Video Article

Selecting and Isolating Colonies of Human Induced Pluripotent Stem Cells Reprogrammed from Adult Fibroblasts

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Abstract

Herein we present a protocol of reprogramming human adult fibroblasts into human induced pluripotent stem cells (hiPSC) using retroviral vectors encoding Oct3/4, Sox2, Klf4 and c-myc (OSKM) in the presence of sodium butyrate [1-3]. We used this method to reprogram late passage (>p10) human adult fibroblasts derived from Friedreich's ataxia patient (GM03665, Coriell Repository). The reprogramming approach includes highly efficient transduction protocol using repetitive centrifugation of fibroblasts in the presence of virus-containing media. The reprogrammed hiPSC colonies were identified using live immunostaining for Tra-1-81, a surface marker of pluripotent cells, separated from non-reprogrammed fibroblasts and manually passaged [4,5]. These hiPSC were then transferred to Matrigel plates and grown in feeder-free conditions, directly from the reprogramming plate. Starting from the first passage, hiPSC colonies demonstrate characteristic hES-like morphology. Using this protocol more than 70% of selected colonies can be successfully expanded and established into cell lines. The established hiPSC lines displayed characteristic pluripotency markers including surface markers TRA-1-60 and SSEA-4, as well as nuclear markers Oct3/4, Sox2 and Nanog. The protocol presented here has been established and tested using adult fibroblasts obtained from Friedreich's ataxia patients and control individuals [6], human newborn fibroblasts, as well as human keratinocytes.

Video Link

The video component of this article can be found at https://www.jove.com/video/3416/

Protocol

1. Virus production and transduction

1. Plate Phoenix Ampho cells at a density of ~7-8x10^6 per 10 cm plate in 10 ml of DMEM medium (DMEM high glucose, 10% FBS heat inactivated, 2 mM L-glutamine, no antibiotics). Place in the incubator and culture overnight at 37°C, 5% CO_2.
2. The next day transfect Phoenix cells using 12 µg of a vector encoding either Oct3/4, Sox2, Klf4, c-myc, or GFP gene (Addgene plasmids 17217, 17218, 17219, 17220) and 35 µl Fugene 6. Prepare transfection mix in 500 µl of DMEM medium (DMEM high glucose, 2 mM L-glutamine, no FBS and antibiotics). Incubate 20 min. Gently pipette DNA complexes into the 10 cm plates containing 70-80% confluent Phoenix cells.
3. Replace media 6 - 8 h post-transfection with DMEM medium (DMEM high glucose, 10% FBS heat inactivated, 2 mM L-glutamine) containing penicillin and streptomycin.
4. Subsequently, collect virus-containing media 4 times in 12 h intervals, combine all portions and filtered using a 0.45 µm filter to remove detached cells and debris (media containing viral particles can be kept in the refrigerator for 2 weeks without losing infectious activity, however freezing is not recommended).
5. For retroviral transduction, plate human adult fibroblasts (passage 10, Coriell Laboratories) from a frozen stock 24 h prior to the last day of retroviral media collection. Seed the cells on 6-well gelatin covered plates at the density 1x10^5 cells per well in DMEM high glucose, 10% FBS, 2 mM L-glutamine, penicillin, streptomycin and non-essential amino acids.
6. The next day substitute DMEM media with media containing viral particles obtained from Phoenix cells. Add viral media (1 ml of each Oct4, Sox2, Klf4, and c-myc media, 4 ml total) supplemented with 6 µg/ml of polybrene into each well of the 6-well plate of the fibroblast cultures and centrifuge at 1600g for 1h at 20°C. Approximately 12 h after the transduction, replace the viral media with fibroblast culture medium. Perform viral infection and centrifugation 3 times in 24 h intervals.
7. Culture fibroblasts in DMEM media for 48 h after last infection. Check the efficiency of the infection by parallel transductions with a retroviral media expressing GFP. Figure 1 illustrates the efficiency of centrifugation-facilitated retroviral infection of human fibroblast.

2. Reprogramming

1. Prepare 6 well plates with γ-irradiated MEF feeder layers by seeding MEF cells at a density of ~$2x10^5$ cells per well of the gelatin treated 6 well plate in fibroblast growth medium. The following day split infected human fibroblasts using 0.05% trypsin/EDTA and seed them at the density of ~$1.2x10^4$ per well in the fibroblasts growth medium.
2. The next day replace media with hES media (DMEM/F12, 20% Knockout Serum Replacement, non-essential amino acids, penicillin/streptomycin, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 20 ng/ml basal Fibroblast Growth Factor (bFGF), supplemented with 0.5 mM sodium butyrate for the initial 7 days of the reprogramming. Change media daily.
3. Monitor morphological changes in transduced cells daily. Colonies of hiPS-like cells will start to emerge approximately 5 - 10 days after transferring the transduced fibroblasts onto feeder cells. The hiPSC colonies are ready to be isolated approximately 14 - 28 days after plating them on the MEF feeder cells.

