Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/39392 holds various files of this Leiden University dissertation

Author: Engels, Marc Christian
Title: Cellular modifications and interventions for the damaged heart
Issue Date: 2016-05-11

CHAPTER 2

Reprogramming of mouse, rat, pig, and human fibroblasts into induced pluripotent stem cells

Kuppusamy Rajarajan[1,†], Marc C. Engels[1,†], Sean M. Wu[1,2]

[†]These authors contributed equally to this work.

[1]Cardiovascular Research Center, Division of Cardiology, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts, USA.
[2]Harvard Stem Cell Institute, Cambridge, Massachusetts, USA.

Adapted from: Current Protocols in Molecular Biology. 2012;Chapter 23: Unit 23.15.

Abstract

The induction of pluripotency in somatic cells by transcription factor overexpression has been widely regarded as one of the major breakthroughs in stem cell biology within this decade. The generation of these induced pluripotent stem cells (iPSCs) has enabled investigators to develop *in vitro* disease models for biological discovery and drug screening, and in the future, patient-specific therapy for tissue or organ regeneration. While new technologies for reprogramming are continuously being discovered, the availability of iPSCs from different species is also increasing rapidly. Comparison of iPSCs across species may provide new insights into key aspects of pluripotency and early embryonic development. iPSCs from large animals may enable the generation of genetically modified large animal models or potentially transplantable donor tissues or organs. This unit describes the procedure for the generation of iPSCs from mouse, rat, pig and human fibroblasts.

Introduction

With the overexpression of just four transcription factors, Oct4, Sox2, Klf-4, and c-Myc, Takahashi and Yamanaka showed that terminally differentiated fibroblasts could be reprogrammed into becoming pluripotent, embryonic stem cell-like cells, which they called induced pluripotent stem cells (iPSCs).[1] These cells are able to proliferate indefinitely and have been shown to differentiate into subtypes of all three germ layers. iPSCs thus harbor potential for disease modeling as well as conducting patient-specific drug screens *in vitro*, without the ethical and technical challenges associated with embryonic stem cells (ESCs). This unit describes the generation of iPSCs from four species: mouse, rat, pig, and human. To lower the barrier for new investigators to enter this exciting area of biology, the focus is on lenti- and retroviral infection-based strategies for somatic cell reprogramming, since these approaches remain the easiest for generating iPSC lines.

Although by no means exclusive, this unit describes in detail the full procedure required for iPSC generation. Basic Protocol 1 describes the isolation and culture of fibroblasts, with a Support Protocol for lentiviral production. Basic Protocol 2 describes the viral infection of fibroblasts, which can be applied to all four species. After this section, the procedure diverges for various species: Basic Protocol 3 describes the establishment and maintenance of mouse iPSCs and Alternate Protocols 1, 2, and 3 respectively describe the same procedure in rat, pig and human cells.

Note: All solutions and equipment coming into contact with cells must be sterile, and aseptic technique should be used accordingly.
Note: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.
Note: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Basic Protocol 1

Isolation and culture of fibroblasts

This protocol describes the isolation and culturing of mouse embryonic fibroblasts (MEFs). However, a slightly modified protocol can be followed for mouse dermal fibroblasts or tail-tip fibroblasts, as well as rat embryonic fibroblasts, rat dermal fibroblasts, rat tail-tip fibroblasts, pig dermal fibroblasts, and human dermal fibroblasts.

Note: When using either skin fibroblasts or tail tip fibroblasts, the same procedure can be followed as described below after finely mincing the sample using a sterile scalpel or razor blade.

Note: For rat samples the same procedure can be followed as described below using pregnant female rats at 14.5 to 15.5 days post coitum (dpc) for embryonic rat fibroblasts. Alternatively, skin samples can be used for dermal fibroblasts or tail tip fibroblasts.

Note: For pig and human samples a punch skin biopsy should be performed to obtain skin tissues. The same procedure can be followed as described below.

Materials

Pregnant female mice
Sterile Dulbecco's PBS (DPBS; with $CaCl_2$ and $MgCl_2$; Invitrogen, cat. no. 14040141)
Penicillin and streptomycin (Invitrogen, cat. no. 15140-155)
0.25% trypsin/0.53 mM EDTA (Invitrogen, cat. no. 25200-056)
MEF medium (see recipe)
Freezing/cryopreservation medium (see recipe)
Liquid N_2
Dissecting equipment including sterile forceps and sterile scalpel blades
100-mm petri dishes (BD Falcon 351029)
15- and 50-mL conical tubes
Centrifuge
Pipettors with 1000-µl (P1000) pipet tips
100-mm cell culture dishes (Corning 430167), pre-coated with 0.1% gelatin (see recipe)
Cryovials
Additional reagents and equipment for counting cells (*Appendix3F*)

Preparation of mouse embryonic fibroblasts

1. Euthanize pregnant female mice at 13.5 to 15.5 dpc by cervical dislocation.[2] Isolate the gravid uterus and briefly wash with DPBS supplemented with antibiotics (penicillin at 5000 IU/mL and streptomycin at 5000 µg/mL final concentration).
2. Dissect through the placenta and uterus until embryos are within view. Separate embryos from their placenta and surrounding fetal membranes with sterile forceps. Carefully remove the head, visceral organs, and gonads from the embryos.
3. Wash bodies of the embryos thoroughly in a 100-mm petri dish containing fresh DPBS supplemented with antibiotics (penicillin and streptomycin at 5000 IU each per mL). Mince well with sterile scalpel blade, digest the embryos in 1 mL of 0.25% trypsin/EDTA in a 15-mL conical tube, and incubate at 37°C for 15 minutes (min). Pipetting up and down with a 1000-µL (P1000) pipet tip, gently triturate intermittently to allow the cells to disperse from the large tissue clumps.
4. Add 9 mL of MEF medium to neutralize the trypsin. Again pipet up and down gently to dissociate as many cells as possible.
5. Incubate the samples for 5 min at room temperature (20° to 25°C) to allow the tissue debris to settle at the bottom and transfer the supernatant into a sterile 50-mL conical tube. Centrifuge 4 min at 200 × *g*, at room temperature, discard the supernatant, and resuspend the pellet in 10 mL fresh MEF medium.
6. Simply count cells with a hemocytometer (see *Appendix 3F*; trypan blue test not needed) and adjust the concentration to 1×10^6 cells per mL with MEF medium (generally, 1×10^7 cells can be obtained from a single embryo). Transfer the cell suspension to gelatinized 100-mm tissue culture dishes (1×10^7 cells per dish) with 10 mL of MEF medium and incubate in a 37°C, 5% CO_2 humidified incubator.
   This is considered passage number 0.
7. Remove non-adherent cells after 48 hours by washing with DPBS (with calcium and magnesium) or MEF medium, then replenish with MEF medium.
   Cells should be replenished with fresh MEF medium every 48 hours until confluent.
8. When the cells have become confluent, remove MEF medium, wash once with DPBS (with calcium and magnesium), and trypsinize with 2 mL of 0.25% trypsin/EDTA for 4 min at 37°C. After detaching, add 9 mL of MEF medium and resuspend the cells by pipetting. Passage to new 100-mL dishes at 1:4 dilution (passage no. 1).
   Now these cells can be used for viral infection to make iPSCs. It is recommended to conduct a mycoplasma test before use.

Important note: For the generation of iPSCs, it is advisable to use MEFs (mouse embryonic fibroblasts) up to and including passage no. 3 to avoid replicative senescence. As an alternative, other easily

accessible tissues can be used for reprogramming, such as adult skin cells, tail tip fibroblasts, blood, and cells from biopsy tissues,[3] but these cells show less reprogramming efficiency than embryonic fibroblasts within the first 5 to 7 passages.

Freezing mouse embryonic fibroblasts (optional)

When working with DMSO-containing freezing medium, it is essential to work as quickly as possible and to transfer the freezing stock rapidly into a –80°C freezer in order to initiate the freezing procedure. To minimize exposure at room temperature, you should pre-label the cryovials and have the freezing container ready.

9. Let cells grow until they reach 90% confluency.
10. Aspirate the cell culture medium and wash cells with 5 mL of sterile DPBS (with calcium and magnesium).
11. Remove DPBS completely, add 2 mL of 0.25% trypsin/EDTA and incubate at 37°C with 5% CO_2 for 4 min.
12. Add 2.5 mL of MEF medium and resuspend the cells by pipetting up and down to a single-cell suspension.
13. Transfer the cell suspension to a 15-mL tube and add MEF medium for a total volume of 10 mL. Count cells using a hemocytometer (*APPENDIX 3F*). Centrifuge 4 min at 200 × *g*.
14. Aspirate the supernatant, resuspend the pellet in freezing/cryopreservation medium (10% DMSO in FBS) to obtain a concentration of 2×10^6 cells per mL.
15. Transfer the cell suspension to prelabeled cryovials at 1 mL per cryovial.
    Labeling should include cell line, passage number, and date.
16. Quickly store the cryovials in a chilled cell-freezing container (4°C or on wet ice) and freeze at –80°C overnight.
    Cells should be transferred to –80°C rapidly, since DMSO is toxic to cells in liquid phase.
17. The next day, remove cryovials from freezing container and store in the gas phase of a liquid nitrogen tank for long-term storage.

Production of lentivirus

This protocol describes the production of viral constructs in a lentiviral system. The protocols for infecting mouse, rat, pig, and human fibroblasts with lenti- and retroviruses are similar. Therefore the following applies to all four animal species.

NOTE: For retroviral production, use PLAT-E cells with FuGENE HD Transfection Reagent.

Materials

293FT cells (Invitrogen, cat. no. R700-07)
293FT medium (see recipe)
FuGENE HD Transfection Reagent (Roche, cat. no. 04 709 705 001)
DMEM/high glucose (Invitrogen, cat. no. 11965092)
VSV-G plasmid (Addgene, cat. no. 8454)
D8.9/psPAX2 plasmid (Addgene, cat. no. 12260)
STEMCCA (OKSM) Lentivirus Reprogramming Kit (Millipore, cat. no. SCR511)
10% bleach disinfectant
Serum-free DMEM medium (e.g., Invitrogen)
100-mm cell culture dishes (Corning, cat. no. 430167) pre-coated with 0.1% gelatin
0.45-µm disposable filters
Ultracentrifugation tubes (Beckman, cat. no. 344058)
Beckman Coulter Optima L-90K ultracentrifuge with SW-32 rotor

1. Culture 293FT cells in a gelatinized 100-mm dish in 293FT medium until cells reach 80% to 90% confluency. Use 1 dish per viral construct or several if harvesting more virus.
2. Pre-warm DMEM/high glucose and FuGENE HD Transfection Reagent to room temperature and combine in a microcentrifuge tube. Use 770 µl DMEM/high glucose and 50 µl FuGENE Reagent per 100-mm dish.
3. Incubate at room temperature for 5 min.
4. For each infection, add 5.5 µL VSV-G (5.5 µg/infection) and 8.25 µL D8.9 (8.25 µg/infection) to each microcentrifuge tube and mix well.
   Listed below are typical infection ratios of lentiviral helper plasmids for production of lentiviral vectors by FuGENE transfection:
   1× VSV-G
   1.5× D8.9
   2× vector

5. Add a total of 11 µg vector DNA (STEMCCA Lentivirus Reprogramming Vector– pluripotent transcription factors at 11 µg/infection).
6. Mix by gently tapping tube several times (do not vortex).
7. Incubate 30 min at room temperature.
8. During incubation, add 10 mL of fresh 293FT medium to 293FT cells.
9. Add entire FUGENE/DNA complex to the 100-mm dish containing the 293FT cells in a dropwise fashion around the dish. Gently rotate the plate to mix the contents. Incubate for 12 to 24 hours (hr) at 37°C with 5% CO_2.

