Generation and Characterization of Human Induced Pluripotent Stem Cells

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ABSTRACT

This unit describes how to generate human induced pluripotent stem (iPS) cells and evaluate the qualities of the generated iPS cells. The methods for establishment and maintenance of human iPS cells are similar to those for mouse iPS cells but not identical. In addition, these protocols include excellent procedures for passaging and cryopreservation of human iPS cells established by ES cell researchers, which result in an easy way to culture human iPS cells. Moreover, we include methods for characterizing iPS cells for further research. RT-PCR and immunocytochemistry for detection of pluripotent cell markers, embryoid body differentiation, and teratoma differentiation are used to determine pluripotency in vitro and in vivo, respectively. *Curr. Protoc. Stem Cell Biol.* 9:4A.2.1-4A.2.25. © 2009 by John Wiley & Sons, Inc.

Keywords: reprogramming • pluripotency • iPS cells

INTRODUCTION

The authors of this unit have reported that the forced expression of four transcription factors, *Oct3/4*, *Sox2*, *Klf4*, and c-*myc*, could reprogram fibroblasts to pluripotent stem cells (Takahashi and Yamanaka, 2006; Yamanaka, 2007). These reprogrammed cells are referred to as induced pluripotent stem (iPS) cells. By using Nanog or Oct3/4 as selection markers, we and others have successfully induced germ line competency with these four factors (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007).

Recently, it was demonstrated that drug selection with pluripotent stem cell markers is not required for establishment of iPS cells (Blelloch et al., 2007; Meissner et al., 2007; Nakagawa et al., 2008). Reprogrammed cells formed round-shaped colonies and could be morphologically distinguished by microscopic observation. The result suggests that iPS cells can be established utilizing somatic cells from genetically unmodified animals, and provides hope of medical applications in human cells with genes that are hard to modify.

The same set of four factors allowed reprogramming human adult fibroblasts to the pluripotent state (Takahashi et al., 2007a; Yu et al., 2007; Lowry et al., 2008; Masaki et al., 2008; Park et al., 2008a). Generation of human iPS cells requires only basic techniques in molecular and cell biology and does not require any special equipment.

In this unit, we introduce not only how to generate iPS cells but also how to evaluate the characteristics of iPS cells. The methods for establishment and maintenance of iPS cells are similar to those for mouse iPS cells, but not identical (Takahashi et al., 2007b; Basic Protocol 1). Support Protocols describe preparation of SNL feeder cells (Support Protocol 1) and preparation of PLAT-E packaging cells (Support Protocol 2). In addition, our protocols include excellent procedures for passaging (Basic Protocol 2) and cryopreservation (Basic Protocol 3) of human iPS cells established by ES cell researchers

Current Protocols in Stem Cell Biology 4A.2.1-4A.2.25
Published online June 2009 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/9780470151808.sc04a02s9
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(Fujioka et al., 2004; Watanabe et al., 2007), which results in an easy way to culture human iPS cells. Moreover, we demonstrate the methods to characterize iPS cell clones for further in-depth research. RT-PCR for detection of pluripotent cell markers (Support Protocol 3), immunocytochemistry for pluripotent cell markers (Support Protocol 4), and in vitro and in vivo differentiations by embryoid body (Support Protocol 5) and teratoma formation (Support Protocol 6) are extensively described.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

BASIC PROTOCOL 1

GENERATION OF iPS CELLS

The first step in generation of iPS cells is to transduce mouse ecotropic retrovirus receptor genes into human skin fibroblasts. These genes are necessary to enhance transduction efficiency of transgenes and increase safety for the experimenters. The next step is to introduce the factors to be tested for their ability to induce iPS cells. See Figure 4A.2.1 for an outline of the procedure.

For gene transduction, we utilized the combination of pMXs retroviral vector and PLAT-E packaging cells, which can produce a much higher titer of retrovirus, serving as a sufficient vector to generate mouse iPS cells. Despite the fact that ecotropic retrovirus that affects exclusively rodent cells is produced, we decided to apply this combined system to human cells for the safety of research personnel. In order to enable ecotropic retroviruses to transduce human cells, we introduced mouse solute carrier family 7 (cationic amino acid transporter, y^+ system) member 1 (*Slc7a1*) gene encoding the ecotropic retrovirus receptor into human cells.

We use SNL cells as feeder cells for maintenance of both mouse and human iPS cells. These cell lines are derived from mouse embryos and express the neomycin resistance gene and the leukemia inhibitory factor (LIF) gene. These cell lines provide two significant merits: they show no remarkable difference between tubes and they have a more extended period of proliferation when compared to primary mouse embryonic fibroblasts (MEFs). We have always used iPS cells before passage 20, but the highest limit on passage number is not known.

CAUTION: All processes involving lentivirus should be performed in a safety cabinet while wearing gloves. All waste must be treated with first with ethanol, then with bleach (hypochlorous acid), and finally autoclaved.

Materials

293FT cells for producing lentivirus (Invitrogen; see manufacturer-provided protocol for culture)
Dulbecco's phosphate-buffered saline without calcium and magnesium (CMF-DPBS: Nacalai Tesque, cat. no. 14249-95)
0.25% trypsin/1 mM EDTA solution (Invitrogen, cat. no. 25200-056)
293FT medium (see recipe)
OPTI-MEM I medium (Invitrogen, cat. no. 31985-062)
ViraPower packaging mix (from ViraPower expression system kit; Invitrogen, cat. no. K4990-00)
pLenti6/UbC containing mouse *Slc7a1* gene (Addgene; *http://www.addgene.org/Shinya_Yamanaka*)
Lipofectamine 2000 (Invitrogen, cat. no. 11668-019)

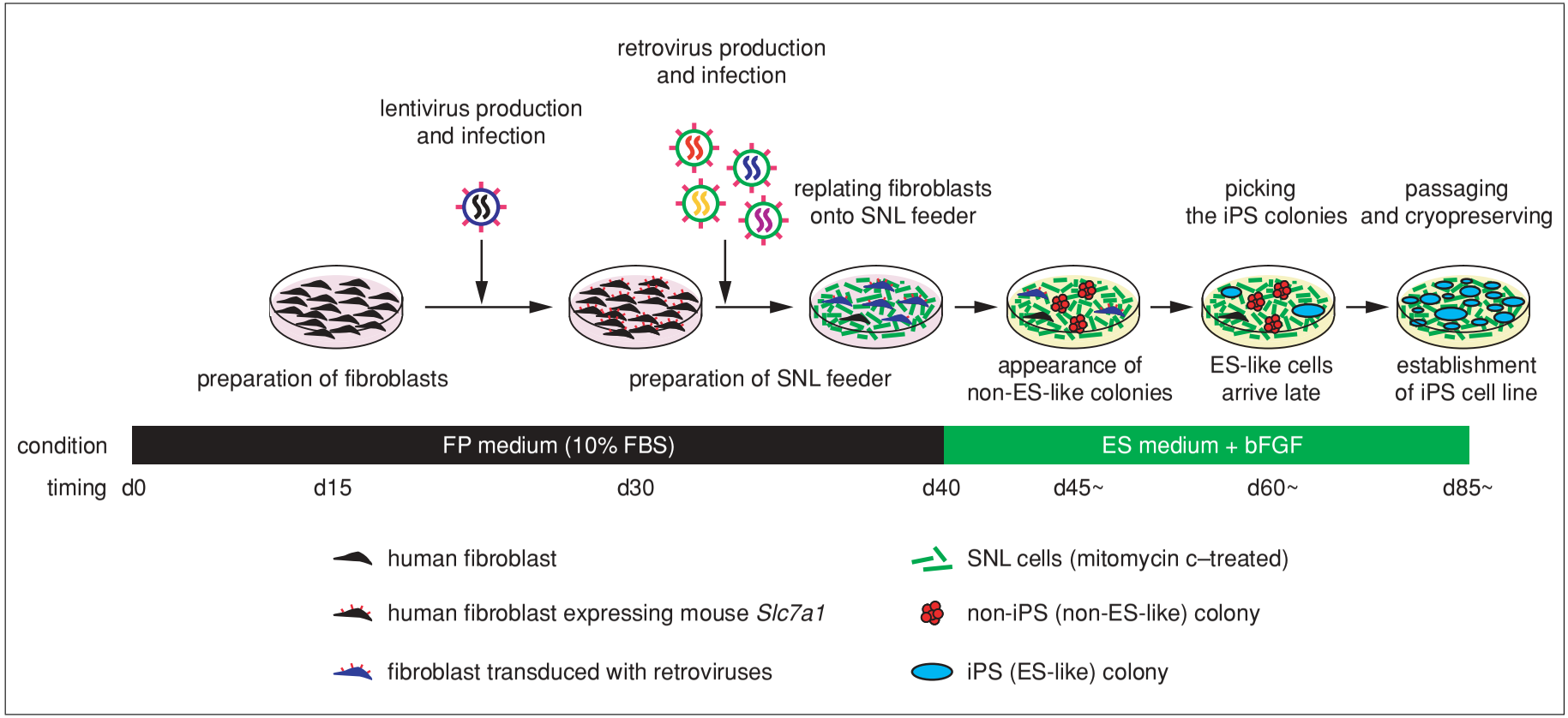

Figure 4A.2.1 Schematic diagram of iPS cell generation. A strategy and approximate time table for human iPS cell generation.

