Video Article

Reprogramming Human Somatic Cells into Induced Pluripotent Stem Cells (iPSCs) Using Retroviral Vector with GFP

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URL: https://www.jove.com/video/3804
DOI: doi:10.3791/3804

Keywords: Stem Cell Biology, Issue 62, Human iPS cells, iPSCs, Reprogramming, Retroviral vectors and Pluripotency

Date Published: 4/3/2012

Citation: Kim, K.Y., Hysolli, E., Park, I.H. Reprogramming Human Somatic Cells into Induced Pluripotent Stem Cells (iPSCs) Using Retroviral Vector with GFP. *J. Vis. Exp.* (62), e3804, doi:10.3791/3804 (2012).

Abstract

Human embryonic stem cells (hESCs) are pluripotent and an invaluable cellular sources for *in vitro* disease modeling and regenerative medicine[1]. It has been previously shown that human somatic cells can be reprogrammed to pluripotency by ectopic expression of four transcription factors (*Oct4, Sox2, Klf4* and *Myc*) and become induced pluripotent stem cells (iPSCs)[2-4] . Like hESCs, human iPSCs are pluripotent and a potential source for autologous cells. Here we describe the protocol to reprogram human fibroblast cells with the four reprogramming factors cloned into GFP-containing retroviral backbone[4]. Using the following protocol, we generate human iPSCs in 3-4 weeks under human ESC culture condition. Human iPSC colonies closely resemble hESCs in morphology and display the loss of GFP fluorescence as a result of retroviral transgene silencing. iPSC colonies isolated mechanically under a fluorescence microscope behave in a similar fashion as hESCs. In these cells, we detect the expression of multiple pluripotency genes and surface markers.

Video Link

The video component of this article can be found at https://www.jove.com/video/3804/

Protocol

1. Reprogramming by Retrovirus Expressing Reprogramming Factors

1. Human fibroblasts are cultured in fibroblast medium (10% FBS in DMEM with Pen/Strep).
2. One day before infection, plate $1x10^5$ human fibroblasts into one well of a 6-well plate.
3. Aspirate medium to remove dead cells and add 2 ml of fresh fibroblast medium. Add protamine sulfate at a final concentration of 5 µg/ml.
4. Carefully add the appropriate amount of each GFP-expressing virus corresponding to a multiplicity of infection (MOI) 5^5.
5. One day after infection, remove the viral supernatant, wash three times with 2 ml PBS, then add 2 ml fibroblast medium.
6. Three days after infection, check the GFP fluorescence and replenish the well with 2 ml fibroblast medium.
7. Four days after infection, plate $1x10^4$ /cm^2 of irradiated mouse embryonic fibroblast (MEFs) feeder cells in fibroblast medium onto a 10-cm Petri dish coated with 0.1% gelatin. Incubate at 37 °C overnight.
8. Five days after infection, detach infected human fibroblasts with 1 ml 0.05% typsin/EDTA for 5 minutes at 37 °C, and centrifuge for 5 minutes at 200 g. Aspirate the medium and resuspend the cells with 10ml of fibroblast medium. Transfer the cells into a pre-coated 10-cm plate.
9. After 24 hours, replace the medium with hESC culture medium (20% Knock-Out serum replacement, DMEM/F12, 0.1 ml Non-essential Amino Acids, 4 ng/ml bFGF, Pen/Strep/Glutamate, beta-merceptoethanol). Change the medium daily. ESC-like colonies will start to appear by day 20-27 after infection.

2. Isolation and Expansion of iPSCs

1. Under a fluorescence microscope, check for the absence of GFP fluorescence in a colony that shows a similar morphology to hESCs.
2. Using a 10 µl pipette, pick individual iPSC colonies and place them into one well of a gelatin- and MEF-coated 12-well plate and supplemented with hESC medium. Change medium daily.
3. For passaging, wash the plate with 1 ml of DMEM/F12, then add 0.5 ml of collagenase, and incubate for 10 minutes at 37 °C.
4. Wash the cells twice with DMEM/F12.
5. Add 2 ml of fresh hESC medium. Using a cell lifter, break up the colonies into small pieces and detach remaining cells from the plate.
6. Transfer the resuspended pieces of colonies into one well of a gelatin- and MEF- coated 6-well plate.