10. After the 12- to 24-hr incubation, change the 293FT medium. Add 10 mL of fresh medium at each medium-replenishing step.
11. After 24 hr, collect all medium into a 50-mL conical tube and store at 4°C until the collection procedure is completed. Add 10 mL of fresh 293FT medium to the dish. Repeat every subsequent 24 hr for a total of three collections. After completion, discard cells using proper viral waste disposal procedures.
    This will allow a collection of 30 mL of virus medium per 100-mm dish.
12. Filter collected viral medium through a 0.45-µm filter.
13. Transfer collected media into ultracentrifugation tubes at 30 mL per tube and centrifuge 1.5 hr at 50,000 × *g* and 4°C. Turn brakes off to avoid disturbance of pellet.
14. Decant supernatant into 10% bleach, then briefly place tube upside down onto tissue paper to remove supernatant completely.
    This will take up to 1 to 2 min.
15. Add 200 µL of serum-free DMEM medium to the pellet and let stand overnight at 4°C.
16. Resuspend by carefully pipetting up and down and aliquot the virus in small volumes (e.g. 20 µl per aliquot). Store aliquots up to 1 year at −80°C.

Basic Protocol 2

Infection of fibroblasts using pluripotency factors

This protocol describes the infection of fibroblasts using viral constructs of the four pluripotency factors. This protocol can be applied to all four species described using the viral method detailed in Basic Protocol 1.

Caution: When working with virus, use a designated laminar flow hood for virus-related work. Always wear protective disposable gowns and double gloves. Make sure to discard any viral waste into designated viral waste bins and rinse any disposables with bleach before disposing of them. The contents of a viral waste bin should be autoclaved in an autoclavable bag for 45 min in a standard autoclave using the sterilization program.

Materials

Embryonic fibroblasts or dermal fibroblasts (passage ≤3; see Basic Protocol 1)
MEF medium (see recipe)
Sterile Dulbecco's PBS (DPBS; with CaCl2 and MgCl2; Invitrogen, cat. no. 14040141)
0.25% trypsin/0.53 mM EDTA (Invitrogen, cat. no. 25200-056)
Polybrene (Millipore TR-1003-G)

Pre-aliquoted virus (see Support Protocol 1)
6-well plate pre-coated with 0.1% gelatin
15-mL conical centrifuge tubes
Additional reagents and equipment for counting cells (APPENDIX 3F)

Note: Using fresh virus gives dramatically better infection efficiency. Virus should not be stored for longer than 1 week at 4°C before use. Virus should never undergo more than 1 freeze/thaw cycle, as multiple freeze/thaw cycles considerably reduce infection efficiency.
Note: For mouse and rat fibroblasts, mouse four factor plasmids are most widely used for making iPSCs. For pig and human fibroblasts it is preferable to use human four-in-one factor constructs, but individual human factors can also be used. A STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit is commercially available (Millipore).
Note: For increased viral infection rate, multiple infections can be performed by aspirating and replenishing medium with fresh virus-containing medium every 12 hr up until 36 hr after initial infection.
Note: As a feeder layer for iPSCs, MEFs can be used for all four species. The MEFs should be growth-inhibited by irradiation or mitomycin C treatment. For best results, a single monolayer of feeder cells should be plated onto gelatinized culture plates (for mouse and rat) or Matrigel-coated culture plates (for pig and human).

1. Culture embryonic fibroblasts or dermal fibroblasts (passage ≤3) to ~90% confluency in a single well of a gelatin-coated 6-well plate in 2 mL MEF medium. *This will yield more than 1 × 10^4 cells per dish.*
2. Aspirate the culture medium from the MEF cells and wash with 2 mL of DPBS.
3. Aspirate PBS, add 0.5 mL per well of 0.25% trypsin-EDTA, and incubate at 37°C for 4 min.
4. Add 2 mL MEF medium to inactivate the trypsin, triturate to obtain a single-cell suspension, and transfer to a 15-mL tube.
5. Centrifuge 4 min at 200 × *g*, at room temperature, discard the supernatant and resuspend the pellet in 1 mL of MEF medium.
6. Simply count the number of cells using a hemocytometer (trypan blue test not needed) and adjust the cell concentration to 1×10^4 cells (see *Appendix 3F*) with MEF medium.
7. Add 1 µL of 8 mg/mL polybrene per mL of cell-containing medium. Mix by gently pipetting up and down. *The final concentration of polybrene in the mixture should be 8 µg/mL.*
8. Thaw and freshly combine all four factor lenti- or retroviruses (Oct-3/4, Sox2, Klf4, and c-Myc) into a cocktail and add freshly into the cell mixture for mouse and rat iPSC generation. Mix by gently pipetting up and down.

For pig and human iPSCs, a four-in-one mono-cassette virus is recommended. If using a doxycycline-inducible TetO viral system, a separate rtTa expressing virus should also be added at this point. Additionally, doxycycline should be added immediately and with each medium change at a final concentration of 1 µg/mL to induce gene expression. The amount of virus added should be calculated by making use of the equation mentioned below, using a Multiplicity of Infection (MOI) of at least 20.

The following equation can be used to determine the volume of virus required to achieve a Multiplicity of Infection (MOI) of at least 20.

Virus volume (µL) required = [(# MEFs seeded for infection)/virus titer (U/mL)] × [(desired MOI)/1 mL] × 1000 µL.

For example, if the number of cells in the well at the time of transduction is 1×10^5 cells, the viral titer is 3×10^8 IFU/mL, and a desired MOI is 20, then the volume of virus required is:
$(1 \times 10^5$ cells)/$(3 \times 10^8$ U/mL) × [20/1 mL × 1000 µL] = 6.6 µL virus required for 1 well of a 6-well plate.

9. Plate 1 mL of the cell/virus mixture onto a single well of a gelatin-coated 6-well plate and incubate the cells for 24 hr at 37°C with 5% CO_2.
10. After 24 hr, wash with warm MEF medium and replenish with 2 mL of fresh MEF medium. Continue incubation at 37°C with 5% CO_2.
11. For post-infection iPSC colony selection and maintenance procedures, see the respective protocol below (Basic Protocol 3 for mouse, Basic Protocol 4 and Alternate Protocol 1 for rat, Alternate Protocol 2 for pig, and Alternate Protocol 3 for human) for each species of interest to be reprogrammed.

Basic Protocol 3

Establishment and maintenance of mouse iPSCs

This protocol describes the establishment and maintenance of mouse iPSCs. See Basic Protocol 2 for the viral infection procedure. *Figure 1* is a schematic diagram showing the process of generating mouse iPSCs.

Figure 1. Schematic diagram of mouse iPSC generation.

Materials

Mouse fibroblasts infected with pluripotency factors (Basic Protocol 2); for timing see *Figure 1*.
MEF medium (see recipe)
0.25% trypsin/0.53 mM EDTA (Invitrogen, cat. no. 25200-056)
Mouse iPSC medium (see recipe) with double concentration of LIF and with normal concentration of mouse LIF as specified in Reagents and Solutions
Doxycyline (Sigma, cat. no. D9891); optional
24-and 6-well plates of inactivated mouse fibroblast feeder cells (*UNIT 23.2*)
Phosphate-buffered saline (PBS) without CaCl2 and MgCl2 (Invitrogen 14190-250)
Freezing/cryopreservation medium (see recipe)
Isopropanol
Liquid N2
70% ethanol
15-mL conical centrifuge tubes
Centrifuge
Light microscope with camera
Marker pen
20-µL (P20) and 1000-µl (P1000) pipet tips
2.0-mL cryovials
Freezing chamber (e.g., Mr. Frosty; Thermo Scientific, cat, no. 5100-0001)
Liquid nitrogen storage tank
Cryogenic handling gloves and eye protectors
Forceps

Additional reagents and equipment for preparing inactivated mouse embryonic fibroblast feeder cells (*Unit 23.2*) and counting cells using a hemocytometer (*Appendix 3F*)

Post-infection Culturing of Infected Fibroblasts

1. After 48 hr of viral infection or when cells reach confluency, aspirate the medium from the infected cells and dissociate by adding 0.5 mL of 0.25% trypsin/0.53 mM EDTA and incubating for 4 min at 37°C with 5% CO_2. Add 2 mL of warm MEF medium, then pipet up and down to obtain a single-cell suspension. Transfer to a 15-mL tube containing 9 mL of warm MEF medium and centrifuge 4 min at 200 × *g* and room temperature.

2. Discard the supernatant and resuspend the pellet in 8 mL of mouse iPSC culture medium. For initial culture use double the concentration of mouse LIF. After 2 to 3 passages, use the regular dose of mouse LIF.
3. Aspirate the medium from four single wells of a 6-well plate that has been gelatin-coated and pre-seeded with irradiated or mitomycin C–treated MEF cells (prepared as described in *UNIT 23.2*). Add 2 mL of the infected cell mixture per well onto the MEF feeder layer so that the infected cells are passaged in a 1:4 ratio. Incubate at 37°C with 5% CO_2.
4. Change iPSC medium supplemented with doxycycline (final concentration, 1 µg/mL if using a TetO lentiviral system) every 24 hr and check for colony formation. *Colonies should start becoming microscopically visible approximately 7 to 10 days post-infection.*
5. Let colonies grow into a reasonable size (roughly 50 to 100 cells/colony).

   This should take approximately 14 or 15 days post-infection.

Picking and establishing mouse iPSC clones

6. One hour before picking iPSC colonies, pre-feed the cells by replenishing with fresh mouse iPSC medium.

   This is especially important when the medium has turned acidic (indicated by yellow color), since pre-feeding will increase cell survival after dissociation.
7. Prepare a microscope inside a laminar hood to maintain sterile conditions for picking colonies (*Figure 2*).
8. Before picking, select up to 40 good colonies by circling the colony with a marker pen on the bottom of the plate to be able to retrieve the selected colonies when the actual picking procedure is started. *Suitable colonies should appear translucent and circular. See Figure 3 for examples of suitable mouse iPSC colonies.*
9. Pipet 50 µL of 0.25% trypsin/0.53 mM EDTA into each of several 15-mL tubes.
10. Use a 20-µL pipet tip for the picking procedure. Pick one individual colony by gently scratching with the pipet tip. Make sure not to touch any neighboring colonies.
11. Transfer each picked colony into an individual 15-mL tube containing 50 µL of 0.25% trypsin/0.53 mM EDTA. Dissociate the colony by gently pipetting up and down and incubate at 37°C in a water bath for 4 min.
12. After 4 min of incubation, add 5 mL of iPSC medium to each 15-mL tube containing one single colony and pipet up and down to dissociate the colony further into a single-cell suspension. Centrifuge 4 min at 200 × *g*, at room temperature.
13. Aspirate the supernatant and resuspend the pellet in 1 mL of mouse iPSC medium. Transfer the cell suspension from each picked colony into a single well of a 24-well plate that has been gelatin-coated and pre-seeded with growth-inactivated MEF cells (prepared as described in *UNIT 23.2*). Incubate at 37°C with 5% CO_2 and replenish medium every 24 hr until nearly confluent. *Cells are now ready to be passaged into a 6-well plate.*

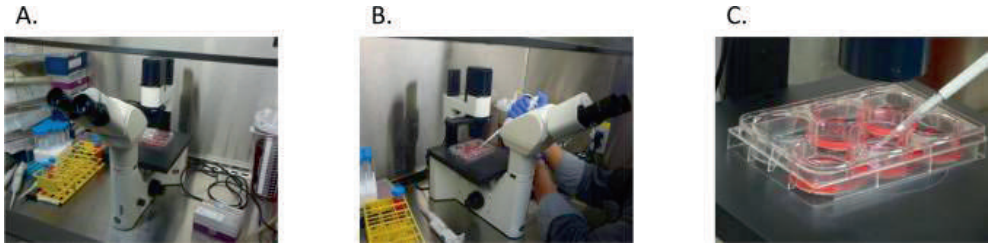

Figure 2. Procedural set up for iPSC colony picking. (**A**) Setup for picking iPSC colonies under a microscope in a laminar flow hood. (**B**) Process of iPSC colony picking. Note that the forearm is fixed against the microscope stage while looking through microscope (**C**) Close-up of picking. Make sure not to touch any neighboring colonies.