10% FBS medium (see recipe)
Human fibroblast cells (available from the following sources:
Cell Applications Inc. (*http://www.cellapplications.com/*)
Lonza (*http://www.lonza.com/group/en.html*)
American Type Culture Collection (ATCC, *http://www.atcc.org/*)
European Collection of Cell Cultures (ECACC; *http://www.ecacc.org.uk/*)
Riken Bioresource Center (*http://www.brc.riken.jp/*)
Japanese Collection of Research Bioresources (*http://cellbank.nibio.go.jp/*)
0.05% trypsin/0.53 mM EDTA solution (Invitrogen, cat. no. 25300-054)
Hexadimethrine bromide (polybrene; Nacalai Tesque, cat. no. 17736-44)
PLAT-E packaging cells (Support Protocol 2)
pMXs retroviral vectors encoding *OCT3/4*, *SOX2*, *KLF4*, and/or c-*myc* (Addgene; *http://www.addgene.org/Shinya_Yamanaka*):
pMXs-hOCT3/4
pMXs-hSOX2
pMXs-hKLF4
pMXs-hc-MYC
pMXs retroviral vector encoding the green fluorescence protein (GFP) to monitor transfection efficiency and serve as a negative control for iPS cell induction (Cell Biolabs, Inc.)
Fugene 6 transfection reagent (Roche, cat. no. 1 814 443)
Mitomycin C–treated SNL feeder cell plates, 100-mm and 24-well (Support Protocol 1)
hES cell medium (see recipe)
Recombinant basic fibroblast growth factor, human (bFGF; Wako, cat. no. 064-04541)

100-mm tissue culture dish (Falcon, cat. no. 353003)
0.45-μm pore size cellulose acetate filter (Whatman, cat. no. FP30/0.45 CA-S)
96-well tissue culture plate (Falcon, cat. no. 351172)
24-well tissue culture plate (Falcon, cat. no. 353047)

Additional reagents and equipment for counting cells (*UNIT 1C.3*) and preparation of PLAT-E packaging cells (Support Protocol 2)

Passage 293FT cells

1. Aspirate the medium from an 80% to 90% confluent culture of 293FT cells growing in a 100-mm tissue culture dish and wash the cells once with 10 ml of CMF-DPBS. Add 1 ml of 0.25% trypsin/1 mM EDTA and incubate for 2 min at room temperature.

2. Add 9 ml of 293FT medium and break the cells into single-cell suspension by pipetting up and down about 10 times.

3. Determine cell number using a hemacytometer (*UNIT 1C.3*), plate 4×10^6 cells on 100-mm dish and incubate overnight at 37°C, in a humidified 5% CO_2 incubator.

Prepare virus

4. Dispense 1.5 ml of OPTI-MEM I medium into a 1.7-ml tube. Add 9 μg of ViraPower packaging mix (including pLP1, pLP2, and pLP/VSVG) and 3 μg of pLenti6/UbC encoding mouse *Slc7a1* gene, and mix them gently.

5. In another 1.7-ml tube, dispense 1.5 ml of OPTI-MEM I medium and add 36 μl of Lipofectamine 2000, and mix gently. Incubate for 5 min at room temperature.

6. Mix Lipofectamine 2000 diluted solution and previous DNA mixture gently and incubate for 20 min at room temperature to form a DNA/Lipofectamine complex.

Produce virus

7. Replace the medium on 293FT cells (see step 3) with fresh 10% FBS medium.
8. Add 3 ml of DNA/Lipofectamine complex (from step 6) to the dish of 293FT cells and rock it back and forth gently. Incubate the dish overnight in a 37°C, 5% CO_2 incubator.
9. At a time point 24 hr after transfection, replace the medium with 10 ml of fresh 10% FBS medium. Incubate the dish for another 24 hr.

Begin culturing human fibroblasts

10. In preparation for infection, plate 5×10^5 human fibroblasts in 100-mm dish with 10 ml of 10% FBS medium. Incubate.

Collect virus

11. On the next day, collect the virus-containing supernatant from the 100-mm dish of transfected 293FT cells with a 10-ml disposable syringe. Filter the supernatant with a 0.45-μm pore size cellulose acetate filter.
12. Use the virus-containing medium immediately (see step 17) or store at −80°C.

Prepare fibroblasts

Fibroblasts should be replated as described below on the day before infection for the sake of better infection efficiency.

13. Remove the 100-mm dish of human fibroblasts (step 10) from the incubator.
14. Aspirate the medium and wash the cells once with 10 ml CMF-DPBS, add 1 ml of 0.05% trypsin/0.53 mM EDTA, and incubate for 10 min at 37°C.
15. Add 9 ml of 10% FBS medium and break the cells into a single-cell suspension by pipetting.
16. Determine cell number (*UNIT 1C.3*), plate 8×10^5 cells on a 100-mm dish, and incubate overnight.

Infect the cells

17. Replace the medium on the fibroblasts with the virus cocktail (entire supernatant from step 11) supplemented with 4 μg/ml polybrene (hexadimethrine bromide). Incubate the dish 5 hr to overnight.
18. After infection, wash the cells with 10 ml of CMF-DPBS (optional), and exchange the medium with 10 ml of fresh 10% FBS medium at room temperature. Return the fibroblast cultures to the incubator.

    Sometimes overnight incubation with lentivirus is toxic to fibroblasts. In that case, dilute virus cocktail by ∼50% with medium or shorten the incubation time to 5 hr.

19. To check the expression of infected genes, use a GFP-encoding vector as a control.

    Infection can also be confirmed by culturing in blasticidin S–supplemented medium (10 μg/ml) because pLenti6/UbC/mSlc7a1 includes the blasticidin S–resistance gene.

    Expression is determined by microscopic examination or flow cytometry.

Prepare retroviruses

20. Prepare a single-cell suspension of PLAT-E cells (Support Protocol 2).

    The protocol is based on 100-mm dish cultures of cells. If you use different sizes of dishes or plates, adjust the cell numbers and volumes according to Table 4A.2.1.

21. Transfer 3.6×10^6 PLAT-E cells to a new 100-mm dish in 10% FBS medium without puromycin or blasticidin S. Prepare one dish per plasmid (the plasmids will be pMXs

Table 4A.2.1 Amounts of Reagents for Generating Plasmids in Different Size Culture Dishes

Reagents	100-mm dish	60-mm dish	6-well plate
PLAT-E cells	3.6×10^6	1.2×10^6	6×10^5
OPTI-MEM I	300 µl	100 µl	50 µl
Fugene 6	27 µl	9 µl	4.5 µl
Plasmid	9 µg	3 µg	1.5 µg

encoding *OCT3/4, SOX2, KLF4*, and/or c-*myc*, as well as GFP as a control). Incubate the dishes overnight.

One plasmid DNA involves one dish of PLAT-E cells and, consequently, introducing genes for four factors and the GFP control requires five dishes of PLAT-E cells.

22. The day after passage of PLAT-E cells, prepare one 1.5-ml microcentrifuge tube per plasmid DNA.
23. Dispense 0.3 ml of OPTI-MEM I into each tube.
24. Add 27 µl of Fugene 6 transfection reagent into each tube of OPTI-MEM I and mix gently with finger tapping. Incubate tubes for 5 min at room temperature.
25. Add 9 µg of the appropriate plasmid DNA to each tube, one plasmid per tube. Mix by tapping and incubate tubes for 15 min at room temperature.
26. Add each DNA/Fugene 6 mixture to one of five separate cultures of PLAT-E cells (see step 21). Incubate the dishes overnight.

    Monitor the efficiency of transfection with GFP-coding pMXs vector. Our laboratory confirms transduction efficiency of more than 60%. High efficiency is essential for iPS induction.

27. On the next day, replace the medium containing DNA and Fugene with fresh 10% FBS medium and return the dishes to the incubator.
28. The day after transfection of the PLAT-E cells, prepare a suspension of mouse *Slc7a1*-expressing human fibroblasts in 10% FBS medium, as described in steps 13 to 16. Count cells and plate 8×10^5 cells per 100-mm dish (see step 16), and incubate the dish overnight.
29. On the next day, collect the virus-containing medium from each of the dishes of transfected PLAT-E cells with a 10-ml disposable syringe and filter it with a 0.45-µm pore size cellulose acetate filter.
30. Mix equal amounts of each of the three or four virus-containing media (*OCT3/4*, *SOX2*, *KLF4*, with or without c-*myc*).

    The virus cocktail should be applied immediately. Do not freeze, or infection efficiency will be lower.

    The virus with the GFP control vector will be applied to a separate plate of human fibroblasts.

31. Replace the medium on fibroblasts expressing *Slc7a1* with the virus cocktail (step 30) supplemented with 4 µg/ml polybrene. Incubate the dish for 4 hr to overnight.
32. At a time point 24 hr after infection, change the medium to 10 ml of fresh 10% FBS medium. Change the medium every second day until reseeding.

