Chapter 7

Reprogramming Human Fibroblasts to Induced Pluripotent Stem Cells Using the GFP-Marked Lentiviral Vectors in the Chemically Defined Medium

Zhicheng Shao, Ricardo Raúl Cevallos, and Kejin Hu

Abstract

Much investigation is needed to understand the underlying molecular mechanisms of iPSC reprogramming and to improve this technology. Lentivirus-mediated iPSC reprogramming remains the most effective method to study human pluripotency reprogramming. iPSC production is more efficient and consistent in the chemically defined medium. Fibroblasts are the most common starting cells for iPSC generation. Here, we provide a detailed protocol for iPSC generation from human fibroblasts using the GFP-expressing lentiviral vectors in the chemically defined medium.

Key words Human induced pluripotent stem cells, Human iPSC, Fibroblasts, Lentiviral vectors, Chemically defined medium, Pluripotent reprogramming, Co-expression of GFP

1 Introduction

Human somatic cells can be converted into pluripotent stem cells (PSCs) by ectopic expression of several reprogramming factors [1, 2]. Such factor-induced PSCs (iPSCs) from patient somatic cells have great potentials in regenerative medicine because human iPSCs (HiPSCs) have the same properties as human embryonic stem cells do. iPSC reprogramming is still inefficient, slow, stochastic, incomplete, and aberrant [3] although significant improvements have been made over the last 13 years. The molecular mechanisms for iPSC reprogramming remain a black box to scientists. Much research is needed to understand this process and to improve the iPSC technology.

Various protocols have been developed to generate human transgene-free iPSCs using non-integrating methods [4–6], but in the research setting virus-based iPSC reprogramming remains the most effective approach for investigation of iPSC reprogramming because of the low efficiency and technical limitations of the

Kejin Hu (ed.), *Nuclear Reprogramming: Methods and Protocols*, Methods in Molecular Biology, vol. 2239,
https://doi.org/10.1007/978-1-0716-1084-8_7,

non-integrating systems [7–9]. Human fibroblasts are the most common starting cells for iPSC generation because of their easy access and simple maintenance. Here, we provide a protocol to reprogram human fibroblasts into iPSCs in the chemically defined medium using the lentiviral vectors to deliver the reprogramming factors. We use the chemically defined medium because it is more efficient and consistent [10]. The defined medium will make it easier to screen for new reprogramming genes and reprogramming-enhancing chemicals [8, 9]. We use the lentiviral constructs with the EF1α promoter to drive expression of the reprogramming factors and co-expression of GFP mediated by the P2A peptide [6, 9]. Use of the EF1α promoter avoids premature silencing of the reprogramming factors as compared to the CMV promoter. The vector design with a GFP co-expression with the reprogramming factors has the following advantages: (1) the functional titers of the reprogramming viruses can be determined easily using flow cytometry. This is critical because proper stoichiometry of the reprogramming factors is an important parameter for efficient reprogramming. (2) The transfection and transduction efficiency can be monitored readily using flow cytometry and/or fluorescent microscopy. (3) Silencing status of the transgenes in the iPSC colonies can be judged readily by the levels of GFP expression. Silencing of the integrated reprogramming factors in the reprogrammed iPSCs is a hallmark of complete reprogramming. Because there is a GFP co-expression with each of the three reprogramming factors in our constructs (Fig. 1a), lack of expression for multiple copies of GFP transgenes integrated in the generated iPSCs will effectively indicate sufficient silencing for all of the transgenes.

This chapter will provide step-by-step procedures for: (1) packaging of the reprogramming lentiviral particles (Subheading 3.1); (2) Concentration of the reprogramming lentiviral particles produced (Subheading 3.2); (3) functional titration of the reprogramming viruses (Subheading 3.3); (4) generation of human iPSCs from fibroblasts in the chemically defined medium using the lentiviral reprogramming cocktails (Subheading 3.4); and (5) expansion/maintenance and long-term storage of the established iPSCs (Subheading 3.5). Our reprogramming protocol uses only three of the four Yamanaka factors since MYC does not have any beneficial effect on iPSC reprogramming in the chemically defined medium [9, 11].

