do not grind them.
13. To adjust the isotype controls, arrange the preparatory experiment cells similarly to the situation of the fluorescence compensation using "control tubes." Once the adjustment is done, there is no need to check for the following experiments.
14. The cells reach 80–90% confluency the following day.
15. We can generate marmoset iPSCs using frozen viral stocks stored for up to 1 month. Do not repeatedly freeze/thaw the viral stocks to avoid reducing the viral titer.
16. Infection rates as assessed by GFP fluorescence should be more than 30% (Fig. 6a).
17. This timing of iPSC colony appearance is consistent with the study on rhesus monkey iPSCs (31). All colonies morphologically resembling marmoset ESCs (32) are GFP negative (Fig. 6b). Although various colony types appear approximately 2 weeks post infection, these are not iPSC colonies. ESC-like, clear-edged colonies should appear within 3–5 weeks post infection.
18. When the differentiated cells occupy the majority of the dishes, collect the undifferentiated colonies using a 20-μl pipette, or remove the large clumps of differentiated cells by filtering the cell suspension using a 100-μm nylon cell strainer.
19. Do not dissociate the cell clumps into single cells because too much dissociation could trigger cell death. Cells are passaged approximately every 5–7 days.
20. Do not dissociate the colonies to small clumps prior to freezing.
21. For biopsy of human dermal fibroblasts, you must obtain a proper informed consent from the donors.
22. Be careful not to plate the cells at low cell density to prevent replicative senescence.
23. Transfection of the lentiviral vector should be done when 293FT cells grow to 70% confluency in a 100-mm culture dish.
24. The overnight incubation with lentivirus is sometimes toxic to fibroblasts. In that case, shorten the incubation duration down to 5 h.

25. To evaluate the successful transduction, use a GFP-encoding lentiviral vector as a control. Alternatively, it is possible to select the infected cells by culturing with 10 μg/ml blastocidin S, because pLenti6/UbC/mSlc7a1 vector carries a blastocidin-resistance gene.
26. Prepare one cell culture dish and one 1.5-ml plastic tube per plasmid. Five cell culture dishes and tubes are required for transfection of *OCT4*, *SOX2*, *KLF4*, *C-MYC*, and *GFP* retrovirus vectors.
27. Do not transfect more than two plasmids into a Plat-E cell culture dish. It causes a low-efficient generation of resultant iPSCs.
28. Use immediately for transduction. Do not freeze the virus supernatants.
29. The efficiency of retroviral transduction markedly decreases when using fibroblasts at older passages. It is recommended to use passage 8–10 fibroblasts for iPSC induction.
30. Fuzzy-edged cell colonies appear approximately 2 weeks after retroviral transduction, but they are not iPSC colonies. Keep the culture until clear-edged, hESC-like colonies are observed.
31. Check the emergence of iPSC colonies carefully, because the timing differs in each experiment even when using the same lot of fibroblasts.
32. We usually pick 20–30 iPSC colonies from a culture dish.
33. Do not dissociate iPSC colonies into single cells.
34. To keep undifferentiated iPSC culture, remove the differentiated colonies by aspirating during passaging procedure. Transfer only undifferentiated iPSC colonies to a new culture dish.
35. To ensure high cell viability, these procedures should be done within 15 s.

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## References


1. Stadtfeld M, Hochedlinger K (2010) Induced pluripotency: history, mechanisms, and applications. Genes Dev 24:2239–2263
2. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
3. Chamberlain SJ et al (2010) Induced pluripotent stem cell models of the genomic imprinting disorders Angelman and Prader-Willi syndromes. Proc Natl Acad Sci U S A 107:17668–17673
4. Horii T et al (2008) Loss of genomic imprinting in mouse parthenogenetic embryonic stem cells. Stem Cells 26:79–88
5. Do JT et al (2009) Generation of parthenogenetic induced pluripotent stem cells from parthenogenetic neural stem cells. Stem Cells 27:2962–2968
6. Takahashi K (2010) Direct reprogramming 101. Dev Growth Differ 52:319–333
7. Humpherys D et al (2001) Epigenetic instability in ES cells and cloned mice. Science 293:95–97
8. Stadtfeld M et al (2010) Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. Nature 465:175–181
9. Liu L et al (2010) Activation of the imprinted Dlk1-Dio3 region correlates with pluripotency levels of mouse stem cells. J Biol Chem 285:19483–19490
10. Pick M et al (2009) Clone- and gene-specific aberrations of parental imprinting in human induced pluripotent stem cells. Stem Cells 27:2686–2690
11. Toyooka Y et al (2003) Embryonic stem cells can form germ cells in vitro. Proc Natl Acad Sci U S A 100:11457–11462
12. Geijsen N et al (2004) Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 427:148–154
13. Hubner K et al (2003) Derivation of oocytes from mouse embryonic stem cells. Science 300:1251–1256
14. Eguizabal C et al (2009) Generation of primordial germ cells from pluripotent stem cells. Differentiation 78:116–123
15. Hayashi K, Surani MA (2009) Self-renewing epiblast stem cells exhibit continual delineation of germ cells with epigenetic reprogramming in vitro. Development 136:3549–3556
16. Lavagnolli TM et al (2009) Presumptive germ cells derived from mouse pluripotent somatic cell hybrids. Differentiation 78:124–130
17. Imamura M et al (2010) Induction of primordial germ cells from mouse induced pluripotent stem cells derived from adult hepatocytes. Mol Reprod Dev 77:802–811
18. Park TS et al (2009) Derivation of primordial germ cells from human embryonic and induced pluripotent stem cells is significantly improved by coculture with human fetal gonadal cells. Stem Cells 27:783–795
19. Kim JB et al (2009) Oct4-induced pluripotency in adult neural stem cells. Cell 136:411–419
20. Honda A et al (2010) Generation of induced pluripotent stem cells in rabbits: potential experimental models for human regenerative medicine. J Biol Chem 285:31362–31369
21. Shimada H et al (2010) Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors. Mol Reprod Dev 77:2
22. Nagy K et al (2011) Induced pluripotent stem cell lines derived from equine fibroblasts. Stem Cell Rev 7(3):693–702
23. Bao L et al (2011) Reprogramming of ovine adult fibroblasts to pluripotency via drug-inducible expression of defined factors. Cell Res 21(4):600–8
24. Tomioka I et al (2010) Generating induced pluripotent stem cells from common marmoset (Callithrix jacchus) fetal liver cells using defined factors, including Lin28. Genes Cells 15:959–969
25. Sumer H et al (2011) NANOG is a key factor for induction of pluripotency in bovine adult fibroblasts. J Anim Sci 89(9):2708–2716
26. Fujioka T et al (2004) A simple and efficient cryopreservation method for primate embryonic stem cells. Int J Dev Biol 48:1149–1154
27. Morikawa S et al (2009) Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. J Exp Med 206:2483–2496
28. Niibe K et al (2011) Purified mesenchymal stem cells Are an efficient source for iPS cell induction. PLoS One 6:e17610
29. Takahashi K et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
30. Watanabe K et al (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25:681–686
31. Liu H et al (2008) Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. Cell Stem Cell 3:587–590
32. Sasaki E et al (2005) Establishment of novel embryonic stem cell lines derived from the common marmoset (Callithrix jacchus). Stem Cells 23:1304–1313

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