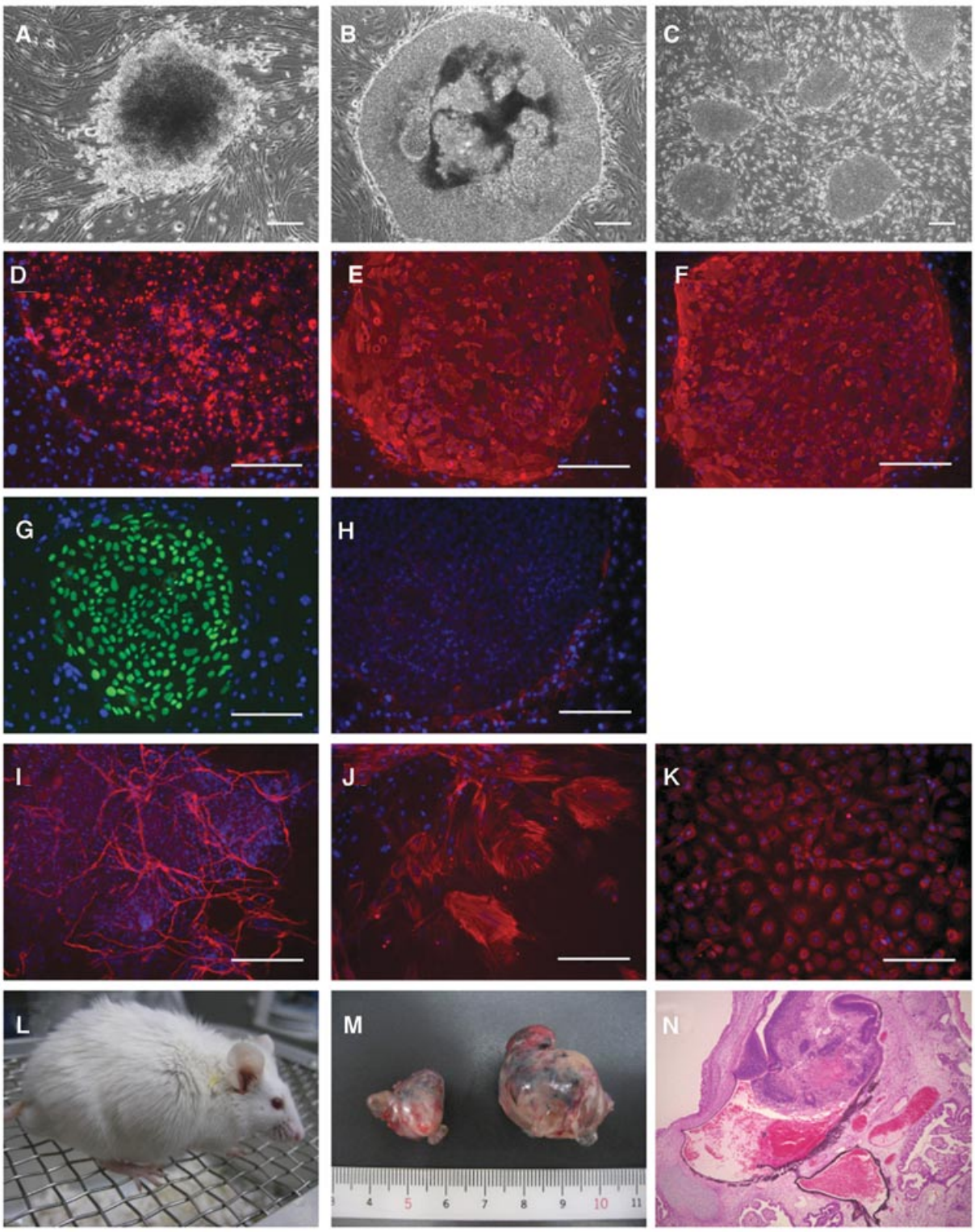

Figure 4A.2.2 Images related to iPS cell induction. (**A, B**) Images of non-iPS and ES-like (iPS) cells, respectively. (**C**) Image of established iPS cells. Images of immunocytochemistry for undifferentiated pluripotent cell markers: (**D**) SSEA3; (**E**) TRA-1-60; (**F**) TRA-1-81; (**G**) Nanog; and (**H**) SSEA1 (negative). Blue indicates nuclei stained with Hoechst 33342. Images of immunocytochemistry for differentiated cell products: (**I**) α-fetoprotein; (**J**) α-smooth muscle actin; and (**K**) βIII-tubulin. Blue indicates nuclei stained with Hoechst 33342. (**L**) Image of SCID mouse that had iPS cells injected into the testes 3 months earlier. (**M**) Image of a dissected teratoma. (**N**) Image of hematoxylin and eosin–stained teratoma section. Bars = 100 μm. Panels D to H illustrate immunohistochemistry for pluripotent cell markers, while panel I illustrates a marker for endoderm, panel J a marker for mesoderm, and panel K a marker for ectoderm.

Reseed fibroblasts on SNL feeder cells

33. At a time point 6 days after infection, aspirate the medium and wash the cells once with 8 ml of CMF-DPBS.
34. Add 1 ml of 0.05% trypsin/0.53 mM EDTA and incubate the dish 10 min at 37°C for 10 min.
35. Add 9 ml of 10% FBS medium to the dish and break up the mass of cells by pipetting.
36. Determine the cell number (*UNIT 1C.3*) and transfer 5×10^4 or 5×10^5 cells to a 100-mm dish with mitomycin C–treated SNL feeder cells (Support Protocol 1). Incubate the dish overnight.
37. The next day and every second day, change the medium to 10 ml of hES cell medium supplemented with 4 ng/ml bFGF.

Pick colonies

38. At a time point 2 to 3 weeks after retroviral infection, examine the dishes for colonies (see Fig. 4A.2.2).

    It takes about 30 days for iPS cell colonies to grow large enough to be picked up.

    Observe the dishes carefully because the timing of colony emergence differs in each experiment even if the same fibroblast clones were induced. Fuzzy-edged colonies appear ~2 weeks after infection; these are not the iPS colonies. Wait another week before ES cell-like, clear-edged colonies begin to be seen; these are the iPS cell colonies that should be picked.

39. Distribute 20 µl of hES cell medium to each well of a 96-well plate. Wash the dish of iPS cell colonies once with 10 ml of CMF-DPBS, and then add another 5 ml of CMF-DPBS.
40. With the 5 ml of CMF-DPBS still in the dish, cut out an iPS colony and separate it from feeder cells under the stereo microscope with a 2- or 10-µl pipet tip and pipettor. Transfer the colony to an individual well of 96-well plate.
41. After picking the colonies add 180 µl of hES medium and break the colonies into small masses of cells but not single cells by pipetting.
42. Transfer the suspension to a well of a 24-well plate with SNL feeder cells. Incubate the plate until the cells grow to 80% to 90% confluency. Continue passaging as in Basic Protocol 2.

SUPPORT PROTOCOL 1

PREPARATION OF SNL FEEDER CELLS

SNL cells are mitomycin C inactivated and used as feeder cells for plating iPS cells. We have always used feeder cells before passage 20, but the highest limit on passage number is not known.

Materials

Frozen vial of SNL feeder cells (McMahon and Bradley, 1990): available from Dr. Allan Bradley of the Sanger Institute (*http://www.sanger.ac.uk/Teams/faculty/bradley/*)
SNL medium (see recipe)
Dulbecco's phosphate-buffered saline without calcium and magnesium (CMF-DPBS: Nacalai Tesque, cat. no. 14249-95)
0.25% trypsin/1 mM EDTA solution (Invitrogen, cat. no. 25200-056)
0.4 mg/ml mitomycin C (see recipe)

Centrifuge
Gelatin-coated 100-mm tissue culture dish (see recipe)
Other gelatin-coated culture vessels (see recipe) as needed

Additional reagents and equipment for counting cells (*UNIT 1C.3*)

1. Place a frozen vial of SNL cells in a 37°C water bath until almost thawed. Resuspend the cells in 9 ml of SNL medium.
2. Centrifuge 5 min at 160 × *g*, room temperature, and discard the supernatant.
3. Resuspend the cells in 10 ml of fresh SNL medium, and transfer to a gelatin-coated 100-mm dish (~1 × 10^6 cells). Incubate the cells in a humidified 37°C, 5% CO_2 incubator until the cells are 80% to 90% confluent.

   Do not make cells overconfluent, or their performance as feeder cells may deteriorate.
4. Aspirate off the medium and wash the cells once with 8 ml of CMF-DPBS. Add 0.5 ml of 0.25% trypsin/1 mM EDTA and incubate for 1 min at room temperature.
5. Add 4.5 ml of SNL medium and break the cells into a single-cell suspension by pipetting up and down several times.
6. Split the cell suspension 1:16, plate on a gelatin-coated 100-mm dishes, and incubate (3 to 4 days) until the cells are 80% to 90% confluent.
7. When the cells reach 80% to 90% confluency, drop 0.3 ml of 0.4 mg/ml mitomycin C solution on the culture of SNL cells and mix by gently shaking back and forth. Incubate 2.25 hr in humidified 37°C, 5% CO_2 incubator.
8. After incubation, aspirate the mitomycin C–containing medium and wash the cells with 5 ml of CMF-DPBS twice.
9. Add 0.5 ml of 0.25% trypsin/1 mM EDTA and incubate for 1 min at room temperature. Add 4.5 ml of SNL medium and break the cells into a single-cell suspension by pipetting.
10. Count cells (*UNIT 1C.3*), and plate 1.5 × 10^6 cells (in 10 ml SNL medium) per gelatin-coated 100-mm dish, 2.5 × 10^5 cells (in 2 ml SNL medium) per well of 6-well plate, or 6.1 × 10^4 cells (in 0.5 ml SNL medium) per well of a 24-well plate.