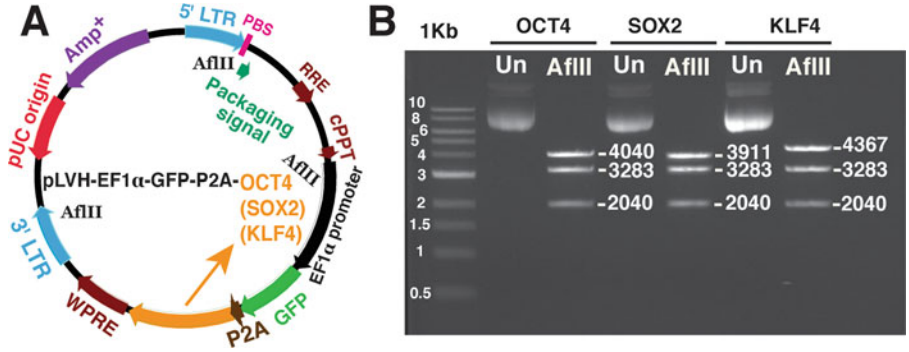

Fig. 1 Lentiviral reprogramming factors with co-expression of GFP. (**a**) Schematic map for the lentiviral reprogramming constructs with GFP co-expression mediated by a P2A peptide. *LTR,* long terminal repeat; *PBS,* primer binding sequence; *RRE,* Rev response element; *WPRE,* woodchuck hepatitis virus post-transcriptional regulatory element; *cPPT,* central polypurine tract; *EF1α,* human EF1α promoter; *pLVH,* lentiviral vector plasmids in Hu laboratory. The locations of the three AflII sites are indicated. (**b**) Confirmation of plasmid integrity for the lentiviral reprogramming constructs. The sizes for each fragment in base pair (bp) are indicated in the gel image. The fragment sizes in kilobase pair (kb) of the 1 kb marker are also indicated. The quality of the plasmids can also be judged by the predominant bands for the supercoiled plasmids without restriction digestion (Un lanes). *Un,* uncut

2 Materials

All reagents should be cell culture grade. Aseptic practice should be observed for all steps. Users of this protocol should have received specific training in handling pathogenic agents. This protocol should be conducted in a biosafety level 2 (BSL2) laboratory because lentiviral vectors are used.

2.1 Reagents

1. Lenti-X 293T, for lentivirus packaging.
2. HeLa cells, for lentiviral titration.
3. Hexadimethrine bromide (Polybrene).
4. Human foreskin BJ fibroblasts or other human fibroblasts as starting cells for iPSC generation.
5. TrypLE.
6. hESC-qualified Matrigel.
7. ROCK inhibitors, Y-27632 or thiazovivin.
8. Polyethylene glycol 6000 (PEG-6000).
9. Tris–HCl buffer: 50 mM Tris, pH 7.4.
10. Bleach.
11. Maxiprep Kits.

2.2 Plasmids

1. Envelope plasmid pMD2.G (*see* **Note 1**).
2. Packaging plasmid psPAX2.

3. pLVH-EF1α-GFP-P2A-OCT4 (Addgene, cat# 130692) (Fig. 1a) (*see* **Note 2**).
4. pLVH-EF1α-GFP-P2A-SOX2 (Addgene, cat# 130693).
5. pLVH-EF1α-GFP-P2A-KLF4 (Addgene, cat# 130694).

2.3 Key Equipment

1. Centrifuge with capacity of refrigeration.
2. Flow cytometer.
3. −80 °C freezer.
4. Freezing container for initial freezing of iPSCs.
5. Liquid nitrogen storage tank for long-term storage of iPSCs.
6. CO_2 incubator (*see* **Note 3**).
7. Laboratory biosafety cabinet.
8. Nanodrop.
9. Sharp container.
10. Personal protective equipment (PPE): gloves, masks, laboratory coats, and safety goggles.
11. Portable liquid-N_2 tank or styrofoam box for snap-freezing of lentiviral preparations.
12. Test-tube cooler for thawing Matrigel inside a 4 °C refrigerator.
13. 0.2-μm filters for sterilization of culture media.
14. 0.45-μm filters with receiver flasks, for virus collection.
15. FACS tubes with cell strainer caps, strainer pore size 35 μM.

2.4 Media

1. Fibroblast growth medium: Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, 1× penicillin-streptomycin (100 U/mL penicillin, and 100 μg/mL streptomycin), 0.1 mM Minimum Essential Medium (MEM) Non-Essential Amino Acids (NEAA), and 4 ng/mL human basic fibroblast growth factor (bFGF, also known as FGF2) (*see* **Note 4**).
2. E8 medium (pH 7.4) for iPSC maintenance: DMEM/nutrient mixture F-12 (F-12) (DMEM/F-12), 1.74 g/L $NaHCO_3$ (*see* **Note 5**), 64 mg/L L-ascorbic acid 2-phosphate sesquimagnesium (*see* **Note 6**), 13.6 μg/L sodium selenium, 4 ng/mL FGF2, 20 μg/mL insulin, 10 μg/mL transferrin, and 2 μg/L TGFβ1 (*see* **Note 7**).
3. 293T growth medium: DMEM, 10% FBS, 1× MEM Non-Essential Amino Acid (MEM NEAA), 1× penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin).

4. HeLa growth medium: DMEM, 10% FBS, 1× penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin).
5. Reprogramming E7 medium: E8 medium without TGFβ1, and with addition of sodium butyrate at the final concentration of 100 μM.
6. iPSC freezing medium: E8 medium supplemented with 10% dimethyl sulfoxide (DMSO).