3. Immunofluorescence Analysis of Pluripotent Markers

1. Wash the cells three times with PBS and fix with 4% paraformaldehyde for 20 min at room temperature.

2. Gently wash the cells three times with PBS and permeabilize with 0.2% Triton X-100 in PBS for 30 min.
3. Block non-specific binding by incubating cells with 3% BSA in PBS for two hours.
4. Incubate the cells with primary antibody overnight at 4 °C.
5. Wash the cells three times with PBS and incubate the cells with specific secondary antibody for one hour at room temperature, shielding from light.
6. Wash the cells three times with PBS and add DAPI during the last wash followed by incubation at room temperature for 5 minutes.
7. Detect the staining with a fluorescence microscope.

4. Quantitative Real-time PCR Assay for the Pluripotent Markers

1. Isolate total RNA from the human iPSCs derived from human fibroblasts using Qiagen's RNeasy kit.
2. Synthesize the first-strand cDNA using SuperScript II Reverse Transcriptase.
3. Perform qPCR to detect pluripotency genes using primers previously reported.[6]

5. Representative Results

1. **Morphological change during reprogramming**
   We infected human fibroblasts BJ1 and Detroit 551 with a cocktail of retroviruses carrying OCT4, SOX2, KLF4 and MYC, and were able to detect morphological changes during reprogramming (**Figure 1**). Twenty-one days after infection, we recognize small iPSC colonies by their hESC-like morphology. Furthermore, we recognize iPSCs by the GFP fluorescence. Pluripotent stem cells, such as ESCs and iPSCs, express the molecular machinery to repress the proviral gene expression[7-9]. Our unique retroviral vector expresses GFP together with reprogramming genes by retroviral LTR. Thus, cells continuously expressing GFP are considered to express transgenes without proviral gene silencing. Faithfully reprogrammed iPSC colonies that acquire the pluripotency molecular network show the absence of GFP expression (**Figure 2**)[10].
2. **Characterization of pluripotency of human iPSCs**
   We analyzed colonies derived from Detroit-551 fibroblasts via immunohistochemistry with Tra-1-81, Tra-1-60, SSEA-4, SSEA-3, OCT4 and NANOG antibodies (**Table 2**). Successfully reprogrammed iPSCs express all of these markers (**Figure 3A**). We also analyzed gene expression via quantitative RT-PCR analysis. We observed that the expression of OCT4, SOX2, KLF4, MYC and NANOG was significantly increased compared to the parental fibroblast cells but commensurate with that of H9 hESCs (**Figure 3B**).