    Cells should be nicely spread with few gaps in between.
11. Incubate the dish overnight.

    The cells should become ready for use by the next day.

    SNL feeder cell–plated dishes should be used within 3 days.

PREPARATION OF PLAT-E PACKAGING CELLS

SUPPORT PROTOCOL 2

PLAT-E packaging cells are used to prepare the viral stocks bearing the plasmids for induction of iPS cells. The PLAT-E packaging cell line is designed for producing ecotropic retrovirus; the cells are derived from HEK293 cells and contain env-IRES-puroR and gag-pol-IRES-bsR cassettes driven by the human elongation factor 1α promoter.

Materials

Frozen vial of PLAT-E packaging cells (Morita et al., 2000): available from Dr. Toshio Kitamura at the University of Tokyo (*kitamura@ims.u-tokyo.ac.jp*) or Cell Biolabs, Inc. (*http://www.cellbiolabs.com/*)
10% FBS medium (see recipe)
Puromycin stock solution (see recipe)

Blasticidin S stock solution (see recipe)
Dulbecco's phosphate-buffered saline without calcium and magnesium (CMF-DPBS: Nacalai Tesque, cat. no. 14249-95)
0.05% (w/v) trypsin/0.53 mM EDTA solution (Invitrogen, cat. no. 25300-054)

100-mm tissue culture dishes
Centrifuge
15-ml conical centrifuge tubes

1. Place a frozen vial of PLAT-E packaging cells in a 37°C water bath until almost thawed. Resuspend the cells in 9 ml of 10% FBS medium.
2. Centrifuge 5 min at 180 × *g*, room temperature, and discard the supernatant.
3. Resuspend the cells in 10 ml of fresh 10% FBS medium, and transfer them to a 100-mm dish. Incubate the cells in a humidified 37°C, 5% CO_2 incubator.
4. On the next day, change the medium to fresh 10% FBS medium supplemented with 1 μg/ml puromycin and 10 μg/ml blasticidin S. Incubate until the cells are 80% to 90% confluent.
5. Aspirate the medium and wash the cells once with 10 ml of CMF-DPBS. Add 4 ml of 0.05% trypsin/0.53 mM EDTA and incubate for 1 min at room temperature.
6. Tap the dish and add 10 ml of 10% FBS medium, then transfer cell suspension to a 15-ml conical tube.
7. Centrifuge 5 min at 180 × *g*, room temperature, and discard the supernatant.
8. Resuspend in 10 ml of 10% FBS medium and break the mass of cells into single-cell suspension by pipetting.
9. Split the cell suspension 1:4 to 1:6, plate on 100-mm dishes, and incubate (2 to 3 days) until the cells are 80% to 90% confluent.

BASIC PROTOCOL 2

PASSAGE OF iPS CELLS

The following protocol is based on the cells of 24-well plate cultures. If you use different sizes of dishes or plates, adjust the volume according to Table 4A.2.2.

Materials

Human iPS cells at 80% to 90% confluency in a 24-well plate (Basic Protocol 1)
Dulbecco's phosphate-buffered saline without calcium and magnesium (CMF-DPBS: Nacalai Tesque, cat. no. 14249-95)
CTK solution (see recipe)
hES medium (see recipe)
6-well plate seeded with mitomycin C–treated SNL cells (Support Protocol 1)

Sterile disposable cell scraper
15-ml conical centrifuge tube

1. Wash 24-well plate of 80% to 90% confluent iPS cells once with 0.5 ml per well of CMF-DPBS.
2. Aspirate CMF-DPBS completely, add 0.1 ml of CTK solution to the dish, and incubate for ~5 min at 37°C.
3. When ~90% of feeder cells detach, wash out CTK solution and feeder cells with 0.5 ml of CMF-DPBS, twice.

Usually, feeder cells detach first from the dish, whereas iPS colonies remain attached.

Table 4A.2.2 Reagent Volumes for Passaging iPS Cells Grown in Different Culture Dishes and Plates

Reagent	100-mm	60-mm	6-well	24-well
PBS	10 ml	4 ml	2 ml	0.5 ml
CTK solution	1 ml	0.5 ml	0.3 ml	0.1 ml
hES medium (ml)	10 ml	4 ml	2 ml	0.5 ml

4. Remove CMF-DPBS completely, and add 0.5 ml of hES medium to the dish.
5. Scrape out the iPS colonies by using sterile disposable cell scraper, and break the colonies into small clumps by pipetting up and down.

   Do not break the colonies up completely into single cells, because too much dissociation might trigger cell death.
6. Transfer the cell suspension to a 15-ml conical tube.
7. Dilute the cell suspension at 1:3 to 1:4 with hES medium, and transfer 2 ml of the suspension to a new well of a 6-well plate seeded with mitomycin C–treated SNL feeders.
8. Incubate in humidified 37°C, 5% CO_2 incubator. Change the medium with fresh hES cell medium every day.

   Cells are passaged approximately every 5 days.

STORAGE OF ESTABLISHED iPS CELLS

BASIC PROTOCOL 3

Established cultures of iPS cells should be frozen at early passages to maintain the stock. When the cells reach confluency (i.e., when the colonies approach each other) in the 100-mm dish, it is time to make cryostocks. This method uses a specific inhibitor for p160-Rho-associated coiled-coil kinase (ROCK), Y-27632, to increase the survivability of the frozen cells.

Materials

10 mM Y-27632 (Wako, cat. no. 253-00513)
Confluent iPS cells (see Basic Protocols 1 and 2) in 100-mm dishes
Dulbecco's phosphate-buffered saline without calcium and magnesium (CMF-DPBS: Nacalai Tesque, cat. no. 14249-95)
CTK solution (see recipe)
hES medium (see recipe)
DAP123 solution (see recipe)
Liquid nitrogen
60-mm dish seeded with mitomycin C–treated SNL feeder cells (Support Protocol 1)
Sterile disposable cell scraper
2-ml cryovials
Liquid nitrogen tank

Prepare cells for freezing

1. Add 10 µl of 10 mM Y-27632 to the medium of a confluent iPS cell culture in a 100-mm dish, and incubate the dish at 37°C for at least 1 hr.
2. Aspirate the medium, and wash the cells with 10 ml of CMF-DPBS.

3. Discard CMF-PBS, add 1 ml of CTK solution, and incubate at 37°C for 2 to 5 min.

   Incubation time may depend on cell density. Check the cells by eye once per minute. After treatment with Y-27632, the cells may become less detachable. In such cases, you can treat the cells with the CTK solution for a longer period of time (~10 min).

4. Wash twice, each time with 10 ml of CMF-DPBS to remove feeder cells and CTK solution.

5. Discard CMF-DPBS, add 12 ml of hES medium, detach the colonies from the dish using a cell scraper, and transfer 4 ml of cell suspension to each of three 15-ml conical tubes.

6. Centrifuge 5 min at 160 × *g*, room temperature.

7. Remove the supernatant.

Freeze the cells

8. Resuspend the pellet in 0.2 ml of DAP213 solution by pipetting a few times with pipettor.

   Do not break up the colonies.

9. Transfer 0.2 ml of the cell suspension to 2-ml cryovials.

10. Put the vials quickly into liquid nitrogen.

    After adding DAP213 to the cells, the suspension must be frozen within 15 sec for viability of the cells.

11. Store the cells in the liquid nitrogen tank.

Thaw frozen stock

12. Prepare 10 ml of prewarmed (37°C) hES medium in a 15-ml conical tube.

13. Remove frozen vial of iPS cells from liquid nitrogen tank.

14. Add 0.8 ml of prewarmed hES medium to the vial and thaw the cells quickly by pipetting up and down with a 1000-µl pipet tip and pipettor.

15. Transfer the cell suspension to the tube prepared in step 12.

16. Centrifuge 5 min at 160 × *g*, room temperature.

17. Discard the supernatant, and add 4 ml of hES medium.

18. Transfer the cell suspension to a 60-mm dish seeded with mitomycin C–treated SNL feeder cells and incubate in a humidified 37°C, 5% CO_2 incubator.

    For the viability of the iPS cells, steps 14 to 16 should be finished as quickly as possible. Do not break up the cell clumps into single cells.

SUPPORT PROTOCOL 3

RT-PCR FOR DETECTION OF PLURIPOTENT CELL MARKERS

RT-PCR for marker genes of pluripotent stem cells is one of the easiest assays to evaluate the quality of iPS cells. The expression of not only endogenous genes but also transgenes from retroviruses can be examined by RT-PCR.