2.5 Coated Culture Dishes/Plates

1. Matrigel-coated dishes for reprogramming or iPSC passaging: Take one aliquot of Matrigel from the −80 °C freezer one day before use, and thaw the Matrigel overnight inside a portable test-tube cooler inside a 4 °C refrigerator or on ice in a small ice box with a cover inside a 4 °C refrigerator. Prepare the diluted solution of Matrigel on ice with ice-cold DMEM/F-12 media at the concentration of 75–150 μg/mL (*see* **Note 8**). Add 2.5 mL of the diluted working Matrigel solution into a 60-mm dish and incubate at 37 °C at least for 30 min before use. Just before use, take the dish with coating Matrigel solution out of the 37 °C incubator, remove the Matrigel solution as completely as possible by aspiration, and immediately add the appropriate amount of media containing the iPS cells or the reprogramming fibroblasts (see the procedure below).
2. Collagen-coated dishes for lentiviral packaging: Dissolve collagen I powder in 0.02 N acetic acid solution to a final concentration of 50 μg/mL collagen. Add 15 mL of the collagen-I solution into one 150-mm tissue culture dish and incubate for 30 min at 37 °C (5 $\mu g/cm^2$). Remove the solution (*see* **Note 9**) and wash the dish with 12 mL of DPBS. Remove as much as possible of DPBS liquid using a Pasteur pipet attached to a vacuum line, and then dry the coated dish by incubating at 37 °C for 1–2 h before use.

2.6 Buffers and Solutions

1. 2× BES-buffered saline (BBS), pH 7.07: 50 mM *N*,*N*-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 mM NaCl, and 1.5 mM $Na_2HPO_4 \cdot 7H_2O$. Mix 16.36 g of NaCl, 10.65 g of BES, and 0.402 g of $Na_2HPO_4 \cdot 7H_2O$. Add pure water up to 900 mL. Dissolve the chemicals, titrate to pH 7.07 with 1 M NaOH at room temperature, and bring the volume to 1 L with pure water. Sterilize by filtration using a 0.2-μm filter. Aliquot into 15-mL sterile tubes and store the BBS aliquots at −20 °C.
2. 2.5 M $CaCl_2$ solution: Dissolve 36.75 g $CaCl_2 \cdot 2H_2O$ in 100 mL ultrapure water. Sterilize with a 0.2-μm membrane filter. Aliquot at 10 mL/tube and store the aliquots at −20 °C.

3. Tris Buffered Saline (TBS) for storage of concentrated viruses: 50 mM Tris–HCl, 150 mM NaCl, pH 7.4.
4. EDTA iPSC-dissociation solution: 0.5 μM ethylenediaminetetraacetic acid (EDTA), 0.18% NaCl (w/v) in PBS. Add 1 mL of 0.5 M EDTA (pH 8.0), and 1.8 g of NaCl into 1 L of calcium/magnesium-free PBS. Sterilize the solution by autoclave or by filtration. Store at room temperature or at 4 °C.
5. Fluorescence-Activated Cell Sorting (FACS) buffer: FACS buffer is the standard PBS buffer (without calcium and magnesium) supplemented with 2% FBS (v/v), 2.5 mM EDTA, and 0.05% sodium azide. Sterilize the buffer by filtration using 0.2-μm membrane filters.

3 Methods

3.1 Packaging Reprogramming Lentiviruses (See Note 10)

1. Prepare high-quality plasmids using a Maxiprep kit as instructed by the manufacturer for all of the five plasmids (*see* **Note 11**). Measure the concentration and evaluate the quality of the plasmid preparations using Nanodrop. Make sure the transfer plasmids are intact by running a standard agarose gel of the digested plasmids with the restriction enzyme AflII (Fig. 1b) (*see* **Note 12**).
2. Into one collagen I-coated 150-mm dish, seed 1.5×10^7 Lenti-X™ 293T cells in 25 mL of the 293T growth medium, and culture at 37 °C, 5% CO_2, for 24 h.
3. The next day, replace the spent media with 20 mL of pre-warmed fresh 293T growth media 1–3 h prior to transfection.
4. For a 150-mm dish, prepare 60 μg of total plasmid DNA mix at a ratio of 10.5 μg envelop plasmid pMD2.G, 19.5 μg packaging plasmid psPAX2, and 30 μg transfer vector (pLVH-EF1-α-GFP-P2A-OCT4, pLVH-EF1α-GFP-P2A-SOX2, or pLVH-EF1α-GFP-P2A-KLF4), and mix with 150 μL of the 2.5 M $CaCl_2$ solution (*see* **Note 13**). Bring the DNA/calcium solution to 1.5 mL using the ultrapure water and mix well in a sterile 15-mL tube.
5. Mix the DNA/calcium preparation with an equal volume (1.5 mL each, 1:1) of 2× HBS (pH 7.07); pipette 15–25 times gently using a 5-mL pipette.
6. Immediately, add the DNA complexes dropwise into the cell culture media and swirl gently to mix with the culture media.
7. Incubate the cells for 16–18 h at 37 °C in 5% CO_2.