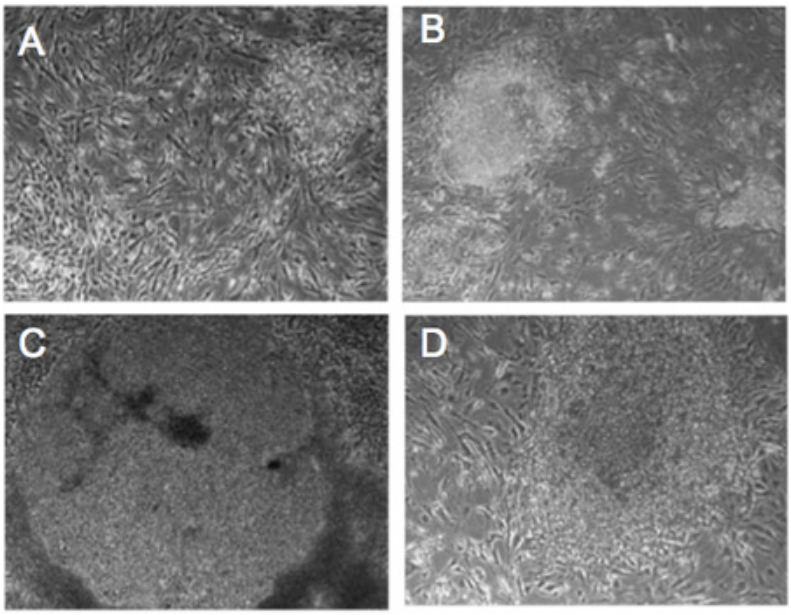

Figure 1. Morphological changes of retrovirus-infected human fibroblasts. (A-D) Progressive morphological change in colonies from Detroit-551 fibroblasts infected with reprogramming factors. Day 5 (A), Day 10 (B), Day 14 (C), day 21 (D). Cells show the hESC-like morphology after 21 days.

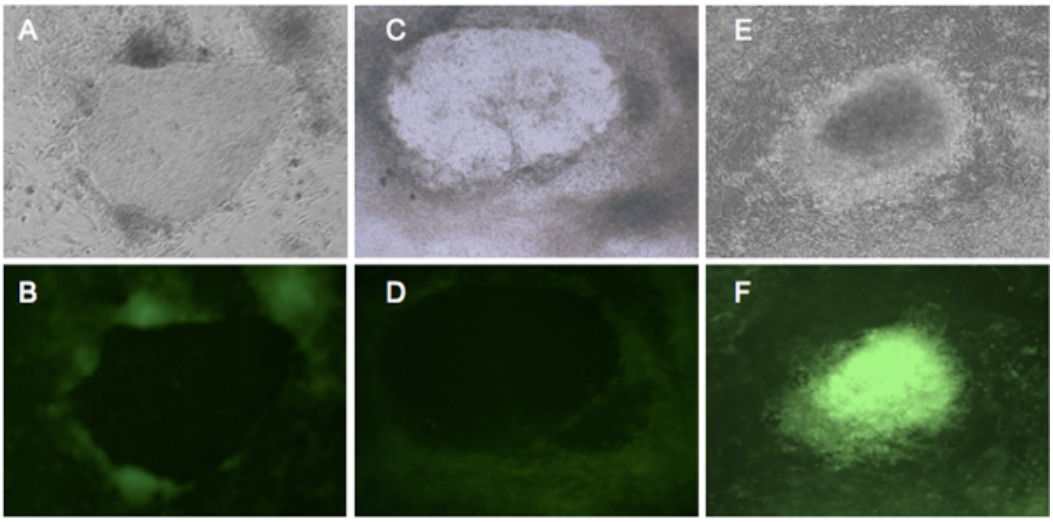

Figure 2. Representative GFP fluorescent expression in cells undergoing reprogramming. BJ and Detroit 551 fibroblasts were infected with retrovirus expressing four reprogramming factors[4], and incubated in hESC medium for four weeks. BJ fibroblasts (A, B) and Detroit 551 (C, D) show similar morphological. From day 21, GFP negative colonies start to form, which represent the bona fide iPSCs[10]. (E, F) show transformed Detroit 551 cells that have not undergone proper reprogramming. (A, C, E) colonies under phase contrast view. (B, D) properly reprogrammed cells that show the GFP silencing. (F) bright GFP expression from transformed colony.