Materials

Human iPS cells cultured in 6-well plate (Basic Protocols 1 and 2), 80% to 90% confluent
Dulbecco's phosphate-buffered saline without calcium and magnesium (CMF-DPBS: Nacalai Tesque, cat. no. 14249-95)
Trizol reagent (Invitrogen, cat. no. 15596-026)

Chloroform (Nacalai Tesque)
Isopropanol (Nacalai Tesque)
70% ethanol in nuclease-free water
Nuclease-free (e.g., Milli-Q) water
Turbo DNA-free Kit (Ambion, cat. no. AM1907) containing:
  10× DNase I buffer
  Recombinant DNase I
  DNase Inactivation Reagent
ReverTra Ace -α- kit (Toyobo, cat no. FSK-101; *http://www.toyobo.co.jp/*) containing:
  5× reverse transcription buffer (containing 25 mM Mg^{2+})
  10 mM dNTPs
  Recombinant ribonuclease inhibitor (10 U/μl)
  Reverse transcriptase
  Oligo dT_{20} primer (10 pmol/μl)
ExTaq kit (Takara, cat. no. RR001A; *http://www.takara-bio.us*) containing:
  ExTaq DNA polymerase (5 U/μl)
  10× ExTaq buffer
  2.5 mM dNTPs
PCR primers for human ES cell markers (Figure 4A.2.3)

15-ml conical tubes (Falcon)
Centrifuge
Nanodrop spectrometer (Thermo Scientific)
Filtered pipet tips 10-μl, 200-μl, and 1000-μl (RNase-free, Watson)
1.5-ml microcentrifuge tubes, RNase-free
0.2-ml PCR reaction tubes (Greiner)
Thermal cycler (Applied Biosystems)

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2000)

NOTE: Use nuclease-free water to make all the reagents. Milli-Q water or equivalent grade of ultrapure water can be used for the experiments with RNA. Wear disposable gloves and mask.

Prepare the cell lysate

1. Wash the cells once with 2 ml of CMF-DPBS.
2. Aspirate CMF-DPBS completely, and add 1 ml of Trizol reagent, and incubate for 5 min at room temperature.
3. Collect the cell lysate in 1.5-ml microcentrifuge tube.

   You can stop the experiment after completing this step. Cell lysates should be stored at −80°C.

Purify the RNA

4. Add 200 μl of chloroform to the thawed lysate and mix vigorously by shaking.
5. Centrifuge 5 min at 15,000 × *g*, room temperature.
6. Transfer the aqueous phase (500 μl) to a new 1.5-ml microcentrifuge tube, add 400 μl of isopropanol, and mix well by inversion for 20 min.
7. Centrifuge tube 5 min at 15,000 × *g*, room temperature.
8. Remove the isopropanol, add 500 μl of 70% ethanol and centrifuge 5 min at 15,000 × *g*, room temperature.
9. Remove the ethanol completely and air dry the pellet at room temperature for 2 to 3 min.

Target	Forward	Reverse	Cycle condition						Cycle number	Additive	Product size (bp)
OCT3/4	GAC AGG GGG AGG GGA GGA GCT AGG	CTT CCC TCC AAC CAG TTG CCC CAA AC	94°C, 1m	94°C, 10s	60°C, 15s	72°C, 30s	72°C, 5m	4°C	27		144
SOX2	GGG AAA TGG GAG GGG TGC AAA AGA GG	TTG CGT GAG TGT GGA TGG GAT TGG TG	94°C, 1m	94°C, 10s	55°C, 15s	72°C, 30s	72°C, 5m	4°C	27		151
NANOG	CAG CCC CGA TTC TTC CAC CAG TCC C	CGG AAG ATT CCC AGT CGG GTT CAC C	94°C, 1m	94°C, 10s	55°C, 15s	72°C, 30s	72°C, 5m	4°C	27		
GDF3	CTT ATG CTA CGT AAA GGA GCT GGG	CCA ACC CAG GTC CCG GAA GTT	94°C, 1m	94°C, 10s	55°C, 15s	72°C, 30s	72°C, 5m	4°C	32	5% DMSO +	625
FGF4	CTA CAA CGC CTA CGA GTC CTA CA	GTT GCA CCA GAA AAG TCA GAG TTG	94°C, 1m	94°C, 10s	55°C, 15s	72°C, 30s	72°C, 5m	4°C	32		371
DPPA4	GGA GCC GCC TGC CCT GGA AAA TTC	TTT TTC CTG ATA TTC TAT TCC CAT	94°C, 1m	94°C, 10s	55°C, 15s	72°C, 30s	72°C, 5m	4°C	32	5% DMSO +	408
ESG1	ATA TCC CGC CGT GGG TGA AAG TTC	ACT CAG CCA TGG ACT GGA GCA TCC	94°C, 1m	94°C, 10s	55°C, 15s	72°C, 30s	72°C, 5m	4°C	36		243
REX1	CAG ATC CTA AAC AGC TCG CAG AAT	GCG TAC GCA AAT TAA AGT CCA GA	94°C, 1m	94°C, 10s	55°C, 15s	72°C, 30s	72°C, 5m	4°C	36		306
TERT	CCT GCT CAA GCT GAC TCG ACA CCG TG	GGA AAA GCT GGC CCT GGG GTG GAG C	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	36	5% DMSO +	446
FOXD3	CAT CGC GCT CAT CAC CAT GGC CAT C	CGC GGG GGA TCT TGA CGA AGC AGT C	94°C, 1m	94°C, 10s	60°C, 10s	72°C, 30s	72°C, 5m	4°C	26		176
DNMT3B	TGC TGC TCA CAG GGC CCG ATA CTT C	TCC TTT CGA GCT CAG TGC ACC ACA AAA C	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	32	5% DMSO +	242
GABRB3	CCT TGC CCA AAA TCC CCT ATG TCA AAG C	GTA TCG CCA ATG CCG CCT GAG ACC TC	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	32	5% DMSO +	227
TDGF1	CTG CTG CCT GAA TGG GGG AAC CTG C	GCC ACG AGG TGC TCA TCC ATC ACA AGG	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	30	5% DMSO +	237
GAL	TGC GGC CCG AAG ATG ACA TGA AAC C	CCC AGG AGG CTC TCA GGA CCG CTC	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	30	5% DMSO +	188
LEFTB	CTT GGG GAC TAT GGA GCT CAG GGC GAC	CAT GGG CAG CGA GTC AGT CTC CGA GG	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	32	5% DMSO +	255
IFITM1	CCC CAA AGC CAG AAG ATG CAC AAG GAG	CGT CGC CAA CCA TCT TCC TGT CCC TAG	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	26	5% DMSO +	229
NODAL	GGG CAA GAG GCA CCG TCG ACA TCA	GGG ACT CGG TGG GGC TGG TAA CGT TTC	94°C, 1m	94°C, 10s	65°C, 10s	72°C, 30s	72°C, 5m	4°C	36	5% DMSO +	234
UTF1	CCG TCG CTG AAC ACC GCC CTG CTG	CGC GCT GCC CAG AAT GAA GCC CAC	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	32	5% DMSO +	171
EBAF	GCT GGA GCT GCA CAC CCT GGA CCT CAG	GGG CAG CGA GGC AGT CTC CGA GGC	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	32	5% DMSO +	274
GRB7	CGC CTC TTC AAG TAC GGG GTG CAG CTG T	TGG GCA GGC TGA GGC GGT GGT TTG	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	32	5% DMSO +	241
PODXL	TCC AGC CCC ACA GCA GCA TCA ACT ACC	CCG GGT TGA AGG TGG CTT TGA CTG CTC	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	26	5% DMSO +	226
CD9	GTG CAT GCT GGG ACT GTT CTT CGG CTT C	CAC GCC CCC AGC CAA ACC ACA GCA G	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	26	5% DMSO +	220
BRIX	CAC CAC GGT ATC ATC CCA AAA GCC AAC C	ACG CCG ATG CAT GTT TGG TGA CTG GTA G	94°C, 1m	94°C, 10s	70°C, 30s		70°C, 5m	4°C	26	5% DMSO +	230

Figure 4A.2.3 PCR primers and reaction conditions for pluripotent cell marker analysis.

10. Resuspend the pellet in 26 μl of RNase-free water.

You can stop the experiment after completing this step. Purified RNA samples should be stored at −80°C.

Remove genomic DNA contamination by DNase treatment

11. Add 3 μl of 10× DNase I buffer and 1 μl of DNase I (from Turbo DNA-free kit) to the RNA sample, mix gently by finger tapping, and incubate for 30 min at 37°C.

12. Add 3 μl of DNase Inactivation Reagent (from Turbo DNA-free kit), and mix well.

13. Incubate for 3 min at room temperature with occasional mixing by finger tapping.

14. Centrifuge 3 min at 15,000 × *g*, room temperature. Transfer the supernatant carefully to a new 1.5-ml microcentrifuge tube.

Determine RNA concentration

15. Use 1 μl of DNase-treated sample to determine concentration of RNA samples by measuring A_{260}/A_{280} with an optical spectrometer (e.g., Nanodrop), and adjust concentration of each sample to appropriate one.

Samples should be >100 ng/μl RNA for RT-PCR.

You can stop the experiment at this step. Purified RNA samples should be stored at −80°C.