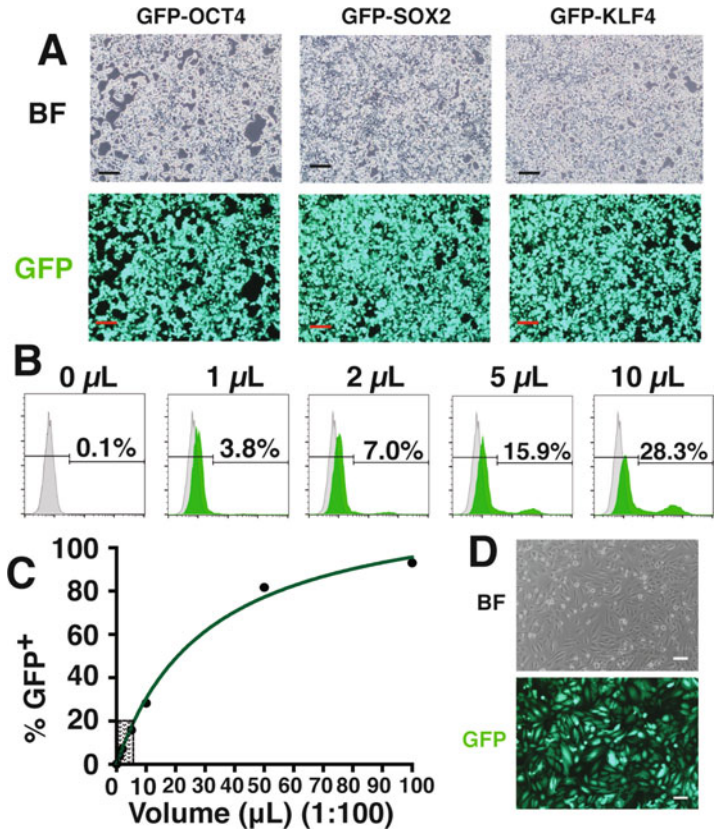

Fig. 2 Viral preparation and titration of the lentiviral reprogramming factors. (**a**) Representative Bright Field (BF) and Fluorescence (GFP) microscopy images of HEK293T cells, 24 h post transfection with the three lentiviral reprogramming factors as indicated. Scale bar = 50 μM. (**b**) Representative histograms of the titration analyses by flow cytometry of HeLa cells. Four different virus volumes from the 1:100 dilution preparation were analyzed along with an uninfected control. (**c**) Saturation curve model of percentage of infected cells versus volume of viruses from the 1:100 dilution preparation. The highly linear region below 20% of GFP^+ cells (boxed) should be used for titer calculation. (**d**) Bright Field (BF) and Fluorescence (GFP) microscopy images of HeLa cells transduced with 1 μL of concentrated viruses of the OCT4 reprogramming factor. Scale bar = 100 μM

8. After 16–18 h, remove the transfection media, and then gently add 25 mL of fresh 293T growth media to the dish. Continue to culture the cells at 37 °C, 5% CO_2. After 24 h post transfection, check the transfection using fluorescence microscopy. Make sure the transfection efficiency is greater than 90% in order to achieve a high titer (Fig. 2a).
9. Collect the virus-containing media between 48 and 72 h post transfection.

3.2 Concentration of Lentiviral Particles by PEG Precipitation

1. Centrifuge the lentivirus-containing media collected above at 3000 × *g* for 10 min at 4 °C to remove the cells debris, and then further clear the viruses of cell debris by filtration using 0.45-μm filters.
2. Transfer the viral supernatants to a new sterile 50-mL centrifuge tube, then add the appropriate amounts of 50% PEG-6000 stock solution and 4 M NaCl solution so that a final concentration of 8.5% PEG-6000, and 0.4 M NaCl will be obtained (*see* **Note 14**).
3. Precipitate the viruses at 4 °C for 3–5 h. Mix the viruses every 20–30 min.
4. Centrifuge at 3,900 × *g* for 30 min to 1 h at 4 °C (*see* **Note 15**).
5. Carefully decant the supernatant and add pre-cooled TBS at 1% the volume of the original virus-containing media (100× dilution) into the tube (*see* **Note 16**).
6. Resuspend the viral pellets by pipetting up and down, and then vortex the tubes vigorously for 20–30 s.
7. Fill a portable liquid-N_2 tank (or a styrofoam container or an ice bucket) with liquid N_2.
8. Using aerosol-resistant filtered sterile pipette tips to transfer the lentiviral suspension into 500-μL microcentrifuge tubes in an aliquot of 10 μL each, and immediately throw each viral aliquot into liquid N_2 prepared at **step 7** above (*see* **Note 17**).
9. Store the viruses at −80 °C immediately after completion of aliquoting.