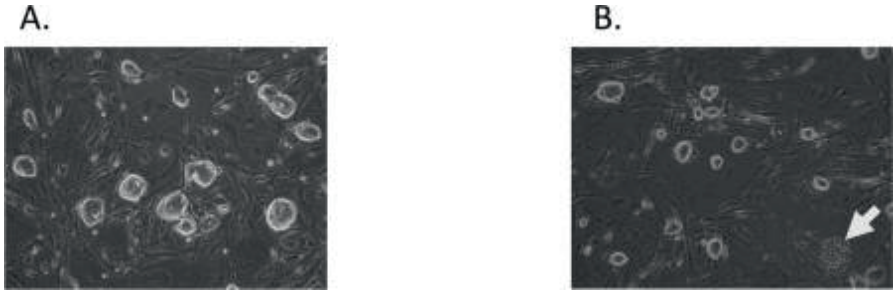

Figure 3. Typical morphology of good and bad mouse iPSC colonies. (**A**) A good mouse iPSC colony. Note the translucent appearance and sharp borders. (**B**) Mouse iPSCs. Notice the one bad mouse iPSC colony (arrow) on the bottom right. This colony looks heterogeneous and differentiated.

In vitro culture and expansion of mouse iPSCs

When reprogramming cells using a doxycycline-inducible system, wean the cells off doxycycline gradually after picking colonies. As a guideline, the following schedule can be applied: use 1/2 concentration of doxycycline after the first passage. Then, gradually lower the concentration with each passage to 1/4 concentration, and finally completely wean off doxycycline. If cell morphology begins to degrade, slow the rate of weaning. Do not change medium conditions at the time of passaging; rather, wait until the cells have attached (usually after 12 to 24 hr) before making changes to the doxycycline concentration.

14. When iPSC colonies reach 80% to 90% confluency in the dish or when individual iPSC colonies become large (approximately >100 cells/colony), aspirate the medium from the dish, and pre-feed the cells with mouse iPSC medium 1 hr before passaging. *For mouse iPSCs, a 1:6 passaging ratio is usually easily tolerated*.
15. After 1 hr aspirate the medium and wash the cells with 2 mL of PBS (no Ca or Mg).
16. Aspirate the PBS, add 0.5 mL of 0.25% trypsin/0.53 mM EDTA and incubate at 37°C with 5% CO_2 for 4 min.
17. Add 1 mL of the mouse iPSC medium and dissociate the colonies by pipetting up and down to create a single-cell suspension.

18. Transfer into a 15-mL tube containing 9 mL of warm mouse iPSC medium and centrifuge 4 min at 200 × *g*, at room temperature.
19. Discard the supernatant and resuspend the pellet in 12 mL of mouse iPSC medium.
20. Aspirate MEF medium from 6-well plates containing mitomycin C growth- inactivated or irradiated MEFs (prepared as described in *UNIT 23.2*) and add 2 mL of cell suspension per well. Incubate the cells at 37°C with 5% CO_2 until cells reach 80% to 90% confluency. Replenish with fresh mouse iPSC medium every 24 hr. *It is advisable to prepare frozen stocks of newly reprogrammed iPSCs at low passage for future use.*

Freezing mouse iPSCs

21. Let cells grow until they reach 80% to 90% confluency. Before beginning the freezing procedure, label 2-mL cryovials with information on cell line, passage number, and date. *Generally two 2-mL cryovials are used per well of a 6-well plate.*
22. Aspirate the cell culture medium from each well and wash cells with 2 mL of sterile PBS.
23. Remove PBS completely, add 0.5 mL of 0.25% trypsin/0.53 mM EDTA and incubate at 37°C and 5% CO_2 for 4 min (for 10-cm plate use 2 mL of 0.25% trypsin/0.53 mM EDTA).
24. Add 2.5 mL of serum-containing mouse iPSC medium and resuspend the cells by pipetting up and down to create a single-cell suspension.
25. Transfer the cell suspension to a 15-mL tube and add in mouse iPSC medium to a total volume of 10 mL. Count cells using a hemocytometer (*APPENDIX 3F*). Centrifuge 4 min at 200 × *g*, at room temperature
26. Aspirate the supernatant, then resuspend the pellet in freezing/cryopreservation medium (10% DMSO in FBS) to obtain a concentration of 2×10^6 cells per mL.
27. Transfer the cell suspension to pre-labeled cryovials at 1 mL per cryovial.

    Labeling should include cell line, passage number, and date. Generally two cryovials are used per well of a 6-well plate.
28. Quickly transfer the cryovials to a chilled isopropanol-containing cell-freezing container (4°C or on wet ice) and freeze at –80°C overnight.

    Cells should be transferred to –80°C rapidly, since DMSO is toxic to cells in liquid phase.
29. On the next day, remove cryovials from freezing container and store in the gas phase of a liquid nitrogen tank for long-term storage (may be kept frozen for many years).

Thawing mouse iPSCs

30. Using cryogenic hand gloves and eye protectors, remove the cryovial from the liquid nitrogen tank using forceps.

    Caution: *These safety measures are required, since cryovials stored in the liquid nitrogen tank may explode unexpectedly when exposed to rapid thawing.*

30. Immerse the vial in a 37°C water bath without submerging the cap.
31. Thaw vial rapidly until just a few ice crystals are left.
32. Record the sample name and passage number and spray 70% ethanol on the outer surface of the vial, then air dry quickly for a few seconds in a sterile laminar flow tissue culture hood.
33. Transfer the thawed cells to a 15-mL conical tube using a pipet with a P1000 pipet tip. Rinse the freezing vial once with 1 mL of mouse iPSC medium and add back to the 15-mL tube.
34. Add 10 mL of cold mouse iPSC medium drop-wise to cells in the 15-mL conical tube. While adding the medium, gently move the tube back and forth to mix the cells. *This step reduces osmotic shock to the cells.*
35. Centrifuge the cells 4 min at 200 × *g*, at room temperature.
36. Aspirate the supernatant and resuspend the cell pellet in 2 mL of mouse iPSC medium.
37. Slowly add the cell suspension in a dropwise fashion to a well of a gelatin-coated 6-well plate, pre-seeded with growth-inhibited MEF cells (prepared as in *UNIT 23.2*).
38. Culture cells as described above in a 37°C, 5% CO_2 incubator.

Alternate Protocol 1

Establishment and maintenance of Rat iPSCs

This protocol describes the establishment and maintenance of rat iPSCs. See Basic Protocol 2 for the viral infection procedure. *Figure 4* is a schematic diagram showing the process of generating rat iPSCs.

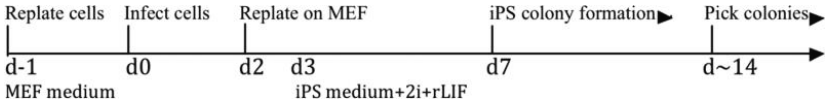

Figure 4. Schematic diagram of rat iPSC generation.

Materials

Rat fibroblasts infected with pluripotency factors (Basic Protocol 2); for timing, see *Figure 4* MEF medium (see recipe)

0.25% trypsin/0.53 mM EDTA (Invitrogen, cat. no. 25200-056)

Rat iPSC medium (see recipe for serum-free rat iPSC medium or KOSR-containing rat iPSC medium) with double concentration of LIF and with normal concentration of mouse LIF as specified in Reagents and Solutions

Doxycyline (Sigma, cat. no. D9891); optional

24-and 6-well plates of inactivated mouse fibroblast feeder cells (*UNIT 23.2*)

Phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ (Invitrogen 14190-250)
Freezing/cryopreservation medium (see recipe)
Isopropanol
Liquid N_2
70% ethanol
15-mL conical centrifuge tubes
Centrifuge
Light microscope with camera
Marker pen
20-µL (P20) and 1000-µL (P1000) pipet tips
2.0-mL cryovials
Freezing chamber (e.g., Mr. Frosty; Thermo Scientific, cat. no. 5100-0001)
Liquid nitrogen storage tank
Cryogenic handling gloves and eye protectors
Additional reagents and equipment for preparing inactivated mouse embryonic fibroblast feeder cells (*Unit 23.2*) and counting cells using a hemocytometer (*Appendix 3F*)

Note: There are two recipes for rat iPSC medium (see Reagents and Solutions): one for serum-free rat iPSC medium and one for KOSR-containing rat iPSC medium. Either of these media can be used for culturing rat iPSCs.

Post-infection Culturing of infected fibroblasts

1. After 48 hr of viral infection, or when cells reach confluency, aspirate the medium from the infected cells and dissociate by adding 0.5 mL of 0.25% trypsin/0.53 mM EDTA and incubating for 4 min at 37°C with 5% CO_2. Add 2 mL of warm MEF medium, then pipet up and down to obtain a single-cell suspension. Transfer to a 15-mL tube containing 9 mL of warm MEF medium and centrifuge 4 min at 200 × *g*, room temperature.
2. Discard the supernatant and resuspend the pellet in 8 mL of MEF medium.
3. Aspirate the medium from four single wells of a 6-well plate that has been gelatin-coated and pre-seeded with growth-inhibited MEF cells (prepared as described in *UNIT 23.2*). Add 2 mL of the infected cell mixture per well onto the MEF feeder layer so that the infected cells are passaged in a 1:4 ratio. Incubate at 37°C with 5% CO_2.
4. After 12 to 24 hr change the medium to rat iPSC culture medium (supplemented with doxycycline at a final concentration of 1µg/mL, if using a TetO lentiviral system). For initial culture conditions, use double the concentration of rat LIF. After two to three passages, use the regular dose of LIF (1000 U/mL). *Colonies should start becoming microscopically visible approximately 7 to 10 days post-infection.*

5. Change rat iPSC medium supplemented with doxycycline (final concentration, 1 µg/mL if using a TetO lentiviral system) every 24 hr and check for colony formation.
6. Let colonies grow into a reasonable size (>50 to 100 cells/colony).
   This should take until approximately day 14 or 15 post-infection.
   Cell death is a common problem when cells are transferred directly from serum-containing medium to serum-free medium or KOSR-containing medium, like rat iPSC medium. This may appear quite dramatic initially, as up to 50% of cells may fail to attach, but abundant viable and proliferative cells should be apparent after medium change.

