# Generation of human-induced pluripotent stem cells

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**Pluripotent cells, such as embryonic stem cells, are invaluable tools for research and can potentially serve as a source of cell- and tissue-replacement therapy. Rejection after transplantation of cells and tissue derived from embryonic stem cells is a significant obstacle to their clinical use. Recently, human somatic cells have been reprogrammed directly to pluripotency by ectopic expression of four transcription factors (Oct4, Sox2, Klf4 and Myc) to yield induced pluripotent stem (iPS) cells. Human iPS cells are a potential source of patient-specific pluripotent stem cells that would bypass immune rejection. iPS cells can also be used to study diseases for which there are no adequate human *in vitro* or animal models. In this protocol, we describe how to establish primary human fibroblasts lines and how to derive iPS cells by retroviral transduction of reprogramming factors. Overall, it takes 2 months to complete reprogramming human primary fibroblasts starting from biopsy.**



## INTRODUCTION

Ectopic expression of transcription factors in human fibroblasts (hFibs) is sufficient to yield induced pluripotent stem (iPS) cells that resemble embryonic stem (ES) cells in their morphology, gene expression and ability to form teratomas in immune-deficient mice[1–5]. At present, reprogramming with defined factors is achieved by retroviral transduction of potentially oncogenic transcription factors, significantly limiting its clinical use. However, these limitations do not preclude the use of iPS cells as *in vitro* models for human development and disease. Because primary human dermal fibroblasts can be readily generated in the laboratory from a small skin biopsy, investigators can create iPS cells from patients or healthy volunteers for research purposes.

Some distinct cocktails of transcription factors have been used to reprogram hFibs. Nakagawa *et al.* demonstrated that OCT4, SOX2 and KLF4 were sufficient to induce reprogramming in adult human dermal fibroblasts; however, the efficiency is much improved with the addition of MYC[3,5]. Other combinations of factors, including novel factors, may also promote reprogramming, and indeed NANOG and LIN28 have been shown to complement OCT4 and SOX2 in reprogramming fetal and newborn hFibs[2]. Currently, no comprehensive data are available to compare which combination of factors is more efficient and/or applicable to reprogram certain cell types. But, it seems that adult primary fibroblasts are more refractory to reprogramming than fetal and neonatal fibroblasts, and our cocktail of six factors (four factors+hTERT and SV40 LT) successfully reprograms adult primary fibroblasts[4].

Here, we describe a detailed protocol to generate iPS cells from primary adult human dermal fibroblasts by ectopic expression of OCT4, SOX2, MYC, KLF4, hTERT and SV40 Large T (**Fig. 1**). This protocol is based on our recent report of iPS cell derivation from adult dermal fibroblasts[4]. In our hands, hTERT and SV40 large T enhance the overall efficiency of iPS generation for unclear reasons, but we speculate that these factors may act indirectly on supportive cells in the culture to enhance the efficiency with which the reprogrammed colonies can be selected. You may choose to exclude these factors, if you find that your efficiency of iPS generation is sufficient without them.

This protocol can serve as an experimental platform for studying pluripotency, complemented with the protocol for reprogramming mouse fibroblasts[6]. By fine-tuning the efficiency of reprogramming and scaling the tissue culture appropriately, one could use this protocol to screen other putative pluripotency factors, as well as libraries of cDNAs and small RNAs. We anticipate the eventual development of direct factor-based reprogramming that does not rely on retroviral transduction. The protocol can be adapted to test transient expression vectors, protein transduction, small molecules and/or combinations of these along with other technologies to study the induction of pluripotency.

The study of human ES (hES) cells carries many limitations: high cost, scarcity of embryos from which new lines can be derived, governmental restrictions, limited availability of lines (especially novel, early passage lines), etc. iPS cells can serve as an attractive alternative for