3.3 Functional Titration of Lentiviral Particles by Flow Cytometry

1. Seed HeLa cells using the Hela growth medium into one 60-mm tissue culture dish. Culture cells until confluence.
2. Passage the cells into at least 28 wells of 24-well plates at a density of 2.5×10^4 cells per well.
3. The next day, quantify the cell number from two of these wells. First, detach the cells with 250 μL per well of TrypLE for 3 min. Stop the reaction with 250 μL of the Hela growth medium. Combine the cell suspension from the two wells (final volume of 1 mL), mix well, and take 10 μL to count the cells with a hemocytometer. Divide the calculated cell number by 2 in order to obtain the cell number per well. Keep a record of the cell number per well for titer calculation later.
4. Thaw one 10 μL-aliquot per lentiviral vector (pLVH-EF1-α-GFP-P2A-OCT4, pLVH-EF1α-GFP-P2A-SOX2, and pLVH-EF1α-GFP-P2A-KLF4) on ice. Take 5 μL of concentrated viruses and dilute with 495 μL of PBS (dilution factor = 100). Mix well and keep the viruses on ice until use (*see* **Notes 18** and **19**).

5. Add 15 mL of the HeLa medium in a 50-mL falcon tube and add Polybrene at 4 μg/mL.
6. Remove the medium from the plate with the HeLa cells and then transfer 500 μL of the Polybrene-supplemented medium to each well.
7. Use four different volumes of the diluted virus: 1, 2, 5, and 10 μL per well with two replicates (8 wells per reprogramming factor). Leave at least 1 well un-transduced as a control for flow cytometry analysis. Mix well (*see* **Note 18**).
8. Incubate the plate overnight at 37 °C in 5% CO_2.
9. The next day, remove the medium carefully and replace it with fresh HeLa medium.
10. On day 3, observe the cells using fluorescent microscope and make sure the Hela cells are expressing GFP (Fig. 2d). After 48 h, remove the medium and detach the cells by TrypLE treatment for 3 min at 37 °C.
11. Inactivate the reaction with 1 mL of FACS buffers or the Hela growth medium. Filter the cells into round-bottom tubes with a cap cell strainer.
12. Centrifuge at 400 × *g* for 5 min at 4 °C.
13. Discard the supernatant and resuspend the cell pellet with 1 mL of FACS buffer.
14. Run flow cytometry to analyze for the percentage of GFP-positive cells.
15. Analyze the data using a flow cytometry software such as FlowJo (Fig. 2b). Consider only the values of two or more volumes that fall between 2% and 20% of GFP-positive cells (Fig. 2b, c) (*see* **Note 20**).
16. Calculate the titer in Transduction Units per milliliter (TU/mL) using the following formula:

$$\mathrm{TU/mL} = \frac{N \times F_{\mathrm{GFP}} \times D_{\mathrm{f}} \times 1000}{V_{\mu\mathrm{L}}}$$

$V_{\mu L}$ = volume of diluted virus in μL.
N = cell numbers at the time of transduction.
F_{GFP} = fraction of GFP-positive cells expressed as percentage.
D_f = is the dilution factor. In this case, it is 100 (*see* **Note 19**).

3.4 Conversion of Human Fibroblast into iPSCs

1. Culture human foreskin BJ fibroblasts in the fibroblast medium (*see* **Note 21**).
2. Seed BJ fibroblasts into a 6-well plate at 1×10^5 cells per well.

3. Twenty-four hours post plating, premix the OSK viruses (OCT4 at 8 Multiplicity Of Infection (MOI); SOX2 at 5 MOI; KLF4 at 5 MOI), and add the viral cocktails into the cells to be reprogrammed using fibroblast medium supplemented with 4 μg/mL of Polybrene (*see* **Note 22**). For an accurate transduction, quantify the cell numbers at the time of transduction (N) from 1 well of the same plate. Calculate the required amount of viruses using the following formula: μL of viruses $= \frac{N \times \mathrm{MOI} \times 1000}{\mathrm{TU}}$.
4. The next morning, remove any residual virus particles by replacing the virus-containing medium with fresh fibroblast medium (*see* **Note 23**).
5. Twenty-four hours post transduction, reseed the transduced cells in the fibroblast growth media from one well of a 6-well plate into one Matrigel-coated 60-mm dish.
6. The next day, replace the fibroblast medium with the reprogramming E7 medium. Observe the reprogramming fibroblasts with fluorescent microscope and make sure that the cells are expressing GFP (Fig. 3a).
7. Refresh the E7 medium daily. Small clusters with epithelial morphology should be seen at around day 10 of reprogramming (Fig. 3b).
8. From day 12–14, start to use the E8 medium for continued reprogramming, as well as expansion and proliferation of the reprogrammed iPSCs.
9. On day 21–25 of reprogramming, iPSC colonies can be picked up for establishment of cell lines, or can be directly stained for analyzing the expression of pluripotency markers such as alkaline phosphatase or TRA-1-60. For establishment of iPSC lines, pick up colonies with low or negative-GFP signals (Fig. 3c) and place one picked colony into one Matrigel-coated well of a 12-well or a 6-well plate (*see* Subheading 2.5 for preparation of Matrigel-coated plates/dishes).
10. Expand the iPSCs that the GFP expression is progressively silenced using procedures described in Subheading 3.5 below (Fig. 3d) (*see* **Note 24**).