Picking and establishing rat iPSC clones

7. One hour before picking iPSC colonies, pre-feed the cells by replenishing with fresh rat iPSC medium. *This is especially important when the medium has turned acidic (indicated by yellow color), since pre-feeding will increase cell survival after dissociation.*
8. Prepare a microscope inside a laminar hood to maintain sterile conditions for picking colonies (*Figure 2*).
9. Before picking, select up to 40 good colonies by circling the colony with a marker pen on the bottom of the plate to be able to retrieve the selected colonies when the actual picking procedure is started. *Suitable colonies should appear translucent and circular. See Figure 5 for examples of suitable rat iPSC colonies.*
10. Add 50 µL of 0.25% trypsin/0.53 mM EDTA into each of several 15-mL tubes.
11. Use a 20-µL (P20) pipet tip for the picking procedure. Pick one individual colony by gently scratching with the pipet tip. Make sure not to touch any neighboring colonies.
12. Transfer each picked colony into an individual 15-mL tube containing 50 µL of 0.25% trypsin/0.53 mM EDTA. Dissociate the colony by gently pipetting up and down and incubate at 37°C in water bath for 4 min.
13. After 4 min of incubation, add 5 mL of rat iPSC medium to each 15-mL tube containing one single colony and pipet up and down to dissociate the colony further into a single cell suspension. Centrifuge 4 min at 200 × *g*, at room temperature.
14. Aspirate the supernatant and resuspend the pellet in 1 mL of rat iPSC medium. Transfer the cell suspension from each picked colony into a single well of a 24-well plate that has been gelatin-coated and pre-seeded with irradiated or mitomycin C–treated MEF cells (prepared as in *UNIT 23.2*). Incubate at 37°C with 5% CO_2 and replenish medium every 24 hr until nearly confluent. *Cells are now ready to be passaged into a 6-well plate.*

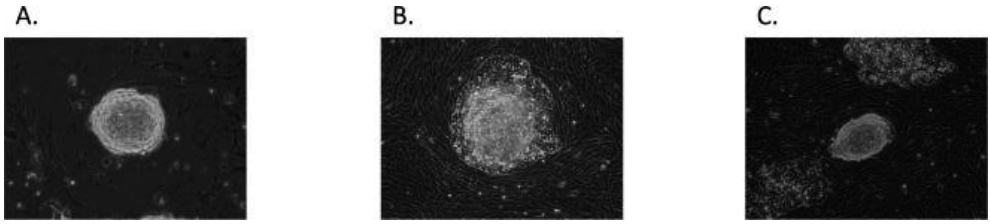

Figure 5. Examples of rat iPSC colonies. (**A**) A good rat iPSC colony. Note the translucent appearance and sharp borders. (**B**) a partially differentiated rat iPSC colony. This colony looks heterogeneous and differentiated. (**C**) One good rat iPSC colony (center) among two differentiated iPSC colonies (top and bottom). Care should be taken when picking the center colony not to touch the differentiated colonies.

In vitro culture and expansion of rat iPSCs

If reprogramming cells using a doxycycline-inducible system, wean the cells off doxycycline gradually after picking colonies. As a guideline, use the following schedule: use 1/2 concentration of doxycycline and then lower the concentration with each passage, to 1/4 concentration, then no doxycycline. If cell morphology begins to degrade, slow the rate of weaning. Do not change medium conditions at the time of passaging; rather, wait until the cells have attached (usually after 12 to 24 hr) before making changes to the doxycycline concentration.

15. When iPSC colonies reach 80% to 90% confluency in the dish or when individual iPSC colonies become large (approximately >100 cells/colony), aspirate the medium from the dish, and pre-feed the cells with rat iPSC medium 1 hr before passaging. *For rat iPSCs, a 1:6 passaging ratio is usually easily tolerated.*
16. After 1 hr aspirate the medium and wash the cells with 2 mL of PBS (no Ca or Mg).
17. Aspirate the PBS, add 0.5 mL of 0.25% trypsin/0.53 mM EDTA, and incubate at 37°C with 5% CO_2 for 4 min.
18. Add 1 mL of rat iPSC medium and dissociate the colonies by pipetting up and down into a single-cell suspension.
19. Transfer into a 15-mL tube containing 9 mL of warm rat iPSC medium and centrifuge 4 min at 200 × *g*, room temperature.
20. Discard the supernatant and resuspend the pellet in 12 mL of rat iPSC medium.
21. Aspirate the medium from 6-well plates containing mitomycin C–inactivated MEF (prepared as in *UNIT 23.2*), and add 2 mL of cell suspension per well. Incubate the cells at 37°C with 5% CO_2 until cells reach 80% to 90% confluency. Replenish with fresh rat iPSC medium every 24 hr. *It is advisable to prepare frozen stocks of newly reprogrammed iPSCs at low passage for future reference.*

Freezing rat iPSCs

22. Let cells grow until they reach 80% to 90% confluency. Before beginning the freezing procedure, label 2-mL cryovials with information on cell line, passage number, and date. *Generally two 2-mL cryovials are used per well of a 6-well plate.*
23. Aspirate the cell culture medium from each well and wash cells with 2 mL of sterile PBS.
24. Remove PBS completely, add 0.5 mL of 0.25% trypsin/0.53 mM EDTA, and incubate at 37°C with 5% CO_2 for 4 min (for 10-cm plate use 2 mL of 0.25% trypsin/0.53 mM EDTA).
25. Add 2.5 mL of serum containing rat iPSC medium and suspend the cells by pipetting up and down to create a single-cell suspension.
26. Transfer the cell suspension to a 15-mL tube and add in rat iPSC medium to a total volume of 10 mL. Count cells using a hemocytometer (*APPENDIX 3F*). Centrifuge 4 min at 200 × *g*, at room temperature.
27. Aspirate the supernatant, then resuspend the pellet in freezing/cryopreservation medium to obtain a concentration of 2×10^6 cells per mL.
28. Transfer the cell suspension to pre-labeled cryovials at 1 mL per cryovial. *Generally two cryovials are used per well of a 6-well plate.*
29. Quickly store the cryovials in a chilled isopropanol-containing cell-freezing container (4°C or on wet ice) and freeze at –80°C overnight. Transfer cells to –80°C rapidly, since DMSO is toxic to cells in liquid phase.
30. On the next day, remove cryovials from freezing container and store in the gas phase of a liquid nitrogen tank for long-term storage (may be kept frozen for many years).

Thawing rat iPSCs

31. Using cryogenic hand gloves and eye protectors remove the cryovial from the liquid nitrogen tank using forceps.

    CAUTION: *These safety precautions are required since cryovials stored in the liquid nitrogen tank may explode unexpectedly when exposed to rapid thawing.*
32. Immerse the vial in a 37°C water bath without submerging the cap.
33. Thaw vial rapidly until just a few ice crystals are left.
34. Record the sample name and passage number and spray 70% ethanol on the outer surface of the vial, then air dry quickly for a few seconds in a sterile laminar flow tissue culture hood.
35. Transfer thawed cells to a 15-mL conical tube using a pipet with a P1000 tip. Rinse freezing vial with 1 mL of rat iPSC medium and add to the 15-mL tube.
36. Add 10 mL of cold rat iPSC medium drop-wise to cells in the 15-mL conical tube. While adding the medium, gently move the tube back and forth to mix the cells. *This step reduces osmotic shock to the cells.*

37. Centrifuge the cells 4 min at 200 × *g*, at room temperature.
38. Aspirate the supernatant and resuspend the cell pellet in 2 mL of rat iPSC medium.
39. Slowly add the cell suspension in a drop-wise fashion into a well of a gelatin-coated 6-well plate, pre-seeded with growth-inhibited MEF cells (prepared as in *Unit 23.2*).
40. Culture cells as described above in a 37°C, 5% CO_2 incubator.

Alternate Protocol 2

Establishment and maintenace of pig iPSCs

This protocol describes the establishment and maintenance of pig iPSCs. See Basic Protocol 2 for the viral infection procedure. *Figure 6* is a schematic diagram showing the process of generating pig iPSCs.

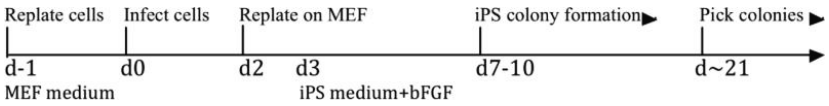

Figure 6. Schematic diagram of pig iPSC generation.

Materials

Pig fibroblasts infected with pluripotency factors (Basic Protocol 2); for timing, see *Figure 6*.
MEF medium (see recipe)
0.25% trypsin/0.53 mM EDTA (Invitrogen, cat. no. 25200-056)
24-and 6-well plates of inactivated mouse fibroblast feeder cells (*UNIT 23.2*)
Matrigel-coated plates (see recipe; optional)
Pig iPSC culture medium (see recipe)
mTeSR1 medium (optional)
Doxycyline (Sigma, cat. no. D9891); optional
Phosphate-buffered saline (PBS) without CaCl2 and MgCl2 (Invitrogen, cat. no. 14190-250)
Collagenase IV
Accutase (optional)
Dispase (optional)
ROCK inhibitor Y27632 (Calbiochem, cat. no. 688000)
TeSR2 (STEMCELL Technologies, cat. no. 05860; optional)
mFreSR (STEMCELL Technologies, cat. no. 05855; optional)
Freezing/cryopreservation medium (see recipe)
Isopropanol
Liquid N2
70% ethanol

15-mL conical tubes
Marker pen
Light microscope with camera
20-µl (P20) and 1000-µl (P1000) pipet tips
Cell lifter (Corning, cat. no. 3008)
Eppendorf Model 5810R benchtop centrifuge (or equivalent)
2.0-mL cryovials
Freezing chamber (e.g., Mr. Frosty; Thermo Scientific)
Liquid nitrogen storage tank
Cryogenic handling gloves and eye protectors
Forceps
Additional reagents and equipment for preparing inactivated mouse embryonic fibroblast feeder cells (*Unit 23.2*) and counting cells using a hemocytometer (*Appendix 3F*)

Post-infection culturing of infected fibroblasts

1. After 48 hr, or when cells reach confluency, aspirate the medium from the infected cells and dissociate by adding 0.5 mL of 0.25% trypsin/0.53 mM EDTA and incubating for 4 min at 37°C with 5% CO_2. Add 2 mL of warm MEF medium, then pipet up and down to obtain a single-cell suspension. Transfer to a 15-mL tube containing 9 mL of warm MEF medium, and centrifuge 4 min at 200 × *g*, at room temperature.
2. Discard the supernatant and resuspend the pellet in 8 mL of MEF medium.
3. Aspirate the medium from four single wells of a 6-well plate that has been gelatin-coated and pre-seeded with growth-inhibited MEF cells as described in *Unit 23.2*.
   Add 2 mL of the infected cell mixture per well on to the MEF feeder layer so that the infected cells are passaged in a 1:4 ratio. Incubate at 37°C with 5% CO_2. *It is recommended to culture pig iPSCs on MEFs initially. Once stable colonies are established, the cells can be transferred to feeder-free conditions. If using MEFs, plate 1 million MEFs per 10-cm plate or approximately 170,000 MEFs per well of a 6-well plate. Use gelatin-coated plates.*
4. After 12 to 24 hr, exchange the medium for pig iPSC culture medium (supplemented with doxycycline at a final concentration of 1 µg/mL if using a TetO lentiviral system).
5. Change pig iPSC medium supplemented with doxycycline (final concentration, 1 µg/mL if using a TetO lentiviral system) every 24 hr and check for colony formation. *Colonies should start becoming microscopically visible approximately 7 to 10 days post-infection.*
6. Let colonies grow into a reasonable size (approximately >50 cells/colony).
   This should take until approximately day 21 post-infection.