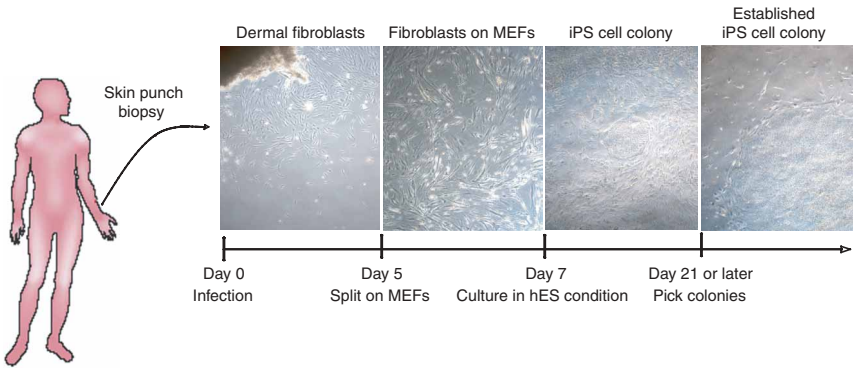


**Figure 1** | Overview of preparing human dermal fibroblasts and isolating induced pluripotent stem (iPS) cells. Specimens from 6-mm skin punch biopsy are dissociated, and plated to expand dermal fibroblasts. Fibroblasts are infected with retroviral vectors expressing transcription factors (OCT4, SOX2, MYC and KLF4) with or without hTERT and SV40 LT. Infected cells are incubated in human embryonic stem (hES) cell medium until iPS cell colonies start to appear. iPS cell colonies are picked and expanded for further use. MEFs, mouse embryo fibroblasts.

investigators affected by these limitations. Although iPS cells resemble ES cells in many ways, they do not replace them. The ES cell remains, at least for now, the standard against which pluripotency is measured. iPS cells serve as an important adjunct to the study of pluripotency and the pathways of lineage specification and differentiation.

Compliance with regulatory agencies that serve to protect human research subjects is of utmost importance. We have included in this protocol the consent form used at our institution to obtain a skin biopsy from healthy adult volunteers to derive pluripotent cells (see **Supplementary Note** online). This protocol describes the derivation of primary fibroblast lines from skin punch biopsy. Instructions on how to perform the biopsy are not included because this should be performed in an appropriate clinical setting by an adequately trained clinician.



## MATERIALS

### REAGENTS

- A 6-mm skin punch biopsy ! CAUTION This should be obtained by a dermatologist or other appropriately trained physician. Procurement of skin tissue for use in reprogramming experiments must be obtained via informed consent under a protocol approved by the Institutional Review Board and Embryonic Stem Cell Research Oversight Committee or similar committees that serve to protect human research subjects. See **Supplementary Note** for the consent form used by our laboratory.
- pMIG, pBABE-puro-SV40LT and pBABE-hygro-hTERT retroviral vectors containing cDNA (see REAGENT SETUP)
- Vesicular stomatitis virus G protein (VSV-G) and Gag-Pol vectors (see REAGENT SETUP)
- 293T cells (American Type Culture Collection, cat. no. CRL11268)
- DMEM (Invitrogen, cat. no. 11965-092)
- Knockout (KO) DMEM (Invitrogen, cat. no. 10829-018)
- DMEM/F12 (Invitrogen, cat. no. 11330-32)
- Minimum essential medium α (MEMα; Mediatech, cat. no. 10-022-CV)
- Heat-inactivated FBS (Gemini Bioproducts, cat. no. 100-106)
- KO serum replacement (KOSR; Invitrogen, cat. no. 10828-028)
- PBS without Ca/Mg (Mediatech, cat. no. 21-040-cv)
- L-GIn (Invitrogen, cat. no. 25030-081)/GlutaMAX (Invitrogen, cat. no. 35050-061)
- Nonessential amino acid solution (Invitrogen, cat. no. 11140-050)
- 2-Mercaptoethanol (Sigma, cat. no. M7522) ! CAUTION When used, avoid inhalation and skin contact.
- Basic fibroblast growth factor (bFGF; Invitrogen, cat. no. 13256-029) (see REAGENT SETUP)
- 7.5% BSA solution (wt/vol; Invitrogen, cat. no. 15260-037)
- Penicillin/streptomycin
- 0.25% Trypsin/EDTA (Invitrogen, cat. no. 25200-056)
- 0.05% Trypsin/EDTA (Invitrogen, cat. no. 25300-054)
- Collagenase type IV (Invitrogen, cat. no. 17104-019)
- Gelatin 0.1% solution (wt/vol; Millipore, cat. no. ES-006-B) (see REAGENT SETUP)
- Protamine sulfate (Sigma, cat. no. P4020)
- Y-27632 (Calbiochem, cat. no. 688001)
- FuGENE 6 transfection reagent (Roche Applied Science, cat. no. 1181509001)
- 293T medium
- Mouse embryo fibroblast (MEF) medium (see REAGENT SETUP)
- hFib medium (see REAGENT SETUP)
- hES cell medium (see REAGENT SETUP)
- Retroviral infection medium (see REAGENT SETUP)
- iPS cell-freezing medium (see REAGENT SETUP)