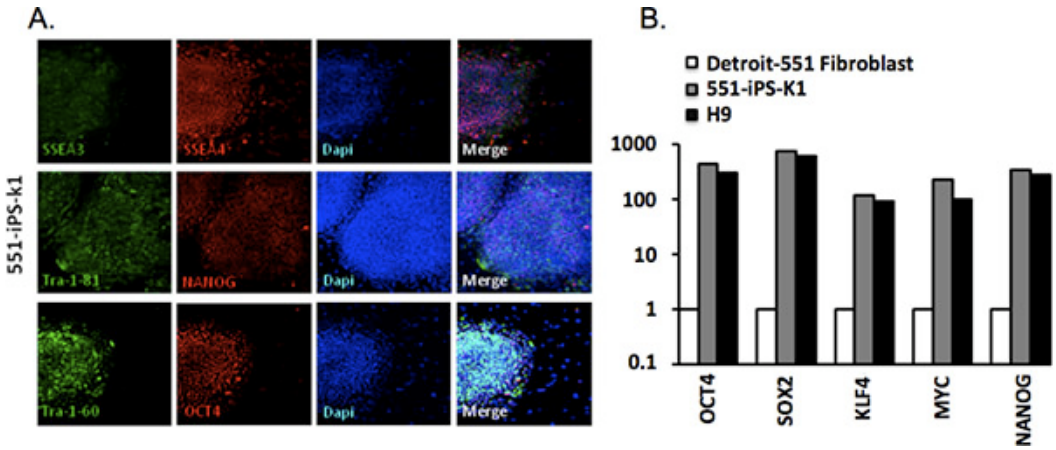

Figure 3. Characterization of the human induced pluripotent stem cells. (A) Human 551-iPS-K1 cell colonies express markers common to pluripotent cells. DAPI staining indicates the total cell content per field. (B) Quantitative real time-PCR (RT-qPCR) for expression of OCT4, SOX2, KLF4, MYC in parental fibroblast, 551-iPS-K1 iPSCs, and H9 human embryonic stem cells (hESCs). Data were normalized against β-actin housekeeping gene and plotted relative to the expression level in the parental fibroblast cells[4].

Discussion

Expression of four transcription factors reprograms human fibroblasts to iPSCs. Many attempts were made to generate human iPSCs using non-integrating or non-genetic approaches to generate clinically safe iPSCs. So far, these methods show extremely low efficiency and require further optimization to improve the reproducibility[11-14]. Retro- or lentiviral methods are readily used to derive and apply iPSCs for human *in vitro* disease models, which are less dependent on the safety issues caused by viral integration. Reprogramming method described here is available for the efficient derivation of human iPSCs. Selection of human iPSCs is primarily based on colony morphology that resembles human ESCs. More importantly, our method takes advantage of the feature of silencing of the retroviral long terminal repeats (LTR) in pluripotent stem cells[7,15]. The retroviral vector used in this protocol contains the GFP gene linked to the reprogramming factors via an Internal Ribosome Entry Sequence (IRES) and[5]. GFP expression is driven by proviral LTR. The fibroblasts infected with these viruses initially show the bright GFP expression. Once fully reprogrammed, iPSCs will lose GFP expression, which is easily visualized under a fluorescence microscope. In this protocol, we described the generation of iPSCs from fetal and neonatal fibroblasts (Detroit 551 and BJ1). However, this retroviral vector has been used to generate iPSCs from normal adult fibroblasts as well as a variety of patients with Mendelian and complex disorders[4,16,17].

Previously we have analyzed the change in cellular surface markers during human somatic cell reprogramming[10]. There is a progressive change in cell surface markers. Fibroblasts express CD13, which is repressed by the expression of reprogramming. Cells undergoing reprogramming start to express SSEA4 together with GFP. Then, they lose the expression of GFP via proviral silecing and express additional pluripotency maker TRA1-60[10]. The expression of TRA1-60 is well correlated with GFP silencing and the well-developed teratoma formation, suggesting that TRA1-60 is a marker for faithfully reprogrammed iPSCs. GFP silencing is the alternative marker for TRA1-60 expression and allows the

identification of iPSC colonies without laborious live cell staining. Using the loss of GFP expression as a marker for iPSCs, stem cell scientists who have no prior experience in reprogramming will readily and consistently isolate iPSCs.

Disclosures

We have nothing to disclose.

Acknowledgements

This work was funded by Yale School of Medicine and Child Health Research Award from Charles Hood Foundation.

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