Perform reverse transcription

16. Prepare 20 μl of reaction mixture by mixing the reagents listed below:

4 μl 5× reverse transcription buffer (from ReverTra Ace kit)
2 μl 10 mM dNTPs (from ReverTra Ace kit)
1 μl ribonuclease inhibitor (from ReverTra Ace kit)
1 μl ReverTra Ace (reverse transcriptase; from ReverTra Ace kit)
1 μl 10 μM oligo dT_{20} primer (from ReverTra Ace kit)
1 μg DNase-treated total RNA (step 14)
Nuclease-free water up to 20 μl.

You should prepare reactions containing no reverse transcriptase as negative controls for each sample.

17. Incubate the mixture in thermal cycler at the condition as follows:

60 min at 42°C
5 min at 95°C
Indefinitely at 4°C.

You can stop the experiment at this step. cDNA samples should be stored at −20°C or lower.

Amplify the products by PCR

18. Prepare 25 μl of PCR mixture by mixing the reagents listed below in a 0.2-ml PCR reaction tube:

2.5 μl 10× ExTaq buffer (from ExTaq kit)
2 μl 2.5 mM dNTPs (from ExTaq kit)
0.5 μl 10 μM forward primer (Figure 4A.2.3)
0.5 μl 10 μM reverse primer (Figure 4A.2.3)
0.5 μl 5 U/μl ExTaq DNA polymerase (from ExTaq kit)
1 μl cDNA sample (step 17)
1.25 μl of DMSO (optional, depends on primer sets)
Nuclease-free water up to 25 μl.

19. Carry out PCR according to the conditions listed in Figure 4A.2.3.

    PCR conditions, particularly the number of cycles, may differ among different thermal cyclers. It is necessary to experiment to find the optimal conditions.

20. After finishing PCR, analyze the by electrophoresis on a 2% agarose gel in 1× TAE buffer using a standard protocol (e.g., Voytas, 2000).

SUPPORT PROTOCOL 4

IMMUNOCYTOCHEMISTRY FOR PLURIPOTENT CELL MARKERS

The expression of pluripotent stem cells marker can be confirmed not only by RT-PCR (Support Protocol 3) but also by immunocytochemistry. Some surface antigens specifically expressed in pluripotent cells such as SSEAs and TRAs were identified by analyses of human embryonic carcinoma (EC) and ES cells. See Figure 4A.2.2 for examples of immunohistochemistry results.

Materials

Human iPS cells (Basic Protocol 1)
6-well plates seeded with mitomycin C–treated feeder cells (Support Protocol 1)
hES medium (see recipe)
Dulbecco's phosphate-buffered saline without calcium and magnesium (CMF-DPBS: Nacalai Tesque, cat. no. 14249-95)
CMF-DPBS containing 10% (v/v) formalin
CMF-PBS containing 1% (w/v) bovine serum albumin, 5% (v/v) normal goat serum (or donkey serum), and 0.2% (v/v) Triton X-100 (omit Triton if staining surface antigens)
Primary antibodies against desired ES markers (perform all dilutions in CMF-DPBS containing 1% v/v bovine serum albumin):
  Anti-Nanog goat polyclonal (R&D Systems, cat. no. AF1997; use at 1:20 dilution)
  Anti-SSEA-1 mouse IgM (Developmental Studies Hybridoma Bank, cat. no. MC480; use at 1:5 dilution)
  Anti-SSEA-3 rat IgM (Developmental Studies Hybridoma Bank cat. no. MC631; use at 1:5 dilution)
  Anti-TRA-1-60 mouse IgM (Chemicon, cat. no. MAB4630; use at 1:50 dilution)
  Anti-TRA-1-81 mouse IgM (Chemicon, cat no. MAB4381; use at 1:50 dilution)
Secondary antibody against IgG or IgM of species in which primary antibody was raised, labeled with Alexa Fluor 488 or Alexa Fluor 546; use at 1:500 dilution in CMF-DPBS containing 1% (w/v) bovine serum albumin
10 mg/ml Hoechst 33342 (H3570, Invitrogen)

1. To prepare cells for immunostaining, seed about 100 to 200 clumps of human iPS cells in hES cell medium in each well of a 6-well plate containing mitomycin-treated SNL feeder cells and incubate for 5 to 7 days prior to fixation.

Fix cells and block nonspecific binding

2. Prior to fixation, aspirate the medium, and wash with 2 ml of CMF-DPBS.
3. Remove CMF-DPBS, add 2 ml of CMF-DPBS containing 10% formalin, and fix the cells by incubating for 10 min at room temperature.
4. After fixation, wash the cells once with 2 ml of CMF-DPBS.
5. Aspirate CMF-DPBS and add 2 ml of CMF-PBS containing 1% (w/v) bovine serum albumin, 5% (v/v) normal goat serum, and 0.2% (v/v) Triton X-100. Incubate 45 min at room temperature.

Omit Triton X-100 when staining for surface antigens. Triton X-100 is not necessary for immunostaining of surface antigens. Treatment with Triton X-100 is required only for anti-Nanog antibody.

For anti-Nanog antibody, substitute normal donkey serum for normal goat serum because anti-Nanog antibody was raised in goat.

Treat cells with primary and secondary antibodies

6. After blocking procedure, incubate the cells 1 ml of primary antibody at the appropriate dilution in CMF-DPBS containing 1% bovine serum albumin, overnight at 4°C.

   Other antibodies should work. Determine the optimal dilution.

7. Wash the cells three times each for 5 min with CMF-DPBS.

8. Add 1 ml of secondary antibody conjugated with Alexa Fluor 488 or 546 to the sample at the appropriate dilution in CMF-DPBS containing 1% bovine serum albumin supplemented with 1 μg/ml of Hoechst 33342 (added from 10 mg/ml Hoechst stock solution), and incubate for 45 min at room temperature in the dark.

9. Wash out secondary antibody with 2 ml CMF-DPBS three times, each time for 5 min.

10. Observe the cells with a fluorescent microscope equipped with the appropriate filters.

ASSESSING PLURIPOTENCY BY IN VITRO DIFFERENTIATION OF iPS CELLS BY EMBRYOID BODY FORMATION

SUPPORT PROTOCOL 5

Embryoid body formation is one of the easiest procedures for in vitro differentiation of ES cells. This also can be applied for differentiation of iPS cells. Our protocol consists of a primary floating culture for 8 days. After 8 days of floating culture, transfer the cells to gelatin-coated plates to induce further differentiation.

After embryoid body formation, differentiation should be confirmed by immunocytochemistry for differentiated markers. Other procedures such as RT-PCR (Support Protocol 3) are also suitable for determination of pluripotency and/or differentiation.

Materials

10 mg/ml HEMA-MMA (see recipe)
Growing human iPS cells (Basic Protocols 1 and 2) at 80% to 90% confluency in 60-mm dish
Dulbecco's phosphate-buffered saline without calcium and magnesium (CMF-DPBS: Nacalai Tesque, cat. no. 14249-95)
CTK solution (see recipe)
hES medium containing no bFGF (see recipe)
CMF-PBS containing 10% (v/v) formalin (Sigma)
CMF-PBS containing 1% (w/v) bovine serum albumin, 5% (v/v) normal goat serum (or donkey serum), and 0.2% (v/v) Triton X-100
Primary antibodies against desired ES markers for immunohistochemistry (perform all dilutions in CMF-PBS containing 1% v/v bovine serum albumin):
  Anti-α-fetoprotein mouse IgG (R&D Systems, cat. no. MAB1368; use at 1:100 dilution)
  Anti-α-smooth muscle actin mouse IgG (Dako, cat. no. N1584; use at 1:500 dilution)
  Anti-βIII-tubulin mouse IgG (Chemicon, cat. no. CB412; use at 1:100 dilution)
Secondary antibody: anti-mouse IgG labeled with Alexa Fluor (use at 1:500 dilution in CMF-DPBS containing 1% w/v bovine serum albumin)
10 mg/ml Hoechst 33342 solution (Invitrogen)

100-mm tissue culture dish
Sterile disposable cell scraper
15-ml conical centrifuge tubes
Gelatin-coated 6-well culture plate (see recipe)

Additional reagents and equipment for immunohistochemistry (Support Protocol 4)

Establish suspension culture

1. Place 5 ml of 10 mg/ml of HEMA-MMA in a 100-mm dish, and incubate at room temperature in a hood with the dish covered with foil until the solution dries up (3 to 5 days).
2. Wash the iPS cells in 60-mm dish once with 4 ml CMF-DPBS.
3. And add 0.5 ml of CTK solution and return the dish to the 37°C incubator.
4. After 5 min incubation, wash twice with 4 ml of CMF-DPBS to remove the CTK solution and detached feeder cells.
5. Add 4 ml hES medium without bFGF to the dish.
6. Detach iPS colonies from the dish by using cell scraper. Collect the cell clumps to a 15-ml conical tube.

   Do not break up the colonies; larger colonies can form embryoid bodies effectively.

7. Add another 5 ml of hES medium without bFGF and transfer the cell suspension to the HEMA-coated 100-mm dish from step 1.
8. Incubate 2 days in humidified 37°C, 5% CO_2 incubator.
9. To change the medium, collect the cell suspension into a 15-ml conical tube and let sit it for 5 min at room temperature.
10. Remove the supernatant (~8 ml) carefully, then add 8 ml of fresh hES medium without bFGF and return the suspension to a HEMA-coated dish prepared as in step 1. Change the medium every other day.