### EQUIPMENT

- Inverted tissue culture microscope with phase contrast microscope (×4, ×10, ×20, ×40 objectives)
- Stereomicroscope (Nikon; SMZ-1500 or similar)
- Biosafety cabinet with aspirator for tissue culture
- Biosafety cabinet with aspirator for tissue culture, fitted for stereomicroscope
- Tissue culture centrifuge
- Tissue culture dish, 100 and 150 mm
- Tissue culture plates, 4, 6 and 12-well
- Filter unit (0.45 μm; Nalgene Labware, cat. no. 165-0045)
- Conical tubes, 15 and 50 ml
- Glass Pasteur pipettes, 9 inches—sterilized by autoclave
- 3-ml Transfer pipette, sterile, individually wrapped (Falcon, cat. no. 357575)
- Plastic disposable transfer pipettes, 1, 5, 10, 25 and 50 ml
- Glass disposable transfer pipettes, 5 ml
- Disposable sterile filter system (0.22 μm, 500 ml; Corning, cat. no. 430758)
- Disposable syringes, 10 and 1 ml
- Hypodermic needle, 27–30G
- Acrodisc filter (0.2 μm, low protein binding; PALL, cat. no. 4602)
- Acrodisc filter (0.2 μm, DMSO safe; PALL, cat. no. 4433)
- Freezing container (Nalgene Labware, cat. no. 5100)
- Aspirator tube assembly (Sigma, cat. no. A5177)
- Micopipette (Mid Atlantic, cat. no. MXL3-STR)
- Cell lifter (Corning, cat. no. 3008)
- Centra, CL2 Benchtop centrifuge (215 4 Place Economy Rotor; Thermo Scientific)
- Beckman, 1 × 3–$\frac{1}{2}$ polyallomer centrifuge tube (Beckman, cat. no. 326823)
- Cell strainer, 70 μm (Falcon; Becton Dickinson, cat. no. 352350)

### REAGENT SETUP

**Retroviral vectors** pMIG is available from Addgene (plasmid 9044, W. Hahn). pMIG containing OCT4, SOX2 and KLF4 are available from Addgene (17225, 17226 and 17227, respectively). pBABE-puro-SV40 is available from Addgene (plasmid 13970, T. Roberts). pBABE-hygro-hTERT is available from Addgene (plasmid 1773, R. Weinberg). VSV-G (plasmid 8454, R. Weinberg) and Gag-Pol vectors (plasmid 8455, R. Weinberg) are available from Addgene. pMIG containing MYC was obtained from J.L. Cleveland (St. Jude Children's Research Hospital, Memphis, TN).

**Gelatin-coated culture dishes** Add enough 0.1% gelatin solution to cover the bottom of the dish. Incubate the dish for at least 30 min at 37 °C. Before using, aspirate excess gelatin solution and wash with PBS. We recommend coating culture dishes right before use.

**bFGF** Prepare PBS with 0.1% BSA, then filter using 10-ml syringe and a 0.2-μm low protein-binding syringe filter. Reconstitute 10 μg bFGF in 1 ml sterile PBS with 0.1% BSA. Store at –20 °C in 250–500 μl aliquots, which can be used for 6 months, if properly stored.

**MEF/hFib media** DMEM containing 10% FBS (vol/vol), 2 mM L-GIn, 50 U $ml^{-1}$ penicillin and 50 mg $ml^{-1}$ streptomycin. To prepare 500 ml of the medium, mix 50 ml FBS, 5 ml L-GIn and 5 ml penicillin/streptomycin, and then fill up to 500 ml with DMEM. The same FBS is used for both MEF and hFib medium. In general, culture of MEFs and growth of fibroblasts are not significantly affected by different batch of FBS. Filter the medium with a bottle-top 0.22-μm filter and store at 4 °C for 1 week.

**Retroviral infection medium** MEMα containing 10% heat-inactivated FBS (IFS) (vol/vol), 50 U $ml^{-1}$ penicillin and 50 mg $ml^{-1}$ streptomycin. To prepare 500 ml of medium, mix 50 ml of IFS, 5 ml of penicillin/streptomycin and then fill up to 500 ml with MEMα. Filter the medium with a bottle-top 0.22-μm filter and store at 4 °C for 1 week.