Picking and establishing pig iPSC clones

7. One hour before picking iPSC colonies, pre-feed the cells by replenishing with fresh pig iPSC medium. *This is especially important when the medium has turned acidic (indicated by yellow color), since pre-feeding will increase cell survival after dissociation.*
8. Prepare a microscope inside a laminar hood to maintain sterile conditions for picking colonies (*Figure 2*).
9. Before picking, select up to 40 good colonies by circling the colony with a marker pen on the bottom of the plate to be able to retrieve the selected colonies when the actual picking procedure is started. *Suitable colonies should appear translucent and circular. See Figure 7 for examples of suitable pig iPSC colonies.*
10. Add 50 µL of 0.25% trypsin/0.53 mM EDTA into each of several 15-mL tubes.
11. Use a 20 µL (P20) pipet tip for the picking procedure. Pick one individual colony by gently scratching with the pipet tip. Make sure not to touch any neighboring colonies.
12. Transfer each picked colony into an individual 15-mL tube containing 50 µL of 0.25% trypsin/0.53 mM EDTA. Dissociate the colony by gentle pipetting and incubate at 37°C in a water bath for 4 min. *Pick up as many colonies as possible within 15 min to limit exposure of picked colonies to trypsin.*
13. Dissociate the colony by gentle mechanical dissociation with the pipet tip and pipetting up and down. *Ideally, colonies should be dissociated into small cell clusters instead of single-cell dissociation.*
14. Once the colony is properly dissociated, add in 1 mL of pig iPSC medium. Transfer the cell suspension from each picked colony into a single well of a 24-well plate that has been gelatin-coated and pre-seeded with growth-inactivated MEF cells as described in *Unit 23.2. Alternatively, use feeder-free conditions using Matrigel-coated plates with mTeSR1 medium.* Incubate at 37°C with 5% CO_2.
15. After 48 hr, replenish the medium every 24 hr until cells reach 80% to 90% confluency. *This should take about 7 to 10 days. Cells are now ready to be passaged into a 6-well plate.*

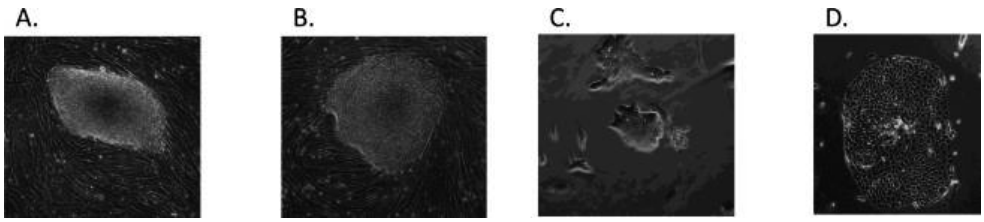

Figure 7. Examples of pig iPSC colonies. (**A**) A developing pig iPSC colony. (**B**) A good pig iPSC colony, cultured on MEFs. Note the translucent appearance and sharp borders. (**C**) A good pig iPSC colony grown on Matrigel. (**D**) A bad pig iPSC colony, grown on Matrigel. This colony looks heterogeneous and differentiated and should be removed from the culture dish.

In vitro culturing and expansion of pig iPSCs

If reprogramming cells using a doxycycline-inducible system, wean the cells off doxycycline gradually after picking colonies. As a guideline, use the following schedule: use 1/2 concentration of doxycycline and then lower the concentration with each passage, so 1/4 concentration, then no doxycycline. If cell morphology begins to degrade, slow the rate of weaning. Do not change medium conditions at the time of passaging; rather wait until the cells have attached (usually after 12 to 24 hr) before making changes to the doxycycline concentration.

16. Before splitting, remove differentiated colonies under a microscope under sterile conditions (i.e., via slow-vacuum aspiration or pipet scraping). Be careful not to leave the plate out too long and make sure cells do not dry out if using the vacuum method. *See Figure 7 for examples of good pig iPSC colonies as well as bad pig iPSC colonies that should be removed from the culture dish.*
17. Wash cells with warm PBS (no Ca or Mg). Aspirate PBS and add 1 mL of 100 U/mL collagenase IV and incubate 4 min at 37°C with 5% CO_2 (for pig iPSCs grown on MEFs). *Alternatively, add 0.5 mL of 0.6 to 2.4 mg/mL Dispase or 0.5 mL of 1 × Accutase and incubate for 0.5 to 1 min at 37°C with 5% CO_2 (for pig iPSCs grown in feeder-free conditions). Collagenase IV is used for passaging cells grown on MEFs. For cells grown on Matrigel, use Dispase or Accutase instead of collagenase IV. Trypsinization is not recommended, since aggressive dissociation into single cells will not be well tolerated. Expect to see visible curling or thickening of colonies around the edges.*
18. Aspirate the enzyme and wash with 2 mL of PBS. After aspirating PBS, add 1 mL of pig iPSC medium supplemented with ROCK inhibitor (at 10 µM final concentration). Using a cell lifter, scrape the entire well to lift the colonies.
19. Transfer colonies into a 15-mL conical tube; wash the well with 1 mL of pig iPSC medium and transfer washings into the 15-mL tube. Centrifuge 4 min at 200 × *g*, at room temperature. Aspirate supernatant and leave the pellet undisturbed.
20. Resuspend the pellet in pig iPSC medium. Use 2 mL of medium per well of a 6-well plate that is going to be seeded. *The ratio depends on cell density prior to passaging. Usually a 1:3 ratio (1 nearly confluent well can be passaged into 3 new wells) serves as a good guideline. For this example, resuspend the cell pellet in 6 mL of medium.*
21. Triturate to obtain medium to small fragments (~50 to 200 cells per fragment). Avoid over-triturating, since this may cause cell death, especially when colonies are broken down to single-cell suspensions.
22. Prewash wells of a gelatin-coated MEF-seeded 6-well plate (prepared as in *Unit 23.2*) or Matrigel-coated wells with 1 mL of warm pig iPSC medium, aspirate medium, and add 2 mL of suspended cell solution into each well.

23. Replenish the cells with 2 mL of fresh pig iPSC medium every 24 hr until cells reach confluency.

    This usually takes about 7 to 10 days. Cells are now ready for passaging. It is advisable to prepare frozen stocks of newly reprogrammed iPSCs at low passage for future use. See below for freezing and thawing instructions. Cell death is a common problem when cells are transferred directly from serum-containing medium to serum-free medium, like mTeSR1 medium. This may appear quite dramatic initially as up to 50% of cells may fail to attach, but abundant viable and proliferative cells should become apparent upon medium change.

Freezing pig iPSCs

For cells grown on Matrigel/mTeSR1, a similar procedure can be followed, except for using 500 µL of mFreSR per 6-well plate. mFreSR is a defined, serum-free cryopreservation medium designed for the cryopreservation of cells. It is very important to minimize the amount of pipetting to ensure later cell survival.

24. Before beginning the freezing procedure, label 2-mL cryovials with information on cell line, passage number, and date. *Generally one cryovial is used per well of a 6-well plate or five cryovials per 10-cm dish.*
25. Dissociate the cells of a nearly confluent well as described above in steps 16 to 19. Use collagenase IV for feeder/serum cultures and Dispase or Accutase for Matrigel/mTeSR1 cultures.
26. Add 500 µL of freezing/cryopreservation medium to the cell pellet.
27. Carefully resuspend the pellet in the freezing medium, keeping cells in chunks that are as large as possible; generally pipetting two times should be enough.
28. Quickly transfer 500 µL of the suspension into a pre-labeled 2-mL cryovial and place inside an isopropanol-containing freezing container. Store 24 to 48 hr at -80°C and then transfer to a liquid nitrogen storage tank. *When working with DMSO-containing freezing/cryopreservation medium, it is key to work as quickly as possible to transfer the freezing stock rapidly into a –80°C freezer to initiate the freezing procedure.*

Thawing pig iPSCs

Each vial should be thawed into 1 well of a 6-well plate. The passage number and the name of the cell line should be noted. Ideally cells should be kept in large clumps to increase survival efficiency, so vigorous pipetting should be avoided.

29. Add 9 mL of cold pig iPSC medium supplemented with 10 µM ROCK inhibitor to a 15-mL tube.
30. Using cryogenic hand gloves and eye protectors remove the cryovial from the liquid nitrogen tank using forceps. Caution: *These safety precautions are required since cryovials stored in the liquid nitrogen tank may explode unexpectedly when exposed to rapid thawing.*

31. Remove the iPSC vial from the liquid nitrogen tank using forceps and immerse the vial in a 37°C water bath without submerging the cap. Thaw vial contents rapidly until just a few ice crystals are left.
32. Record the sample name and spray 70% ethanol on the outer surface of the vial then air dry quickly a few seconds in the sterile laminar hood.
33. Transfer the thawed cell mixture in the tube containing 9 mL of cold pig iPSC medium supplemented with ROCK inhibitor. Rinse the cryovial once with pig iPSC medium and transfer to tube.
34. Centrifuge 4 min at 200 × *g*, at room temperature.
35. Meanwhile, wash one well of a 6-well gelatin-coated and MEF-seeded (growth inhibited) with PBS. *Skip this step when using Matrigel plates.*
36. Add 2 mL of pig iPSC medium. *It is highly recommended that 10 μM ROCK inhibitor Y-27632 be added for the first 24 hr to improve survival efficiency. Do not add ROCK inhibitor to medium replenishing steps that do not involve passaging or thawing.*
37. Aspirate the medium from the cell pellet and gently resuspend in 1 mL of pig iPSC medium supplemented with ROCK inhibitor. Pipet up and down slowly once or twice, avoiding disruption of the cell chunks. Transfer to one well of a 6-well plate.
38. Change the medium after 24 hr.
39. Feed cells daily with 2 mL medium. *Colonies may not develop the next day, but may emerge at any point from 3 to 10 days after plating.*
40. Perform the first passage mechanically, without the use of collagenase IV or Dispase. See steps 13 to 15 above for details. After this initial passage, cells can be cultured as described in steps 16 to 23. *It is highly recommended that a mycoplasma test (see Appendix 3F) be performed upon thawing.*

Alternative Protocol 3

Establishment and maintenance of human iPSCs

This protocol describes the establishment and maintenance of human iPSCs. See Basic Protocol 2 for the viral infection procedure. *Figure 8* is a schematic diagram showing the process of generating human iPSCs.

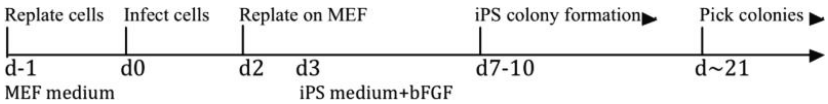

Figure 8. Schematic diagram of human iPSC generation.

Materials

Human fibroblasts infected with pluripotency factors (Basic Protocol 2); for timing, see *Figure 8*.
MEF medium (see recipe)
0.25% trypsin/0.53 mM EDTA (Invitrogen, cat. no. 25200-056)
24- and 6-well plates of inactivated mouse fibroblast feeder cells (*Unit 23.2*)
Human iPSC culture medium (see recipe)
Doxycyline (Sigma, cat. no. D9891; optional)
Matrigel-coated plates (see recipe; optional)
mTeSR1 medium (optional)
Phosphate-buffered saline (PBS) without CaCl2 and MgCl2 (Invitrogen 14190-250)
Collagenase IV
Accutase (optional)
Dispase (optional)
ROCK inhibitor Y27632 (Calbiochem, cat. no. 688000)
TeSR2 (STEMCELL Technologies, cat. no. 05860; optional)
mFreSR (STEMCELL Technologies, cat. no. 05855; optional) Freezing/
cryopreservation medium (see recipe)
Isopropanol
Liquid N_2
70% ethanol
15-mL conical tubes
Marker pen
Light microscope with camera
20-µL (P20) and 1000-µL (P1000) pipet tips
Cell lifter (Corning, cat. no. 3008)
Eppendorf Model 5810R benchtop centrifuge (or equivalent)
2.0-mL cryovials
Freezing chamber (e.g., Mr. Frosty; Thermo Scientific)
Liquid nitrogen storage tank
Cryogenic handling gloves and eye protectors
Forceps
Additional reagents and equipment for preparing inactivated mouse embryonic fibroblast feeder cells (*Unit 23.2*) and counting cells using a hemocytometer (*Appendix 3F*)

Post-infection culturing of infected fibroblasts

1. After 48 hr, or when cells reach confluency, aspirate the medium from the infected cells and dissociate by adding 0.5 mL of 0.25% trypsin-EDTA and incubating for 4 min at 37°C

with 5% CO_2. Add 2 mL of warm MEF medium, then pipet up and down to obtain a single-cell suspension. Transfer to a 15-mL tube containing 9 mL of warm MEF medium and centrifuge 4 min at 200 × *g*, at room temperature.