3.5 Culture Expansion and Freezing of the Established iPSCs

1. Refresh the E8 medium daily for the iPSCs established from one single colony at **steps 9** and **10** of Subheading 3.4, and culture iPSCs at 37 °C, 5% CO_2 until the neighboring colonies start to merge.
2. One hour before passaging, coat the plates with Matrigel and incubate the plates in a 37 °C incubator (*see* Subheading 2.5 for preparation of Matrigel-coated plates).

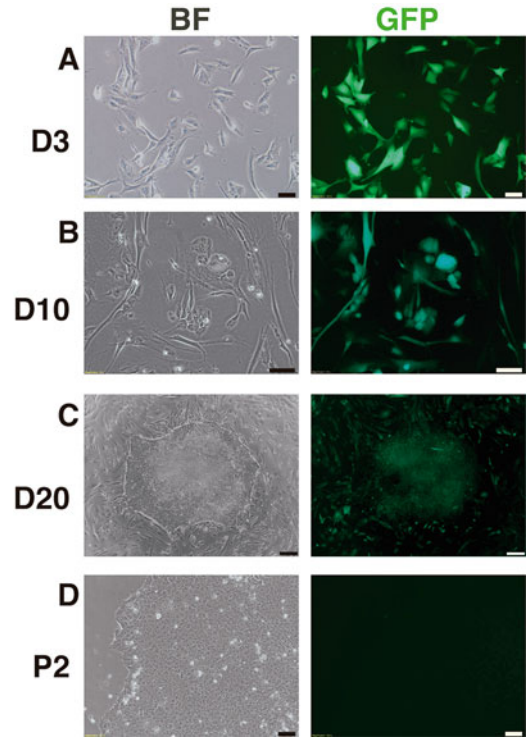

Fig. 3 Changes in morphology during reprogramming, and efficient silencing of transgenes in the established iPSCs. (**a**, **b**) Morphology of reprogramming fibroblasts on day 3 (**a**) and day 10 (**b**) (left panels), and transgene expression in the reprogramming fibroblasts on day 3 (**a**) and day 10 (**b**) as indicated by GFP expression (right panels). (**c**) A representative human iPSC colony (left panel) in the reprogramming vessel on day 20, and faint GFP expression (right panel) at this point indicating significant silencing in the iPS cells. (**d**) A representative iPSC colony at passage 2 showing complete silencing of transgenes in the established iPSC line (right panel). Scale bars, 50 μM in (**a**, **b** and **d**); 200 μM in (**c**). D3, day 3; P2, passage 2

3. Warm up E8 medium and the EDTA iPSC-dissociation solution to room temperature before passaging.
4. Remove and discard the spent E8 medium.
5. Add 1 mL of EDTA-dissociation solution into one well of a 6-well plate (0.5 mL for one well of a 12-well plate) and incubate in a 37 °C incubator for 3 min.
6. Add 1 mL per well of E8 medium for neutralization (0.5 mL for one well of a 12-well plate). Wash colonies off the plate by pipetting the medium.
7. Centrifuge the cells at 300 × *g* for 5 min and remove the supernatant medium by vacuum aspiration.

8. Plate the appropriate amount of cells in E8 media into the freshly prepared Matrigel-coated wells/dishes (*see* **Note 25**). To promote attachment of iPSCs to the vessel, ROCK inhibitor Y-27632 should be used at a final concentration of 10 μM (*see* **Note 26**).
9. Early the next morning, replace the spent media with fresh E8 media to remove the ROCK inhibitors (*see* **Note 27**).
10. Culture iPSCs for 5–7 days with daily change of media until 80% of confluence.
11. For freezing the cells, after **step 7**, resuspend the cells in the freezing medium at a concentration of 0.5–1 $\times$ 10^6 cells/mL and transfer 1 mL of cells into one cryogenic vial (*see* **Note 28**). Place the vials with iPSCs in a cryogenic container and immediately transfer the freezing container into a −80 °C freezer, and pre-freeze overnight.
12. The next day, transfer the iPS cells to liquid nitrogen for long-term storage (*see* **Note 29**).