**hES cell medium** DMEM/F12 containing 20% KOSR (vol/vol), 5–10 ng $ml^{-1}$ bFGF, 1 mM L-GIn, 100 μM nonessential amino acids, 100 μM 2-mercaptoethanol, 50 U $ml^{-1}$ penicillin and 50 mg $ml^{-1}$ streptomycin. To prepare 500 ml of the medium, mix 100 ml KOSR, 2.5 ml L-GIn, 5 ml nonessential amino acids, 3.5 μl 2-mercaptoethanol, 5 ml penicillin/streptomycin, 250–500 μl bFGF and then fill up to 500 ml with DMEM/F12. Filter the medium with a bottle-top 0.22-μm filter and store at 4 °C for 1 week.

**2× iPS cell-freezing medium** 20% DMSO (vol/vol), 60% FBS (vol/vol) and 20% hES medium (vol/vol). Filter the medium with 0.22-μm filter and store at 4 °C for 2 weeks.

## PROCEDURE

### Preparation of human dermal fibroblasts ● TIMING 4 weeks

1| Obtain a 6-mm skin punch biopsy.

! CAUTION This should be obtained by a dermatologist or other appropriately trained physician.

**2|** Place the biopsy specimen immediately in hFib medium on ice for transport to the laboratory. Perform all subsequent steps in a tissue culture hood.

**3|** Using sterile forceps and dissecting scissors, mince the skin biopsy into 0.5–1-mm size pieces.

**4|** Place the pieces in the center of a well of a 6-well plate that contains enough hFib media to cover the bottom.

**5|** Carefully lower a sterile cover slip onto the pieces to hold them in place against the bottom of the plate and then add hFib media to a total of 3 ml.

**6|** Incubate at 37 °C, 5% $CO_2$.

**7|** By 7–10 d dense outgrowths of fibroblast should appear. Aspirate the media and wash two times with PBS. Use sterile forceps to dislodge, and lift the cover slip to ensure the media has been washed away with PBS. Fibroblasts will be adhered to the cover slip; take care not to aspirate these cells.

**8|** Add 0.5 ml of 0.05% trypsin/EDTA. Use sterile forceps to lift the cover slip to ensure that trypsin reaches the underside of the cover slip to which fibroblasts are adhered. Incubate for 5 min at 37 °C.

**9|** Using sterile forceps, flip the cover slip over and incubate for an additional 5 min at 37 °C.

**10|** Using a 5-ml pipette add 2.5 ml of hFib media to inactive the trypsin. Disperse all the cells by aspirating up and down gently, and by scraping the bottom of the well and cover slip. Collect cells in a 15-ml conical tube.

**11|** Wash the well and the cover slip two times with 3–5 ml of hFib media to collect all the cells.

**12|** Pass the cells through a 70-μm cell strainer to remove large chunks of tissue.

**13|** Centrifuge the cells at 200*g* (1,000 r.p.m. using most tissue culture centrifuges) at room temperature for 4 min using Centra CL2 centrifuge and discard the supernatant.

**14|** Resuspend the cells with 5 ml hFib medium and plate into T-25 tissue culture flask (passage 2).

**15|** Passage at a ratio of 1:3 every 5–7 d or until the cells have reached 80% confluence. For ease of use, we use 150-mm tissue culture dishes for expansion.

**16|** For subsequent passages, aspirate medium, wash once with PBS, trypsinize with 0.05% trypsin for 5 min at 37 °C. Continue to passage at a ratio of 1:3. Cells can be split up to passage 10. If desired, freeze cells as described in **Box 1**.

■ **PAUSE POINT** hFib cells can be frozen in LN2 indefinitely.

**Preparing 293T cells** ● **TIMING 2 d**

**17|** Thaw a vial of 293T cells by swirling in 37 °C water bath until most of, but not all, the contents are thawed.

**18|** Spray vial with 70% ethanol and wipe dry before placing in tissue culture hood.

**19|** Gently add 1 ml 293T medium, mix with contents of cryovial and transfer into 15-ml conical tube containing 4 ml 293T medium.

**20|** Centrifuge the cells at 200*g* at room temperature for 4 min.

**21|** Discard the supernatant, resuspend the cells with 5 ml 293T medium into 50-ml conical tube.

**22|** Determine the number of 293T cells using a hemacytometer and adjust the concentration to $2 \times 10^5$ cells $ml^{-1}$.

**23|** Transfer 10 ml of cell suspension ($2 \times 10^6$ cells) into a 100-mm dish.

▲ **CRITICAL STEP** Transfection for retrovirus production is performed in 293T cells at 70% confluence in a 100-mm dish. We recommend preparing 5 × 100 mm dish for each retrovirus. To prepare all six retroviruses, 30 dishes will be required.