2. Discard the supernatant and resuspend the pellet in 8 mL of MEF cell culture medium**.**
3. Aspirate the medium from four single wells of a 6-wells plate that has been gelatin-coated and pre-seeded with growth-inhibited MEF cells as described in UNIT 23.2. Add 2 mL of the infected cell mixture per well onto the MEF feeder layer so that the infected cells are passaged in a 1:4 ratio. Incubate at 37°C with 5% CO_2. *It is recommended to culture human iPSCs on MEFs initially. Once stable colonies are established, the cells can be transferred to feeder-free conditions.*
4. After 12 to 24 hr, substitute the medium with human iPSC culture medium (supplemented with doxycycline if using a TetO lentiviral system).
5. Change human iPSC medium every 24 hr and check for colony formation. *Colonies should start becoming microscopically visible approximately 7 to 10 days post-infection.*
6. Let colonies grow to a reasonable size (approximately >50 cells/colony). *This should take until approximately day 21 post-infection.*

Picking and establishing human iPSC clones

7. One hour before picking iPSC colonies, pre-feed the cells by replenishing with fresh human iPSC medium. *This is especially important when the medium has turned acidic (indicated by yellow color), since pre-feeding will increase cell survival after dissociation.*
8. Prepare a microscope inside a laminar hood to maintain sterile conditions for picking colonies (*Figure 2*).
9. Before picking, select as many good colonies as necessary by circling the colony with a marker pen on the bottom of the plate to be able to retrieve the selected colonies when the actual picking procedure is started. *Suitable colonies should appear translucent and perfectly circular. See Figure 9 for examples of suitable human iPSC colonies.*
10. Place 50 µL of 0.25% trypsin/0.53 mM EDTA into each of several 15-mL tubes.
11. Use a 20-µL pipet tip for the picking procedure. Pick one individual colony by gently scratching with the pipet tip. Make sure not to touch any neighboring colonies.
12. Transfer each picked colony into an individual 15-mL tube containing 50 µL of 0.25% trypsin/0.53 mM EDTA. Dissociate the colony by gentle mechanical dissociation with the pipet tip and pipetting up and down. *Ideally, colonies should be dissociated into small cell clusters instead of single-cell dissociation.*
13. Once the colony is properly dissociated, add in 1 mL of human iPSC medium. Transfer the cell suspension from each picked colony into a single well of a 24-well plate that has been gelatin-coated and pre-seeded with growth-inactivated MEF cells as described in *Unit*

23.2. Incubate at 37°C with 5% CO_2. *Alternatively, use feeder-free conditions using Matrigel-coated plates with mTeSR1 medium.*

14. After 48 hr replenish human iPSC medium every 24 hr until cells reach 80% to 90% confluency. This should take about 7 to 10 days. *Cells are now ready to be passaged into a 6-well plate.*

Figure 9. Examples of human iPSCs. (**A**) A good human iPSC colony grown on Matrigel. Note the translucent appearance and sharp borders. (**B**) A bad human iPSC colony grown on Matrigel. This colony looks heterogeneous and differentiated and should be removed from the culture dish. Images kindly provided by Dr. Emil M. Hansson at MGH Cardiovascular Research Center.

In vitro culture and expansion of human iPSCs

If reprogramming cells using a doxycycline-inducible system, wean the cells off doxycycline gradually after picking colonies. As a guideline, you can use the following schedule: use 1/2 concentration of doxycycline and then lower the concentration with each passage, so 1/4 concentration, then no doxycycline. If cell morphology begins to degrade, slow the rate of weaning. Do not change medium conditions at the time of passaging; rather wait until the cells have attached (usually after 12 to 24 hr) before making changes to the doxycycline concentration.

Note: If using MEFs plate 1 million MEFs per 10-cm plate or approximately 170,000 MEFs per well of a 6-well plate. Use gelatin-coated plates.

15. Before splitting, remove differentiated colonies under the microscope in sterile conditions (i.e., via slow-vacuum aspiration or pipet scraping). Be careful not to leave the plate out too long and make sure cells do not dry out if using the vacuum method. *See Figure 9 for examples of good human iPSC colonies as well as bad human iPSC colonies that should be removed from the culture dish.*
16. Wash cells with warm PBS (no Ca or Mg). Aspirate PBS and add 1 mL of 100 U/mL collagenase IV and incubate 4 min at 37°C with 5% CO_2 (for human iPSCs grown on MEFs). Alternatively, add 0.5 mL of 0.6 to 2.4 mg/mL Dispase or 0.5 mL of 1 × Accutase and incubate for 0.5 to 1 min at 37°C with 5% CO_2 (for human iPSCs grown in feeder-free conditions). Expect to see visible curling or thickening of colonies around the edges. *Collagenase IV is used for passaging cells grown on MEFs. For cells grown on Matrigel, use Dispase or Accutase in place of collagenase IV. Trypsinization is not recommended.*

17. Aspirate the enzyme and wash with 2 mL of PBS. After aspirating off PBS, add 1 mL of human iPSC medium supplemented with ROCK inhibitor (final concentration, 10 μM). Using a cell lifter, scrape the entire well to lift the colonies.
18. Transfer colonies into a 15-mL conical tube; wash the well with 1 mL of human iPSC medium and transfer washings into the 15-mL tube. Centrifuge 4 min at 200 × *g*, at room temperature. Aspirate supernatant and leave the pellet undisturbed.
19. Resuspend the pellet with human iPSC medium. Use 2 mL of medium per well of a 6-well plate that is going to be seeded. *The ratio depends on cell density prior to passaging. Usually a 1:3 ratio (1 nearly confluent well can be passaged into 3 new wells) serves as a good guideline. For this example, resuspend the cell pellet in 6 mL of medium.*
20. Triturate to obtain medium-to-small fragments (~50 to 200 cells per fragment). Avoid over-triturating since that will lead to cell death, especially when colonies are broken down to single-cell suspensions.
21. Pre-wash gelatin-coated and MEF-seeded wells or Matrigel-coated wells with 1 mL of warm human iPSC medium, aspirate medium, and add 2 mL of resuspended cell solution into each well.
22. Replenish the cells with 2 mL of fresh human iPSC medium every 24 hr until cells reach confluency. *This usually takes about 7 to 10 days. Cells are now ready for passaging. It is advisable to prepare frozen stocks of newly reprogrammed iPSCs at low passage for future use. Cell death is a common problem when cells are transferred directly from serum-containing medium to serum-free medium, like mTeSR1 medium. This may appear quite dramatic initially as up to 50% of cells may fail to attach, but abundant viable and proliferative cells should be able to become apparent upon medium change.*

Freezing human iPSCs

For cells grown on Matrigel/mTeSR1, a similar procedure can be followed as described below, except for using 500 μL of mFreSR per 6-well. mFreSR is a defined, serum-free cryopreservation medium designed for the cryopreservation of human embryonic and induced pluripotent stem cells (hESCs and hiPSCs). Together with mTeSR1 or TeSR2, mFreSR eliminates the use of feeders and serum. hESCs cryopreserved in mFreSR have thawing efficiencies 5-to 10-fold higher than reported conventional thawing methods using serum.

Note: It is very important to minimize the amount of pipetting to ensure cell survival.

23. Before beginning the freezing procedure, label cryovials with information on cell line, passage number and date. Generally use one cryovial per well of a 6-well plate or five cryovials per 10-cm dish.
24. Dissociate the cells of a nearly confluent well as described above in steps 15-18 and use collagenase IV for feeder/serum cultures and Dispase or Accutase for Matrigel/mTeSR1 cultures.

25. Add 500 µL of freezing/cryopreservation medium to the cell pellet.
26. Carefully resuspend the pellet in the freezing medium, keeping cells in as large of chunks as possible; generally pipetting two times should be enough.
27. Quickly transfer 500 µL into a labeled cryovial, and place inside isopropanol-containing freezing container. Store 24 to 48 hr at −80°C, and then transfer to liquid nitrogen.

Thawing human iPSCs

Each vial should be thawed into 1 well of a 6-well plate. The passage number and the name of the cell line should be noted. Ideally, cells should be kept in large clumps to increase survival efficiency, so avoid vigorous pipetting.

28. Add 9 mL of cold human iPSC medium supplemented with 10 µM ROCK inhibitor into one 15-mL tube.
29. Using cryogenic hand gloves and eye protectors remove the cryovial from the liquid nitrogen tank using forceps
    Safety precautions are required since cryovials stored in the liquid nitrogen tank may explode unexpectedly when exposed to rapid thawing.
30. Immerse the vial in a 37°C water bath without submerging the cap. Thaw vial rapidly until just a few ice crystals are left.
31. Record the sample name and spray 70% ethanol on the outer surface of the vial then air dry quickly a few seconds in the sterile laminar hood.
32. Transfer the thawed cell mixture in the tube containing 9 mL of cold human iPSC medium supplemented with ROCK inhibitor. Rinse the cryovial once with medium and transfer the rinse solution to the tube.
33. Centrifuge 4 min at 200 × *g*, at room temperature.
34. Meanwhile, wash one well of a 6-well plate that has been pre-coated with gelatin and seeded with irradiated or mitomycin C–treated MEF (prepared as in *UNIT 23.2*) with PBS. Skip this step when using Matrigel-only plates.
35. Aspirate the PBS and add 2 mL of human iPSC medium containing ROCK inhibitor (final concentration, 10 µM) to each well. *It is highly recommended to add 10 µM ROCK inhibitor Y-27632 for the first 24 hr to improve survival efficiency. ROCK inhibitor should not be added to any subsequent medium-replenishing steps that do not involve passaging or thawing. The ROCK Inhibitor Y-27632 enhances the survival rate of human embryonic stem cells following cryopreservation.*[4]
36. Aspirate the supernatant from the cell pellet after centrifugation and gently resuspend in 1 mL of human iPSC medium supplemented with ROCK inhibitor (at 10 µM final

concentration). Pipet slowly once or twice, avoiding disruption of the cell chunks. Transfer to one well of a 6-well plate as prepared in step 34.

37. Change the medium after 24 hr.
38. Feed cells daily with 2 mL medium. *Colonies may not develop the next day, but may emerge anywhere from 3 to 10 days.*
39. Perform the first passage mechanically, without the use of collagenase IV or Dispase, as described in steps 7 to 14. After this initial passage, cells can be cultured as described in steps 15 to 22. *It is highly recommended to perform a mycoplasma test upon thawing.*

Reagents and solutions

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***Appendix 2****; for suppliers, see* ***Appendix 4****. Unless otherwise noted, all solutions and media are filter sterilized using a 0.22-µM filter. All media must be stored at 4°C and should be used within 10 days of preparation, unless otherwise specified.*

293FT Cell culture medium

450.00 mL DMEM (high glucose, Invitrogen, cat. no. 11965-092; 90% final)
50.00 mL fetal bovine serum (BenchMark FBS; Gemini Bioproducts, cat. no. 100-106; 10% final)
Store up to 1 month at 4°C

Cell culture dishes, pre-coated with 0.1% gelatin

Prepare 0.1% gelatin in sterile water. Incubate the culture dish (for 100-mm dish use Corning, cat. no. 430167) with the 0.1% gelatin for at least 30 min at room temperature. We recommend coating dishes right before use. Aspirate the excess gelatin and allow the plate to dry for 20 min.