4 Notes

1. The envelope and packaging plasmids are widely used. If these two plasmids are not available in the user's laboratory, plasmids pMD2.G and psPAX2 are available from Addgene with catalog numbers of 12259 and 12260, respectively.
2. These three reprogramming factors are used in this protocol because the co-expression of GFP provides a convenience for viral titration using flow cytometry, monitoring of transfection and transduction efficiency, as well as a marker for transgene silencing in the reprogrammed iPSCs. Other lentiviral reprogramming constructs available in Addgene or in the users' laboratory may be used.
3. Reprogramming can be carried out in an ordinary CO_2 tissue culture incubator. iPSC reprogramming can be enhanced in a hypoxia condition (5% oxygen) [12] if a hypoxia incubator is available.
4. FGF2 in the fibroblast growth medium is beneficial to reprogramming because it maintains a healthy state of fibroblasts and promotes the expression of OCT4 in fibroblasts even in the absence of reprogramming factors [13], but the FGF2 supplement is not essential for the maintenance of fibroblasts.
5. The osmolarity of E8 medium should be 340 mOsm and the pH is 7.4. Adjust the pH to 7.4 using 10 N NaOH. The amount of sodium bicarbonate are based on the specific DMEM/F-12 discussed below. The components of DMEM/F-12 vary in terms of HEPES, L-glutamine, and sodium

bicarbonate. We use the powder form with HEPES but without sodium bicarbonate (Thermo Fisher Scientific, Cat# 12400024). Use this DEME/F-12, we do not adjust the osmolarity and pH when we add the specified amount of sodium bicarbonate here. This is helpful when the osmometer is not available in the users' laboratory. The liquid form with HEPES can also be used (Thermo Fisher Scientific, Cat# 11330–032).

6. Vitamin C is not stable. This protocol uses the stabilized, synthetically derived version of vitamin C, L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate. Do not use the conventional vitamin C that might be available in the chemical stock of the users' laboratory. The molecular weights of these two versions are also different.
7. E8 medium can be prepared in large volume (e.g., 10 L) as base media without the unstable components. This base medium does not need sterilization by filtration if frozen immediately at −20 °C as 500-mL aliquots in used but clean plastic medium bottles. Do not use glass bottles for this purpose because glass bottles may crack during storage in very low temperature. The unstable components (FGF2, TGF beta, insulin, and transferrin) are added to prepare the fresh working complete E8 medium before use. The fresh complete E8 medium should be sterilized by filtration and stored at 4 °C. It is not encouraged to use the E8 media after 14 days of storage at 4 °C. To maintain potency of the E8 media, it is encouraged to use the genetically modified heat-stable FGF2. Antibiotics can be added, but are not necessary when cares are properly exerted. The E8 medium is commercially available at a higher cost.
8. Upon arrival, Matrigel stock should be aliquoted in a small volume, and stored at −80 °C. Avoid multiple rounds of thawing-freezing of Matrigel. Prepare Matrigel on ice with pipette tips and tubes that are pre-cooled at −20 °C. The diluted Matrigel should be used immediately. The coated plates/dishes should be used the same day. Coated plates/dishes should be stored at 4 °C if not used the same day. Coated plates/dishes stored at 4 °C should be warmed up at 37 °C for at least 30 min before use.
9. The collagen solution can be reused. Collect the coating collagen solution from the coated plates/dishes aseptically, and put it back into the collagen-solution bottle for reuse.
10. Your laboratory should have the approval to use lentiviral vectors. Extreme care should be observed when working with viral particles. Proper PPE should be used when handling lentiviral vectors. It is encouraged to wear double gloves when handling lentiviral particles. All laboratory materials that come into contact with viral preparations should be treated with bleach solution to kill the residual viruses.

11. Endotoxin-free Maxiprep kits are preferred but are not essential. Because of instability of the transfer plasmids, it is encouraged that the *E. coli* strain Stbl3, which reduces the chance of homologous recombination of the resident plasmids, should be used as the host strain of the lentiviral plasmids.
12. Because of repetitive sequences of the long terminal repeats (LTR), the transfer plasmids tend to lose one LTR. There is one AflII site in each of the two LTRs, and a third AflII site within the EF1α promoter sequence. A diagnostic restriction with AflII can quickly examine the integrity of the plasmids (Fig. 1b).
13. Multiple dishes can be transfected simultaneously to prepare a large batch of viruses. Scale up the DNA/calcium based on number of dishes for virus packaging.
14. For convenience of volume calculation, PBS buffer can be used to adjust the total volume of the precipitation preparation. Our experience is that the concentration of NaCl can vary within 0.3–0.4 M without negative effect on virus yields. But, the concentration of PEG-6000 should be 8.5% for good results.
15. To pellet the viral particles, centrifuge the virus-containing liquid for 10 min at 7,000 × *g* is recommended. But, many laboratories do not have a centrifuge that can run a rotor for large tubes (50- and 500-mL tubes) at 7,000 × *g*. We found that longer centrifugation at lower force can pellet the viruses. At 3,900 × *g*, 30 min to 1 h of centrifugation works well. After centrifugation, a white pellet should be visible. Always keep the tubes with the viruses on ice after this step.
16. Remove PEG-NaCl solution as completely as possible with a Pasteur pipet attached to a vacuum line. Most of the PEG-NaCl solution leftovers or drops stay on the wall of the tubes; therefore, it is recommended to roll the pipette tip around the wall to suck out the residual PEG-NaCl liquids. But, do not let the sucking pipette to touch the viral pellet. It is advised to keep the viral pellet in TBS buffer on ice for 1 h to promote resuspension of viruses.
17. Avoid high temperature during this procedure. Keep the master tube containing the resuspended viruses on ice during the entire aliquoting process. The viral aliquots should be snap-frozen immediately after each aliquot. To shorten the time for aliquoting, two lab mates can work together.
18. Use fresh pipette tips to distribute the different viruses when titrating different reprogramming factors. Treat the unused viruses with bleach solutions.
19. A dilution factor of 100 is generally appropriate for titers of around 10^8 TU/mL.