## BOX 1 | FREEZING HUMAN DERMAL FIBROBLASTS

1. When human fibroblast (hFib) cells have reached 80% confluence, aspirate medium; wash once with PBS; cover cells with 0.05% trypsin and incubate for 5 min at 37 °C.
2. Inactivate trypsin with hFib medium and collect cells in 50-ml conical tube.
3. Centrifuge the cells at 200*g* at room temperature for 4 min and discard the supernatant.
4. Resuspend the cells in 5 ml hFib medium and determine cell number using hemacytometer.
5. Dilute cell suspension with hFib medium to $5 \times 10^6$ cells per ml.
6. Add equal volume of 2× freezing medium dropwise to cell suspension and aliquot 0.5 ml per cryovial (1 vial = $2.5 \times 10^6$ cells per ml).
7. Freeze hFib cells overnight in −80 °C freezer, then transfer to LN2 the following day.

**24|** Incubate the cells at 37 °C, 5% $CO_2$ overnight. Cells will be 60–70% confluent in 1–2 d.

**Retrovirus production ● TIMING 4 d**

**25|** Day 1: aspirate medium from 293T cells and replace with 10 ml fresh 293T medium.

**26|** For each 10-cm plate, add 20 µl FuGENE 6 transfection reagent, 300 µl of DMEM, and mix; incubate at room temperature for 5 min.
▲ **CRITICAL STEP** To avoid adversely affecting transfection efficiency, do not allow undiluted FuGENE 6 transfection reagent to come into contact with plastic surfaces other than pipette.

**27|** Add 2.5 µg retroviral vector, 0.25 µg VSV-G and 2.25 µg Gag-Pol. In order to transfect 5–10 plates, increase FuGENE 6, DMEM and vectors accordingly.
! **CAUTION** Retrovirus produced in VSV-G envelope can infect most mammalian cells including human cells. Production, maintenance and infection using VSV-G pseudotype retrovirus must be performed under the containment of BL2 biosafety.

**28|** Mix and incubate at room temperature for 15 min.

**29|** Add the transfection reagent: DNA complex to the cells in a dropwise manner. Swirl the dish to ensure distribution over the entire plate surface.

**30|** Incubate at 37 °C, 5% $CO_2$ for 48 h.
▲ **CRITICAL STEP** Avoid letting 293T cells grow to 100% confluence during transfection step. This can adversely affect the viral titer.

**31|** Day 3: collect retrovirus-containing medium and pass through 0.45-µm filter unit.

**32|** Transfer retrovirus-containing medium into 38.5-ml Beckman centrifuge tube and centrifuge at 70,000*g* at 4 °C for 90 min using Beckman XL-90 ultracentrifuge.

**33|** Remove supernatant and cover viral pellet in 1 ml DMEM with gentle shaking. Store overnight at 4 °C to allow the pellet to dissolve.

**34|** Day 4: if necessary, pipette the DMEM up and down with 100-µl pipette tip until the pellet is completely dissolved.

**35|** Calculate the titer of each virus and determine the multiplicity of infection (MOI). For a detailed procedure, refer to *Nature Protocols* for production and purification of lentivirus[7].
▲ **CRITICAL STEP** Virus can be contaminated during preparation steps which can affect subsequent procedures. Take additional care in the steps involved in measuring the weight of viral suspension through to resuspending the viral pellets with DMEM.
■ **PAUSE POINT** Store retrovirus in 100 µl aliquots in cryovials at 80 °C until use. Retrovirus can be stored at 80 °C for several months without loss of infectivity.
? **TROUBLESHOOTING**

**Retrovirus infection of human dermal fibroblast ● TIMING 6 d**

**36|** Infection day 1: when hFib cells have reached 80% confluence, aspirate medium, wash once with PBS, cover cells with 0.05% trypsin, incubate for 5 min at 37 °C.