Set up attached culture

11. Collect the iPS cell suspension into a 15-ml conical tube, and let sit for 5 min at room temperature. Remove the supernatant and resuspend the cells in 12 ml of hES medium without bFGF.
12. Transfer 2 ml of cell suspension into wells of a gelatin-coated 6-well culture plate, and incubate at 37°C, 5% CO_2.
13. Change the medium every other day.
14. After 8-day attached culture, perform immunocytochemistry for differentiated cell markers (see Support Protocol 4).

    We routinely observe the expression of α-fetoprotein for endoderm, α–smooth muscle actin for mesoderm, and βIII-tubulin for ectoderm. Other antibodies and markers may also be used for this purpose.

ASSESSING PLURIPOTENCY BY IN VIVO DIFFERENTIATION BY TERATOMA FORMATION

SUPPORT PROTOCOL 6

Teratoma formation is another well known, important test of pluripotency. In general, mouse ES and iPS cells can produce teratomas easily. However, it is hard to form tumors derived from either human ES or iPS cells by subcutaneously injection into immunodeficient mice, including NOD-SCID mice. Therefore, in this protocol we inject

stem cells into testes of SCID mice. This change improves the efficiency of tumor formation to more than 80%.

Materials

10 mM Y-27632 (Wako, cat. no. 253-00513)
Growing iPS cells (Basic Protocols 1 and 2) at 80% to 90% confluency in 60-mm dish
Dulbecco's phosphate-buffered saline without calcium and magnesium (CMF-DPBS: Nacalai Tesque, cat. no. 14249-95)
CTK solution (see recipe)
hES medium (see recipe)
DMEM/F12 medium (e.g., Invitrogen) supplemented with 10 μM Y-27632
1.2% tribromoethanol (Avertin): dissolve 2.5 g tribromoethanol in 5 ml butanol, then add 200 ml distilled water; store at 4°C in the dark
SCID mice, (7- to 8-weeks, male)
70% ethanol
CMF-PBS containing 10% formalin

Sterile disposable cell scrapers
15-ml conical centrifuge tubes
Centrifuge
Hamilton syringe
25-G to 26-G needle (Terumo)
Suture needle with thread

Additional reagents and equipment for intraperitoneal injection (Donovan and Brown, 2006a) and euthanasia of the mouse (Donovan and Brown, 2006b), paraffin embedding and sectioning of tissue, and hematoxylin/eosin staining of tissue sections (*UNIT 2A.5*)

NOTE: All protocols involving live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to government regulations for the care and use of laboratory animals.

Prepare cell suspension

1. Add 10 μM Y-27632 (from 10 mM stock) to the medium of a confluent culture of iPS cells, and incubate at 37°C for at least 1 hr.

   Y-27632 is a specific inhibitor for p160-Rho-associated coiled-coil kinase (ROCK).

2. Wash the cells with 4 ml of CMF-DPBS, and add 0.5 ml of CTK solution. Incubate ~5 min at room temperature.

   After treatment with Y-27632, the cells may become less detachable. In such cases, you can treat the cells with CTK solution for longer period of time (~10 min).

3. Wash out CTK solution and detached feeder cells with 4 ml of CMF-DPBS, twice, and add 4 ml of hES medium.

4. Detach iPS cells from the dish with a cell scraper, and break the colonies into small clumps by pipetting up and down several times.

5. Collect the cell suspension to a 15-ml conical tube, and centrifuge 5 min at 200 × *g*, room temperature.

6. Aspirate the supernatant, and resuspend cells in 300 to 500 μl of DMEM/F12 supplemented with 10 μM of Y-27632.

Inject cells

7. Inject 0.8 ml of 1.2% tribromoethanol solution intraperitoneally (Donovan and Brown, 2006a) into SCID mouse (0.12 ml for 10 g weight).
8. Wash the lower abdominal/groin area with 70% ethanol.
9. Dissect out the testes and remove from the body. Dissect the lower abdominal/groin area and withdraw the inguinal canal and then the testes. Leave spermatic cord intact.
10. Inject 30 μl of iPS cell suspension into a testes, under the capsule, using a Hamilton syringe and a 25-G to 26-G needle, as gently as possibly.
11. Return the testes to the original interperitoneal location, and close the incision with stitches. Return mouse to colony within 2 hr.
12. About 3 months later, observe the mice for teratoma formation (Fig. 4A.2.2K).

    Mice may appear to be pregnant, indicating the presence of a teratoma.

Dissect the tumors

13. Euthanize mice (Donovan and Brown, 2006b) bearing teratomas and dissect out the tumors.
14. Fix the tumors in ~50 ml of CMF-DPBS containing 10% formalin and incubate overnight at room temperature with agitation.
15. After fixation, embed the tumor in paraffin.
16. Slice the tumor into 4- to 5-μm sections and mount on slides.
17. Stain the sections with hematoxylin and eosin using a standard protocol (e.g., *UNIT 2A.5*).
18. Examine the entire set of sections for a tumor, scoring for the presence of derivatives of all three germ layers such as cartilage, pigmented epithelium, and gut-like epithelium (see Fig. 4A.2.2M).

    If the tumor contains derivatives of all three germ layers, the iPS cell line is pluripotent.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

10% FBS medium

DMEM (e.g., Invitrogen) containing:
10% fetal bovine serum (FBS)
50 U/ml penicillin
50 μg/ml streptomycin

To prepare 500 ml of 10% FBS medium, mix 50 ml FBS and 2.5 ml of 100× penicillin/streptomycin (containing 10,000 U penicillin and 10,000 mg/ml streptomycin). Make up to 500 ml with DMEM. Store at 4°C up to 1 week.

For Plat-E cells (see Support Protocol 2), add 1 μl of 10 mg/ml puromycin stock (see recipe) and 10 μl of 10 mg/ml blasticidin S stock (see recipe) to 10 ml of 10% FBS medium.

293FT medium

DMEM (e.g., Invitrogen) containing:
10% fetal bovine serum (FBS)
2 mM L-glutamine
1×10^{-4} M nonessential amino acids
1 mM sodium pyruvate
50 U penicillin
50 μg/ml streptomycin
0.5 mg/ml G418

To prepare 500 ml of the medium, mix 50 ml of FBS, 5 ml of 200 mM (100×) L-glutamine, 5 ml 100× nonessential amino acids, 5 ml of 100 mM sodium pyruvate, and 2.5 ml of 100× penicillin/streptomycin (containing 10,000 U penicillin and 10,000 mg/ml streptomycin). Make up to 500 ml with DMEM. Store at 4°C up to 1 week. Just before use, add 100 μl of 50 mg/ml G418 to 10 ml 293FT medium.

Blasticidin S stock solution

Dissolve blasticidin S hydrochloride (Funakoshi Chemical Company; *http://www.funakoshi.co.jp*) in distilled water at 10 mg/ml and sterilize through a 0.22-μm filter. Aliquot and store at −20°C.

CTK solution

5 ml 2.5% (w/v) trypsin
5 ml 1 mg/ml collagenase IV
0.5 ml 0.1 M $CaCl_2$
10 ml Knockout Serum Replacement (KSR; Invitrogen)
30 ml distilled water
Store up to 1 month at −20°C
Do not repeat freeze/thaw cycles

DAP213 solution

To 5.37 ml hES medium (see recipe) add:
1.43 ml DMSO
1 ml 10 M acetamide
2.2 ml of propylene glycol
Store up to 1 month at −80°C

Gelatin coating of culture vessels

Dissolve 1 g of gelatin powder (Sigma, cat. no. G-1890) in 100 ml of distilled water, autoclave, and store at 4°C as the 10× gelatin stock solution. To prepare 0.1% (1×) gelatin solution, thaw the 10× gelatin stock in a microwave and/or autoclave, then add 50 ml of the 10× solution to 450 ml of distilled water. Filter the solution with a 0.22-μm filter unit and store at 4°C. To coat culture dishes, add appropriate volume of 0.1% (1×) gelatin solution to cover the entire area of the dish bottom. For example, 1, 3, or 5 ml of gelatin solution is used for a 35-, 60-, or 100-mm dish, respectively. Incubate the dishes for at least 30 min at 37°C in a sterile environment. Before using, aspirate off the excess gelatin solution.

Gelatin stock is prepared as 10× concentration (1% w/v) stocks.

hES medium

DMEM/F12 medium containing:
20% Knockout Serum Replacement (KSR)
2 mM L-glutamine

continued

1×10^{-4} M nonessential amino acids
1×10^{-4} M 2-mercaptoethanol
50 U penicillin
50 μg/ml streptomycin

To prepare 500 ml of the medium, mix 100 ml KSR, 5 ml of 200 mM (100×) L-glutamine, 5 ml 100× nonessential amino acids, 1 ml 2-mercaptoethanol, and 2.5 ml of 100× penicillin/streptomycin (containing 10,000 U penicillin and 10,000 mg/ml streptomycin). Make up to 500 ml with DMEM/F12. Add 200 μl of 10 μg/ml bFGF into 500 ml of the medium just before use. For differentiation experiments (e.g., Support Protocol 5), do not add bFGF. Store at 4°C up to 1 week.