20. Our experience is that values below 20% of GFP^+ cells fall into the linear stage of the titration curve, in which it is less likely that there are more than one viral integrations per cell. Values below 1% may not predict the titers reliably. Therefore, we do not include values less than 2% or more than 20% in our calculation of viral titers.
21. For best results, use human fibroblasts at a low passage number as the starting cells for reprogramming. This protocol has been tested using fibroblasts with a passage number below 10.
22. The stoichiometry of the three reprogramming viruses is critical. Higher MOI for OCT4 is needed. We empirically use an MOI ratio of (8–10):5:5 for OCT4:KLF4:SOX2.
23. The spent media should be treated with bleach to kill any residual live viral particles. The sucking pipette tips used should also be decontaminated using bleach solution.
24. It may take several passages for the GFP to be completely silenced in iPSCs.
25. For the first EDTA passaging, the cells can be seeded into one well of a 6-well plate because there are not sufficient cells. For the established iPSC lines, the culture can be split every 5–7 days at 1:8–1:12 ratio.
26. In place of Y-27632, thiazovivin can be used at a final concentration of 1 μM [5].
27. ROCK inhibitors trigger differentiation of iPSCs, but iPSCs can recover if treatment of ROCK inhibitors is short. Do not include the ROCK inhibitors in the iPSC culture media for too long. To this end, passage iPSCs in the late afternoon before leaving the laboratory on the day of cell passage. The next day, the first thing to do is to remove the ROCK inhibitors by changing media.
28. With E8 system, the human iPSCs survival rate is much higher than that with the traditional feeder-based culture system. We usually freeze cells from one well of a 6-well plate into three vials. In the feeder culture system, cells from one well are frozen into one vial.
29. Do not store the cells at −80 °C for more than 24 h before transfer into liquid N_2.

Acknowledgments

The research in Hu laboratory is supported by NIH (1R01GM127411-01A1) and the American Heart Association (17GRNT33670780).

References

1. Takahashi K et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872. https://doi.org/10.1016/j.cell.2007.11.019
2. Yu J et al (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920. https://doi.org/10.1126/science.1151526
3. Hu K (2014) All roads lead to induced pluripotent stem cells: the technologies of iPSC generation. Stem Cells Dev 23:1285–1300. https://doi.org/10.1089/scd.2013.0620
4. Yu J et al (2009) Human induced pluripotent stem cells free of vector and transgene sequences. Science 324:797–801. https://doi.org/10.1126/science.1172482
5. Hu K et al (2011) Efficient generation of transgene-free induced pluripotent stem cells from normal and neoplastic bone marrow and cord blood mononuclear cells. Blood 117:e109–e119. https://doi.org/10.1182/blood-2010-07-298331
6. Hu K (2014) Vectorology and factor delivery in induced pluripotent stem cell reprogramming. Stem Cells Dev 23:1301–1315. https://doi.org/10.1089/scd.2013.0621
7. Kang L et al (2016) The universal 3D3 antibody of human PODXL is pluripotent cytotoxic, and identifies a residual population after extended differentiation of pluripotent stem cells. Stem Cells Dev 25:556–568. https://doi.org/10.1089/scd.2015.0321
8. Shao Z et al (2016) Reprogramming by de-bookmarking the somatic transcriptional program through targeting of BET bromodomains. Cell Rep 16:3138–3145. https://doi.org/10.1016/j.celrep.2016.08.060
9. Shao Z et al (2016) The acetyllysine reader BRD3R promotes human nuclear reprogramming and regulates mitosis. Nat Commun 7:10869. https://doi.org/10.1038/ncomms10869
10. Chen G et al (2011) Chemically defined conditions for human iPSC derivation and culture. Nat Methods 8:424–429. https://doi.org/10.1038/nmeth.1593
11. Xu Y et al (2013) Proliferation rate of somatic cells affects reprogramming efficiency. J Biol Chem 288:9767–9778. https://doi.org/10.1074/jbc.M112.403881
12. Yoshida Y, Takahashi K, Okita K, Ichisaka T, Yamanaka S (2009) Hypoxia enhances the generation of induced pluripotent stem cells. Cell Stem Cell 5:237–241. https://doi.org/10.1016/j.stem.2009.08.001
13. Jez M et al (2014) Expression and differentiation between OCT4A and its pseudogenes in human ESCs and differentiated adult somatic cells. PLoS One 9:e89546. https://doi.org/10.1371/journal.pone.0089546