**37|** Inactivate trypsin with hFib medium, collect cells in a 50-ml conical tube.

**38|** Centrifuge the cells at 200*g* at room temperature for 4 min and discard the supernatant.

**39|** Resuspend the cells in 1 ml hFib medium and determine cell number using hemacytometer.

**40|** Dilute cell suspension with hFib medium to $5 \times 10^4$ cells $ml^{-1}$.

**41|** Transfer 2 ml hFib suspension (totally $1 \times 10^5$ cells) per well of 6-well plate.

**42|** Incubate at 37 °C, 5% $CO_2$, for 6 h.

**43|** Aspirate the medium, replace with retroviral infection medium and add 5 µg $ml^{-1}$ protamine sulfate.

**44|** Add OCT4, SOX2, KLF4, MYC virus with an MOI of 5 and hTERT and SV40 LT with an MOI of 1. Infect one well with empty vectors and follow the same procedure as a nontransduced control for reprogramming.

**45|** Incubate at 37 °C, 5% $CO_2$, for 24 h.

**46|** Day 2: aspirate medium, wash cells three times with 3 ml PBS, add 2 ml fresh retroviral infection medium.

**47|** Incubate at 37 °C, 5% $CO_2$, for 72 h.

**48|** Day 4: prepare gelatin-coated tissue culture plates/dishes as described in REAGENT SETUP.

**49|** Thaw one vial of irradiated MEFs (iMEFs, isolated from CF1 mice) cryopreserved in LN2 by swirling gently in 37 °C water bath until most of, but not all, the contents are thawed. One vial of iMEFs ($2 \times 10^6$ cells) is sufficient to prepare 2 × 6-well plate or 2 × 100-mm dish or 2 × 12-well plate or 16 × 4-well plate (~120 $cm^2$). For a detailed protocol to prepare iMEFs, please refer to ref. 8.

**50|** Spray vial with 70% ethanol and wipe dry before placing in tissue culture hood.

**51|** Gently add 1 ml prewarmed MEF medium, mix with contents of cryovial and transfer into 15-ml conical tube containing 4 ml prewarmed MEF medium.

**52|** Centrifuge the cells at 200*g* at room temperature for 4 min and discard the supernatant.

**53|** Resuspend the iMEFs in 12 ml MEF medium. If using a 6-well plate: add 1 ml of iMEF suspension to each well of the 6-well plate containing 1 ml fresh MEF media per well. If using a 10-cm tissue culture dish: add 6 ml of iMEF suspension to 10-mm tissue culture dish containing 6 ml fresh MEF media. If using a 12-well plate: add 0.5 ml iMEF suspension to each well of 12-well plate containing 1 ml fresh MEF media per well. For iPS cell isolation, we use 10-mm tissue culture dishes coated with gelatin.

**54|** Incubate the cells in 37 °C, 5% $CO_2$, overnight.
▲ **CRITICAL STEP** When moving the iMEFs from the tissue culture hood to incubator, take care not to swirl the medium, as this tends to cause the cells to accumulate in the center. Immediately after placing the plates in the incubator, slide the plates forward and backward (2–3 cm) two times, then left to right (2–3 cm) two times to ensure equal distribution of the cells. Use within 5–7 d.

**55|** Day 5: split all infected cells (~$2.5 \times 10^5$ to $5 \times 10^5$ cells) into plate with iMEFs. Aspirate retroviral infection medium from cells incubated in Step 47, wash with PBS and add 0.5 ml of 0.05% trypsin. Incubate at 37 °C, 5% $CO_2$, for 5 min.

**56|** Inactivate medium with 3 ml retroviral infection medium, and collect cells in 15-ml conical tube.

**57|** Centrifuge at 200*g* at room temperature for 4 min.

**58|** Aspirate MEF medium from iMEF 100-mm dish (cells incubated in Step 54), add 5 ml of retroviral infection medium and return to incubator.

**59|** Aspirate supernatant (of cells in Step 57), resuspend cells in 5 ml retroviral infection medium, add to retroviral infection medium on iMEFs.

**60|** Incubate iMEFs at 37 °C, 5% CO2, for 48 h.

**61|** Day 6: aspirate medium and replace with hES medium supplemented with Y-27632 (10 μM final concentration).

**Culturing infected human dermal fibroblast on iMEFs** ● **TIMING 3 weeks**

**62|** Day 1: feed cells (aspirate medium and replace with fresh prewarmed medium) daily with 10 ml hES cell medium.
▲ **CRITICAL STEP** Timing of splitting infected cells depends on the proliferation rate of cells. Slow-growing cells do not need to be split until iPS cell colonies appear. Prepare to split cells when cells become overconfluent.

**63|** When infected cells become overconfluent and before iPS cells appear, prepare fresh iMEF 100-mm dishes to perform 1:3 split for each 100-mm dish of infected hFib cells (see Step 49).