Freezing/cryopreservation medium

900 µL fetal bovine serum (BenchMark FBS; Gemini Bioproducts, cat. no. 100-106)
100 µl dimethylsulfoxide (DMSO; Sigma D-2650)
Store up to 1 month at 4°C

For pig iPSCs or human iPSCs grown on Matrigel (BD Biosciences, cat. no. 354277) and mTeSR1 medium (STEMCELL Technologies 05850), it is advisable to use mFreSR medium (STEMCELL Technologies 05855) for cryopreservation.

Human iPSC medium

400 mL DMEM/F12 medium (Invitrogen, cat. no. 11330-057)
100 mL Knockout Serum Replacer (Invitrogen, cat. no. 10828-028)
5mL L-glutamine (Invitrogen, cat. no. 25030-081)
5 mL MEM-NEAA (Invitrogen, cat. no. 11140-050)
5 mL penicillin/streptomycin solution (Invitrogen, cat. no. 15140-155)
3.5 µL 2-mercaptoethanol (Sigma, cat. no. M7522)
5 to 10 µg human bFGF (Invitrogen, cat. no. PHG0021)
Store up to 1 month at 4°C

Note: Knock-Out DMEM medium (Invitrogen, cat. no. 10829) can be used in place of DMEM/F12 medium. bFGF final concentration should be between 4 and 10 ng/mL. Addition of human bFGF will help maintain cells in an undifferentiated state.

Matrigel-coated culture dishes

Matrigel (BD Biosciences, cat. no. 354277) should be thawed and aliquoted on ice. Aliquots can be stored at −80°C. Mix the Matrigel and ice-cold Knock-Out DMEM medium (Invitrogen, cat. no. 10829) in a ratio of 1:100 on ice. Pipet to mix thoroughly and apply immediately to culture dishes, making sure to coat the entire bottom of the plate. Use 2 mL/well of a 6-well or 1 mL/well of a 24-well plate. Incubate the plate at 37°C for 1 hr. Plates can be used immediately or wrapped with Parafilm and stored at 4°C up to 15 days. Before use aspirate the Matrigel and wash once with cell culture medium.
Store up to 1 month at 4°C.

MEF medium

500.00 mL DMEM (high glucose; Invitrogen, cat. no. 11965-092; 81% final)
94.00 mL fetal bovine serum (BenchMark FBS; Gemini Bioproducts, cat. no. 100106; heat inactivated; 15% final)
12.50 mL pen/strep (5000 penicillin/5000 mg/mL streptomycin; Invitrogen, cat. no. 15070-063; 2% final)
6.25 mL 200 mM L-glutamine (Invitrogen, cat. no. 25030-081; 1% final)
6.25 mL 10 mM (100× MEM nonessential amino acids (Invitrogen, cat. no. 11140050; 1% final)
Store up to 1 month at 4°C

Mouse iPSC medium (CJ7 medium)

500.00 mL DMEM (high glucose; Invitrogen, cat. no. 11965-092; 81% final)

94.00 mL fetal bovine serum (BenchMark FBS; Gemini Bioproducts, cat. no. 100106; heat inactivated; 15% final)
12.50 mL penicillin/streptomycin solution (5000 penicillin/5000 mg/mL streptomycin; Invitrogen, cat. no. 15070-063; 2% final)
6.25 mL 200 mM L-glutamine (Invitrogen, cat. no. 25030-081;1% final)
6.25 mL 10 mM (100× MEM nonessential amino acids (Invitrogen, cat. no. 11140050; 1% final)
4.4 µL 2-mercaptoethanol (2-ME; Sigma, cat. no. M-6250; $\sim 10^{-4}$ M final)
62.5 µL mouse LIF (10^7 U/mL stock; Chemicon, cat. no. ESG1107; 10^3 U/mL final)
Store up to 1 month at 4ºC

Complete medium is stable for ~2 weeks. Medium older than this should be re-supplemented with fresh L-glutamine and mouse LIF.

Pig iPSC medium
400 mL DMEM/F12 Medium (Invitrogen, cat. no. 11330-057)
100 mL Knockout Serum Replacement (Invitrogen, cat. no. 10828-028)
5 mL L-glutamine (Invitrogen, cat. no. 25030-081)
5 mL of 10 mM(100×) MEM nonessential amino acids (Invitrogen, cat. no. 11140050)
5 mL penicillin/streptomycin solution (5000 penicillin/5000 mg/mL streptomycin; Invitrogen, cat. no. 15070-063)
3.5 µL 2-mercaptoethanol (Sigma, cat. no. M7522)
5 to 10 µg human bFGF (Invitrogen, cat. no. PHG0021)
Store up to 1 month at 4°C

Note: Knock-Out DMEM medium (Invitrogen, cat. no. 10829) can be used in place of DMEM/F12 medium. bFGF final concentration should be between 4 and 10 ng/mL. Addition of human bFGF will help maintain cells in an undifferentiated state.

Rat iPSC medium containing KOSR
400 mL Knockout DMEM (Invitrogen, cat. no. 10829)
100 mL Knockout Serum Replacer (Invitrogen, cat. no. 10828-028)
5 mL L-glutamine (Invitrogen, cat. no. 25030-081)
5 mL of 10 mM 100× MEM nonessential amino acids (Invitrogen, cat. no. 11140050)
5 mL penicillin/streptomycin solution (5000 penicillin/5000 mg/mL streptomycin; Invitrogen, cat. no. 15070-063)
3.5 µL 2-mercaptoethanol (2-ME; Sigma, cat. no. M7522)
Store up to 1 month at 4°C

Rat iPSC medium, serum-free

Prepare DMEM/F12-N2 medium: To 100 mL DMEM-F12 Medium (Invitrogen, cat. no. 11330-057), add 1 mL 100× N2 supplement (Invitrogen, cat. no. 17502048). Store at 4°C and use within 1 month.

Prepare neurolbasal/B27 medium: To 100 mL Neurobasal Medium (Invitrogen, cat. no. 21103), add 2 mL B27 (Invitrogen, cat. no. 17504-044), and 0.5 to 1 mL of 200 mM L-glutamine (Invitrogen, cat. no. 25030-081). Store at 4°C and use within 1 month.

Prepare the serum-free rat iPSC medium: Mix DMEM/F12-N2 medium with Neurolbasal/B27 medium in a ratio of 1:1. Add 2-mercaptoethanol (Sigma, cat. no. M7522) to a final concentration of 0.1 mM. Store at 4°C and use within 1 month.

For establishing and culturing Rat iPSCs, rat iPSC medium should always be supplemented with 0.5 μM MEK inhibitor (e.g., PD0325901, Stemgent, cat. no. 04-0006; *http://www.stemgent.com*), 3 μMGSK3β inhibitor (e.g., CHIR99021, Stemgent, cat. no. 04-0004), and 1000 U/mL of Rat LIF (Millipore, cat. no. LIF3005). Add freshly before use. These supplements help maintain rat iPSCs in an undifferentiated state.

Commentary

Background information

Regenerative medicine has gained significant promise in recent years for treating chronic and debilitating diseases.[5] As the aging population increases in the U.S., it has become a medical and scientific imperative to find ways to alleviate the suffering of patients with some of the most challenging illnesses such as Alzheimer's disease, congestive heart failure, and emphysema. While the route to success in treating these diseases is far from clear at the moment, a number of scientific advances have enabled us to envision how cell replacement therapy may be accomplished. One such approach involves the use of pluripotent stem cells (PSCs), which exhibit both the ability to self-renew indefinitely and differentiate spontaneously into a wide variety of different cell types. Initial PSC studies in the 1970s employed a germline tumor called teratocarcinoma, which exhibits features of pluripotent stem cells but with the propensity for unchecked growth.[6] Due to its cancer-like features, teratocarcinoma was relegated to nothing more than a developmental oddity. Then, in the early 1980s, with the isolation of mouse embryonic stem cells (ESCs),[7] it became possible for investigators to engineer genome modifications that could be studied by *in vitro* differentiation or, when introduced into the germline, could even generate knock-out mice for *in vivo* studies. Since mouse ESCs were karyotypically normal, they contributed efficiently to all adult tissues when

injected into a developing blastocyst stage embryo. Furthermore, the creation of live-born animals exclusively from injected ESC in a tetraploid complementation assay validated the true developmental competency of these mouse ESCs.[8-9] This has enabled the generation of countless numbers of genetic mouse models of human disease. However, despite these technical advances for deriving mouse ESCs, it took yet another 18 years for the generation of ESCs from discarded human embryos.[10]

Although mouse and human ESCs are now providing a platform for studies of early development, the use of human fetal-derived cells has been highly controversial. Furthermore, for a cell-based therapy to be successful, it would ideally involve immunohistocompatible donor cells with immunocompetent host individuals to avoid the need for immunosuppressants. In order to circumvent these issues, it would be desirable to have a source of pluripotent stem cell that can be derived easily from somatic cells. Early seminal work by Jon Gurdon and others demonstrated that the introduction of somatic cell nuclei from frogs into an enucleated oocyte can induce pluripotent gene expression in the nucleus of the injected somatic cell.[11] This principle was subsequently used to generate "cloned" animals such as Dolly the sheep.[12] Despite the success with nuclear cloning of multiple animal species, including primates,[13-14] this approach has not led to successful human nuclear cloning thus far. The reason for this is likely due to the limited availability of donor human eggs. The challenging social climate for human embryo work has also not been conducive to significant progress in this area.

To overcome these challenges in human somatic cell nuclear transfer, Takahashi and Yamanaka set out to prove that direct reprogramming by means of transcription factor overexpression could be feasible.[1] In their seminal work, they systematically screened 24 transcription factors that are highly expressed in undifferentiated ESCs to find a combination of as few as four factors (Sox2, Oct4, Klf-4, and c-Myc) that could reprogram mouse embryonic fibroblasts into ESC-like cells. These induced pluripotent stem cells (iPSCs) express Nanog, Rex1, Oct4, and Sox2 from their endogenous loci and are able to silence the expression of exogenously supplied transcription factors to enable their differentiation *in vitro*. Following this groundbreaking work, many investigators from around the world have been able to reproduce these findings.[15-17] Furthermore, the developmental competency of a subset of iPSCs lines were validated by their ability to generate "all-iPSC" mice in tetraploid complementation assays.[18-21]

Remarkable advances have since been made to improve the efficiency of iPSC generation. While the use of viral vectors has established the feasibility of transcription factor-based reprogramming, concerns regarding the potential tumorigenicity from viral-mediated genome modification have spurred the development of reprogramming strategies that are virus-free.

Such methods include non-integrating adenoviral vectors,[22] naked plasmid transfection,[23] mini-circle plasmids,[24] proteins,[25] modified RNA,[26] and genome-editing enzymes such as Cre recombinase[27-28] and transposases.[29-30] While these non- integrating strategies induce minimal genome perturbation, their overall reprogramming efficiency is usually 100 to 1000 times lower than viral-based strategies. With the availability of lentiviruses carrying polycistronic cassettes encoding all four reprogramming factors, the efficiency of reprogramming has increased significantly.[31-32]

Given the high degree of conservation of pluripotency gene function, iPSCs have been derived from a number of different popular species, including mouse,[1] rat,[33-34] sheep,[35] pig,[36] rhesus monkey,[37] and human,[38-40] as well as endangered species such as the drill and the nearly extinct northern white rhinoceros.[41] This has been a remarkable feat, since the derivation of true ESC lines from some of these species (e.g., rat, sheep, pig) has been technically challenging. The availability of iPSCs from these species now allows creation of genetic models in large animals that may exhibit a more similar disease phenotype as human.