All abovementioned components are available from Invitrogen. Primate ES cell medium from ReproCELL (http://www.reprocell.net/) may be used as an alternative.

Mitomycin C, 0.4 mg/ml

Dissolve 2 mg of mitomycin C (Kyowa Hakko Kirin; *http://www.kyowa-kirin.co.jp/english/*) in 5 ml of CMF-DPBS (Nacalai Tesque, cat. no. 14249-95). Store up to 1 month at −20°C in the dark.

CAUTION: *Because of its toxicity, the solution must be treated exclusively in a safety cabinet with gloves and lab coat and disposed of in accordance with the rules each institution stipulates.*

Poly(hydroxyethyl methacrylate-co-methyl methacrylate; HEMA-MMA), 10 mg/ml

Add 0.3 g of HEMA-MMA (Sigma, cat. no. P-3932) to a tube containing 30 ml ethanol. Incubate at 37°C overnight with agitation. Prepare fresh for each experiment.

Puromycin

Dissolve puromycin (Sigma, cat. no. P-8833) in distilled water at 10 mg/ml and sterilize through a 0.22-μm filter. Divide into aliquots and store up to 1 month at −20°C.

SNL medium

DMEM (e.g., Invitrogen) containing:
7% fetal bovine serum (FBS)
2 mM L-glutamine
50 U penicillin
50 μg/ml streptomycin

To prepare 500 ml of the medium, mix 35 ml FBS, 5 ml 200 mM (100×) L-glutamine, and 2.5 ml of 100× penicillin/streptomycin (containing 10,000 U penicillin and 10,000 mg/ml streptomycin). Make up to 500 ml with DMEM. Store at 4°C up to 1 week.

This medium is used for fibroblasts and PLAT-E cells.

COMMENTARY

Background Information

Although it is commonly known that nuclei of differentiated cells can be reprogrammed back to embryonic states by means of nuclear transfer into oocytes or fusion with ES cells, the mechanism of inducing nuclear reprogramming has yet to be revealed. The fact that somatic cells can be reprogrammed by fusion with ES cells implies that ES cells contain factors that can induce reprogramming.

We hypothesized that factors which play important roles in ES cells also play pivotal roles in induction of nuclear reprogramming. Pluripotency and tumor-like proliferation are

the most exquisite properties of ES cells. Three transcription factors—Oct3/4, Sox2, and Nanog—have been found to be essential in the maintenance of pluripotency in both early embryos and ES cells. While a handful of laboratories have demonstrated that several tumor-related gene products, such as ERas, c-*myc*, and Stat3, contribute to long-term maintenance of ES cells in culture, we have identified several genes that are specifically expressed in ES cells by analyzing expressed sequence tag (EST) databases. After selecting the most promising 24 gene products as candidates for potential factors that could induce reprogramming, we narrowed these to four transcription factors (Oct3/4, Sox2, Klf4, and c-*myc*) that have been shown to convert fibroblasts back to pluripotent state. The identification of these factors was an important breakthrough that has revealed a mechanism of nuclear reprogramming and let us create pluripotent cells directly from skin biopsy specimens.

One year later, other groups succeeded in generating iPS cells from human somatic cells. Recently, two research groups have reported that various disease-specific iPS cells from a patient's own somatic cells have been successfully reprogrammed (Dimos et al., 2008; Park et al., 2008b). Now, iPS cell technology can be used in conjunction with or in place of ES cell technology to shed light on understanding pathogens, in drug discovery, and most of all, to develop regenerative medicine applications. Encouraging broad use of iPS cell technology will facilitate the development of practical applications. These protocols should provide guidance to scientists who share our objectives.

Troubleshooting

In some cases, lentivirus is toxic to fibroblasts. Depending on the different cell lines, lentiviral transduction may lead to loss or growth arrest of fibroblasts due to their sensitivity to the virus. As some fibroblasts are more vulnerable to lentivirus than common cells, they should be treated with a double dilution of the virus-containing supernatant in fresh medium or by shortening the exposure time from overnight to 5 hr. For our purposes, the expression of mouse *Slc7a1* gene is sufficient for generation of iPS cells despite lower infection efficiency.

When no ES-like (iPS) colonies appear in fibroblast cultures after introduction of the four factors, the following causes should be considered. First, the titer of retrovirus may be too low. Transduction efficiencies of retroviruses for reprogramming factors are critical for iPS cell colony formation as described above. The retrovirus must be prepared fresh for every experiment. Do not use frozen stock retroviruses because freezing causes reduction of the titer.

Growth properties of the fibroblasts are also important for iPS cell generation. Efficiency of retroviral transduction is markedly reduced when senescent fibroblasts are used for transduction. We strongly recommend banking stocks of fibroblasts at early passages and using fresh fibroblasts of early passage for iPS cell production.

The number of cells that are plated onto SNL feeder cells after retroviral transduction is important. Overgrowth of fibroblasts might make cells peel off from the edge of the dish like a sheet, inhibiting formation of iPS cell colonies. Although this may be overcome by reducing the cell number, too small a number of cells could lead to no colony appearance. The optimal conditions differ for each individual cell type. We recommend that you seed at least in two or three different dishes with different densities when first plating the transduced cells.

In addition, the qualities of feeder cells are crucial not only for generation of iPS cells, but also for maintenance of them. If feeder cells are too old, cells may peel off the substrate during the reprogramming or maintenance. SNL feeder cells more than 3 days after mitomycin C treatment cannot survive the stimulation by bFGF in hES medium (as bFGF may have a toxic effect on older feeder cells). It is recommended that SNL feeder cells be used within 3 days after inactivation.

Some problems may arise after iPS cells are generated. For example, iPS cells can change characteristics and potential, depending on the line, with long-term culture. Human iPS cells, like human ES cells, may become adapted in a long-term culture. We recommend that large amounts of iPS cell stocks be frozen at early passages to support long-term experimentation. iPS cells are relatively unstable during early passage period so that spontaneous differentiation in daily culture may also happen. When the number of differentiated colonies increases, select undifferentiated colonies and transfer them by aspiration to a new dish of SNL feeder cells. After this procedure is repeated two to three times, the majority of the dish will consist of undifferentiated colonies. In addition, the qualities of feeder cells, such as density and freshness, are also important.

Anticipated Results

The efficiency of lentiviral transduction to fibroblasts should be >90%. You can estimate the efficiency of infection with the GFP-encoding lentivirus. On the other hand, because retroviruses can be transfected only into dividing cells, the transduction efficiency may stay at ~30% to 60%.

From 10 days to 2 weeks after retroviral transduction, some granule colonies usually appear (Fig. 4A.2.2, panel A). However, these colonies are not iPS cells. Generally, clear-edged colonies are produced at 3 weeks post transduction (panel B). They can be expanded after being picked up and transferred to another plate. Established iPS cells show hES-like morphologies on feeder cells (panel C). When the cells reach this stage, you should passage once a week.

The expression of markers in pluripotent stem cells can be detected in iPS cells as similar level to ES cells. iPS cells typically express SSEA3 (panel D), TRA-1-60 (panel E), TRA-1-81 (panel F), and Nanog (panel G), but not SSEA1 (panel H).

Differentiation potentials of iPS cells can be determined easily by embryoid body formation. After a 16-day induction, the expression of differentiation markers such as α-fetoprotein (panel I), α-smooth muscle actin (panel J) and βIII-tubulin (panel K) can be confirmed by immunocytochemistry. Another assay for determination of pluripotency, teratoma formation, is also important. Generally, around 3 months after injection of iPS cells into the testes of SCID mice, the mice may appear to be pregnant (panel L). In some cases, black-colored pigment cells can be observed in dissected tumors by the naked eye (panel M). Staining of tumors with hematoxylin and eosin may show that many types of all three germ layers exist in the teratoma if parental iPS cells are pluripotent (panel N).

Treatment of human iPS cells with Y-27632, which is an inhibitor for p160-Rho-associated coiled-coil kinase (ROCK), before harvesting, may improve the survival rate. If you have trouble with frail viability of iPS cells, you can treat the cells at least an hour before harvesting.

Time Considerations

It takes 1 week to successfully transduce the fibroblasts with the lentiviral vector and to verify transduction by microscopic examination or flow cytometry. Then it requires an additional 5 days to prepare the retrovirus vectors and transduce the fibroblasts. Once plated on SNL feeder cells, it takes ~3 weeks for iPS cell colonies to appear and additional time for them to grow to a size where they can be passaged. iPS cells are fed every other day and passaged once a week. Overall, it takes over 3 months to establish an iPS cell line.

Acknowledgements

We thank Dr. Tetsuya Ishii, Kanon Takeda, and Yuko Shimazu for reading the manuscript, and Tomoko Ichisaka and Noriko Tsubooka and other members of Yamanaka laboratory for valuable discussion and support. Thanks too to Rie Kato, Ryoko Iyama, Eri Nishikawa, Noriyo Maruhashi and the member of CiRA for administrative support. We are also grateful to Drs. Toshio Kitamura for retroviral system, Peter Andrews for antibodies, and Yoshiki Sasai for instruction on teratoma experiments.

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