**64|** Aspirate MEF medium from prepared iMEF dish, wash with 1–2 ml DMEM/F12, add 5 ml hES cell medium to each dish, place back in incubator.

**65|** Aspirate medium from infected hFib cells and wash dish with 10 ml DMEM/F12.

**66|** Add 5 ml collagenase (1 mg $ml^{-1}$ in DMEM-F12) solution to each dish.

**67|** Incubate the plate at 37 °C for 10 min.

**68|** Using a 5-ml glass pipette scrape off cells horizontally, vertically and circularly.

**69|** When all the colonies are detached from the surface, pool the suspension into a sterile 15-ml conical tube.

**70|** Wash each well with 5 ml DMEM/F12 media and add media to the 15-ml conical tube (total volume per 100-mm dish is 10 ml).

**71|** Centrifuge at 200*g* at room temperature for 4 min.

**72|** Remove 100-mm dishes of iMEFs with hES cell medium from incubator and label appropriately.

**73|** Aspirate supernatant and gently resuspend pellet in 15 ml hES cell medium.

**74|** Transfer 5 ml cell suspension per 100-mm dish of iMEFs with hES cell medium.

**75|** Incubate at 37 °C, 5% $CO_2$.

**76|** Repeat Steps 63–75 as needed based on confluency of the culture. By days 21–30 after infection of hFib cells, hES cell-like colonies should start to appear. These are homogenous clusters of cells that have the morphology of hES cells—high nuclear/cytoplasmic ratio, prominent nucleoli—that have pushed aside the surrounding heterogeneous cells. When this 'colony' has reached ~50–150 cells in size (2–3 mm in diameter), it is ready to be picked.
**? TROUBLESHOOTING**

**Picking and expanding iPS cell colonies ● TIMING 7 d**

**77|** Day 1: 1–2 d before picking colonies, prepare a 12-well plate (or 4-well plates) with iMEFs. See directions in Steps 49–54.

**78|** Aspirate MEF medium from iMEFs, wash with 1.5 ml DMEM/F12, add 1.5 ml hES cell medium to each well, place plates back in incubator.

**79|** Using a 20-µl pipettor set to 10 µl, pick individual iPS cell colonies and deposit one colony per well of iMEF 12-well (or 4-well) plate with hES cell medium.

**80|** Incubate the cells in 37 °C, 5% $CO_2$, overnight. Leave undisturbed for 48 h.

**81|** Day 4: feed (aspirate medium and replace with fresh prewarmed medium) daily with 2 ml hES cell medium.

**82|** After 7 d, the colony will become large enough to be passaged. Because only a single colony is present in each well, we recommend using mechanical passage into a well of a 12-well (or 4-well) plate with fresh iMEFs. After another 7 d the subsequent passage can be into a well of 6-well plate.

**83|** Once derived, iPS cells should be treated like hES cells. Instruction on how to passage, freeze and characterize these cells can be found in ref. 8.

**● TIMING**
Steps 1–16, preparation of human dermal fibroblasts: 4 weeks
Steps 17–24, preparing 293T cells: 2 d
Steps 25–35, retrovirus production: 4 d
Steps 36–61, retrovirus infection of human dermal fibroblasts: 6 d
Steps 62–76, culturing infected human dermal fibroblasts: 3 weeks
Steps 77–83, picking and expanding iPS cell colonies: 7 d

**? TROUBLESHOOTING**
Troubleshooting advice can be found in **Table 1**.

**TABLE 1 |** Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 35 | Low viral titer | Poor-quality 293T cells, low-quality plasmids | Thaw out and use new 293T to make virus. Prepare fresh plasmids |
| 76 | No human embryonic stem cell-like colonies appear | High-passage fibroblasts, refractory fibroblasts, bad retrovirus, bad-quality irradiated mouse embryo fibroblasts (iMEFs) | The quality of fibroblast is important to get induced pluripotent stem (iPS) cell colonies. Consider repeating experiment with fibroblasts of lower passage number. The age of the donor for fibroblasts seems important for iPS cell formation. In general, fibroblasts from older donors tend to be refractory to reprogramming. When you do not see any iPS cell colonies, consider starting with fibroblasts from a younger donor. The infectivity of retrovirus is critical for consistent iPS colony formation. Make an aliquot of retrovirus for storage and do not repeat freeze/thaw step. If the quality of virus is suspected, determine the titer again, and make fresh virus if the titer is low ($<0.5 \times 10^6$ cells per ml). Quality of iMEFs are important. When poor-quality iMEFs are used, small starting iPS cell colonies begin to differentiate and no distinct colonies can be found |