Critical parameters

The following guidelines should be followed to maximize reprogramming efficiency. Make sure to use fresh chemicals and unexpired media ingredients that have been handled and stored under recommended storage conditions. Fibroblasts should be of low passage number (ideally below p3) to avoid replicative senescence. Cells should never be allowed to become overconfluent, since this affects their reprogramming amenability. Similarly, do not let the feeder cells become overconfluent, or their abilities as feeder cells may decrease. When cells have been successfully reprogrammed into iPSCs, it is also important not to let cells become overconfluent since this will affect their pluripotent state and cells will start differentiating or die. To achieve this, cells should be cultured in fresh feeder and iPSC medium that should be replenished regularly (ideally every 24 hr), and cells should be passaged when reaching approximately 80% to 90% confluency.

At the virus-producing step, it is advisable to transfect the virus-producing cells with a suitable control to monitor the transfection efficiency. For this purpose, generally a lentiviral GFP or pMXs retroviral GFP vector can be used. This can be assessed by flow cytometry of the virus-producing cells. We routinely obtain 70% to 80% efficiency. High-efficiency transfection is crucial for iPSC induction.

For viral overexpression of transcription factors, it is important to use a high-titer virus with a high infection rate. Preferably use fresh virus and avoid multiple freeze/thaw cycles, as this

decreases the viral titer. When choosing a viral system, it is recommended to use a doxycycline-inducible lentiviral system, since this provides better control over transgene expression and silencing.

It is imperative for cell health and reprogramming efficiency to replenish with fresh medium regularly. If the cell medium color changes to yellow (indicator of medium acidity), change the medium immediately. It is always better to pre-feed 1 hr before passaging and/or freezing, since this reduces excessive cell death. For long-term storage, keep frozen cells in the gas phase of a liquid nitrogen tank. The recovery of iPSCs after freezing is about 50%.

Troubleshooting

This protocol has proven to have an extremely high success rate in our hands, generating good iPSC colonies almost 100% of the time. In case no or inefficient reprogramming is achieved, there are a few issues that need to be considered before repeating. First, make sure to use fresh fibroblasts of low passage number (passage <3). High-passage-number cells have significantly lower reprogramming efficiency. Second, it is of eminent importance that the virus used for reprogramming be of good quality in order for there to be a good viral infection rate. Only use fresh virus and limit freeze/thaw cycles to not more than one. It is worth considering checking whether the viral transfection rate in virus-producing cells is efficient by using a GFP control vector (see Critical Parameters for details). In case of small numbers of colonies after viral infection, one can also increase the MOI empirically to increase reprogramming efficiency.

If iPSCs are showing decreased survival or are not attaching to the feeder after passaging or colony picking, it may be due to excessive exposure to trypsin or vigorous pipetting. Make sure to pipet the cells gently when triturating and limit the exposure time to trypsin.

If the colonies are differentiating or disintegrating, this may be due to partial reprogramming or improper culturing conditions. Pick only iPSC clones of good morphology. Make sure to use freshly prepared medium at all times, with fresh reagents, to support the pluripotent conditions (especially LIF). Proof of pluripotency can be checked by analyzing the quantitative expression of pluripotent markers, teratoma assay in SCID mice and by chimeric mouse assays. It is advisable to do regular periodic mycoplasma testing (once a month) to rule out an underlying mycoplasma infection as the reason for unsuccessful reprogramming or culturing.

Anticipated results

The infection efficiency of fresh high titer virus in fibroblasts is more than 60%. Efficiency of iPSC clone derivation from infected (integrated) is approximately 1% for MEFs and 0.1% for TTFs.

After 24 hr of viral infection, many fibroblasts may die due to viral cytotoxicity, but a sufficiently large number of fibroblasts may proliferate abundantly and become confluent within 2 days. High-quality iPSC clones mimic the ESC-like proliferation and morphology (e.g., round shape, large nucleoli and translucent cytoplasm). Poor-quality iPSC clones do not exhibit ESC morphology and will not proliferate continuously when cultured in mouse iPSC medium.

Time considerations

Isolation and culture of embryonic fibroblasts generally takes up to 10 days.

Pause point: The mitomycin C-treated mouse feeder cells can be kept in the incubator for up to a week before use, but every 3 days fresh MEF medium should be replenished.

Virus can be harvested within 2 days after transfecting virus-producing cells with viral plasmids. It is recommended to do this in advance, since reprogramming virus can be stored at −80°C up to 1 year until ready for use. After successful infection, colonies start forming after 10 to 21 days, depending on the species. Picking and establishing iPSC colonies can take an additional 3 to 4 weeks.

It is recommended to freeze early-passage iPSCs (immediately after complete doxycycline weaning in case of using an inducible viral system). If necessary, cells can also be cultured and frozen at early passage for future use. For long-term storage, keep frozen cells in the gas phase of a liquid nitrogen tank. The recovery of iPSCs after freezing is about 50%.

Acknowledgments

This work was supported by grants from the Netherlands Organisation for Scientific Research (M.C.E.), NIH (OD004411, HL081086, HL100408 to S.M.W.) and the Harvard Stem Cell Institute (S.M.W.).

References

1. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;126:663-676.
2. Donovan J, Brown P. Euthanasia. *Curr Protoc Immunol.* 2006;73:1.8.1-1.8.4.
3. Park IH, Lerou PH, Zhao R, Huo H, Daley GQ. Generation of human- induced pluripotent stem cells. *Nat Protoc.* 2008;3:1180-1186.
4. Li X, Meng G, Krawetz R, Liu S, Rancourt DE. The ROCK inhibitor Y-27632 enhances the survival rate of human embryonic stem cells following cryopreservation. *Stem Cells Dev.* 2008;17:1079-1085.
5. Wu SM, Hochedlinger K. Harnessing the potential of induced pluripotent stem cells for regenerative medicine. *Nat Cell Biol.* 2011;13:497-505.
6. Hogan BL. Changes in the behavior of teratocarcinoma cells cultivated in vitro. *Nature.* 1976;263:136-137.
7. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature.* 1981;292:154-156.
8. Nagy A, Gócza E, Diaz EM, Prideaux VR, Iványi E, Markkula M, Rossant J. Embryonic stem cells alone are able to support fetal development in the mouse. *Development.* 1990;110:815-821.
9. Eggan K, Akutsu H, Loring J, Jackson-Grusby L, Klemm M, Rideout WM 3rd, Yanagimachi R, Jaenisch, R. Hybrid vigor, fetal overgrowth, and viability of mice de- rived by nuclear cloning and tetraploid embryo complementation. *Proc Natl Acad Sci U S A.* 2001;98:6209-6214.
10. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998;282:1145-1147.
11. Gurdon JB. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *J Embryol Exp Morphol.* 1962;10:622-640.
12. Campbell KH, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. *Nature.* 1996;380:64-66.
13. Chan AW, Dominko T, Luetjens CM, Neuber E, Martinovich C, Hewitson L, Simerly CR, Schatten GP. Clonal propagation of primate offspring by embryo splitting. *Science.* 2000;287:317-319.
14. Byrne JA, Pedersen DA, Clepper LL, Nelson M, Sanger WG, Gokhale S, Wolf DP, Mitalipov SM. Producing primate embryonic stem cells by somatic cell nuclear transfer. *Nature.* 2007;2:497-502.
15. Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R, Plath K, Hochedlinger K. Directly reprogrammed fibroblasts show global epigenetic re-modeling and widespread tissue contribution. *Cell Stem Cell.* 2007;1:55-70.
16. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature.* 2007;448:313-317.
17. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature.* 2007;448:318-324.
18. Boland MJ, Hazen JL, Nazor KL, Rodriguez AR, Gifford W, Martin G, Kupriyanov S, Baldwin KK. Adult mice generated from induced pluripotent stem cells. *Nature.* 2009;461:91-94.
19. Kang L, Wang J, Zhang Y, Kou Z, Gao S. iPS cells can support full-term development of tetraploid blastocyst-complemented embryos. *Cell Stem Cell.* 2009;5:135-138.
20. Zhao XY, Li W, Lv Z, Liu, L., Tong, M., Hai, T., Hao, J., Guo, C.L., Ma, Q.W., Wang, L., Zeng, F., and Zhou, Q. iPS cells produce viable mice through tetraploid complementation. *Nature.* 2009;461:86-90.
21. Stadtfeld M, Apostolou E, Akutsu H, Fukuda A, Follett P, Natesan S, Kono T, Shioda T, Hochedlinger K. Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. *Nature.* 2010;465:175-181.

22. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. *Science.* 2008;322:945-949.

23. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science.* 2008;322:949-953.

24. Jia F, Wilson KD, Sun N, Gupta DM, Huang M, Li Z, Panetta NJ, Chen ZY, Robbins RC, Kay MA, Longaker MT, Wu JC. A nonviral minicircle vector for deriving human iPS cells. *Nat Methods.* 2010;7:197-199.

25. Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Schöler HR, Duan L, Ding, S. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell.* 2009;4:381-384.

26. Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schlaeger TM, Rossi DJ. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell.* 2010;7:618-630.

27. Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature.* 2009;458:771- 775.

28. Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M, Isacson O, Jaenisch R. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell.* 2009;136:964-977.

29. Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hämäläinen, R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A. PiggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature.* 2009;458:766- 770.

30. Yusa K, Rad R, Takeda J, Bradley A. Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat Methods.* 2009;6:363-369.

31. Carey BW, Markoulaki S, Hanna J, Saha K, Gao Q, Mitalipova M, Jaenisch R. Reprogramming of murine and human somatic cells using a single polycistronic vector. *Proc Natl Acad Sci U S A.* 2009;106:157-162.

32. Sommer CA, Stadtfeld M, Murphy GJ, Hochedlinger K, Kotton DN, Mostoslavsky G. Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells.* 2009;27:543-549.

33. Li W, Wei W, Zhu S, Zhu J, Shi Y, Lin T, Hao E, Hayek A, Deng H, Ding S. Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell.* 2009;4:16-19.

34. Kobayashi T, Yamaguchi T, Hamanaka S, Kato-Itoh M, Yamazaki Y, Ibata M, Sato H, Lee YS, Usui J, Knisely AS, Hirabayashi M, Nakauchi H. Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. *Cell.* 2010;142:787-799.

35. Li Y, Cang M, Lee AS, Zhang K, Liu D. Reprogramming of sheep fibroblasts into pluripotency under a drug-inducible expression of mouse-derived defined factors. *PLoS One.* 2011;6:e15947.

36. Ezashi T, Telugu BP, Alexenko AP, Sachdev S, Sinha S, Roberts RM. Derivation of induced pluripotent stem cells from pig somatic cells. *Proc Natl Acad Sci U S A.* 2009;106:10993-10998.

37. Liu H, Zhu F, Yong J, Zhang P, Hou P, Li H, Jiang W, Cai J, Liu M, Cui K, Qu X, Xiang T, Lu D, Chi X, Gao G, Ji W, Ding M, Deng H. Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. *Cell Stem Cell.* 2008;3:587-590.

38. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka, S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131:861-872.

39. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science.* 2007;318:1917- 1920.

40. Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley, GQ. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature.* 2008;451:141-146.

41. Friedrich Ben-Nun I, Montague SC, Houck ML, Tran HT, Garitaonandia I, Leonardo TR, Wang YC, Charter SJ, Laurent LC, Ryder OA, Loring JF. Induced pluripotent stem cells from highly endangered species. *Nat Methods.* 2011;8:829-831.