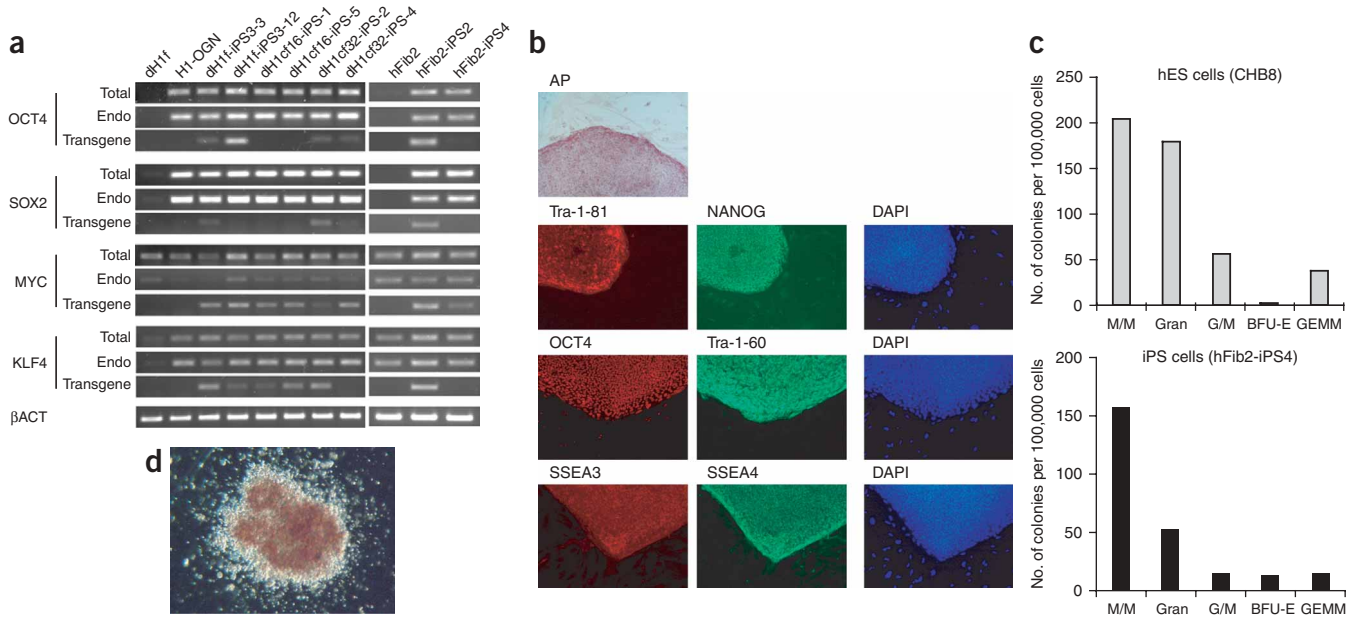


**Figure 2** | Induced pluripotent stem (iPS) cells have characteristics resembling those of human embryonic stem (hES) cells. (**a**) hES cells (H1-OGN) and iPS cells derived from differentiated H1-OGN, and iPS cells from adult human dermal cells express pluripotent genes. Endogenous genes were reactivated, while transgenes in some clones are silenced. (**b**) iPS cells show the expression of surface markers of hES cells. For detailed information about protocols for staining, refer to ref. 4, from where the figures were reproduced. (**c**) iPS cells (hFib2-iPS4) form hematopoietic colonies as comparable numbers as hES (CHB8) cells when cultured in MethoCult after differentiation as embryoid body[9]. (**d**) iPS cells form BFU-E (burst forming unit-erythroid). Data in **a** and **b** were reproduced with permission from ref. 4.

## ANTICIPATED RESULTS

hES-like colonies will appear between 21 and 30 d after infection, if viruses are good and infected cells are appropriately maintained. You would see between 5 and 50 colonies per plate (Step 76). The isolated iPS cells are morphologically very similar to hES cells, share similar gene and surface-antigen expression and developmental potential (**Fig. 2**).

*Note: Supplementary information is available via the HTML version of this article.*

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# ERRATUM: Generation of human-induced pluripotent stem cells

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In Figure 2a of the PDF version of this article initially published online, five labels (OCT4, SOX2, MYC, KLF4 and βACT) were omitted from the left side of the figure.

The error has been corrected in the PDF version of the article.