3. Isolation of hiPSC colonies

1. A day before picking the hiPSC colonies prepare 24-well plates with γ-irradiated MEF feeder cells (~$4x10^4$ cells per well) in fibroblasts growth medium. At this stage hiPSC colonies can also be transferred to feeder free culture using Matrigel and mTeSR1 medium.
2. Prior to isolation of hiPSC colonies prepare MEF plates by removing fibroblasts growth medium and rinsing MEFs with PBS to remove traces of FBS. Subsequently add 0.5 ml hES medium (or mTeSR1 medium in the case of Matrigel coated wells) with 10 μM ROCK inhibitor Y27632 to each well [7,8].
3. If necessary, remove the fibroblasts surrounding the hiPSC colonies with a 21 gauge needle under a microscope mounted in a laminar flow hood (Figure 2A - C). Rinse the plates with PBS and add fresh hES media containing the Tra-1-81 StainAlive specific antibody (1:200, Stemgent). After 30 min., replace media containing the antibody with fresh hES media supplemented with 10 μM ROCK inhibitor. Examine the plates under the fluorescent microscope and mark Tra-1-81 positive colonies using an objective marker (Figure 2D).
4. Cut the Tra-1-81 positive hiPSC colonies under a microscope in a laminar hood, into several small pieces using a 21 gauge needle as shown in Figure 2E and F.
5. Using an automatic pipette (P200) transfer fragments of hiPSC colonies into individual wells of the 24-well plate with MEFs or Matrigel. Avoid transferring non-hiPS cells. Place the plates in a 37°C, 5% CO_2 incubator and allow the hiPSC colonies to attach for 24-36 h.
6. Change hES or mTeSR1 (for colonies grown on Matrigel) media daily. The hiPSC colonies with correct hES-like morphology will be visible 48 h after the initial transfer onto a 24-well plate.
7. Manually passage hiPSC colonies every 6 - 8 days onto a 12-well and subsequently on a 6-well plate.
8. After clonal expansion and establishing the hiPSC lines, analyze expression of pluripotency markers TRA-1-60, SSEA-4, Oct3/4, Sox2 and Nanog using immunocytochemistry as shown in Figure 3. Evaluate genomic integrity and differentiation potential of the obtained lines using karyotype and teratoma formation analyses [6,9].

4. Representative Results

Efficient transduction with retrovirus-containing media is critical for successful reprogramming. It is recommended to conduct the entire transfection/infection procedure using a GFP expressing virus every single reprogramming experiment to monitor the efficiency as shown in Figure 1. The titer of the GFP expressing virus determined, as described in [10], through the transduction of human fibroblasts using non-concentrated viral media was typically in the range of 0.5 - 5 x 10^7 viral particles per ml (vp/ml).

Fibroblasts change morphology as early as 2 days after the last infection. Trypsinized human fibroblasts should be carefully counted prior to seeding them on the MEF feeder cells. It is recommended to seed cells at 3 different densities ($6x10^3$, $1.2x10^4$, $2.5x10^4$ per single well of a 6 well plate) since each cell line demonstrates different growth characteristic and seeding density is critical for the efficiency of reprogramming. Sodium butyrate used for the initial 7 - 14 days of reprogramming increases the efficiency of hiPSC formation approximately 5 fold. Frequently, especially when infected fibroblasts were seeded at higher density, the fibroblast-like cells can overgrow a culture dish and cover hiPSC colonies as shown in Figure 2A. In this case, the fibroblast layer can be carefully lifted and removed to uncover hiPSC colonies (Figure 2B). Subsequently, hiPSC colonies should be rinsed with hES media and stained with Tra-1-81 antibody. Depending on the fibroblast cells, approximately 20 - 40% of colonies demonstrating with iPSC-like morphology do not stain with Tra-1-81 antibody. Identified hiPSC colonies can be transferred from a plate within next 12 - 24h. Prolonged incubation will result in rapid differentiation of hiPSCs. Colonies can be manually passaged onto either MEF feeder cells or Matrigel-coated plates. After expansion established hiPSC clones should be tested using immunocytochemistry (ICC) for the expression of pluripotency markers as demonstrated in Figure 3. Additionally, a detailed molecular characterization of the generated iPS cell lines should include: analyses of the pluripotency gene expression using RT-PCR, demonstration of the DNA demethylation at the promoters of pluripotency genes and analyses of the transgenes silencing [9].

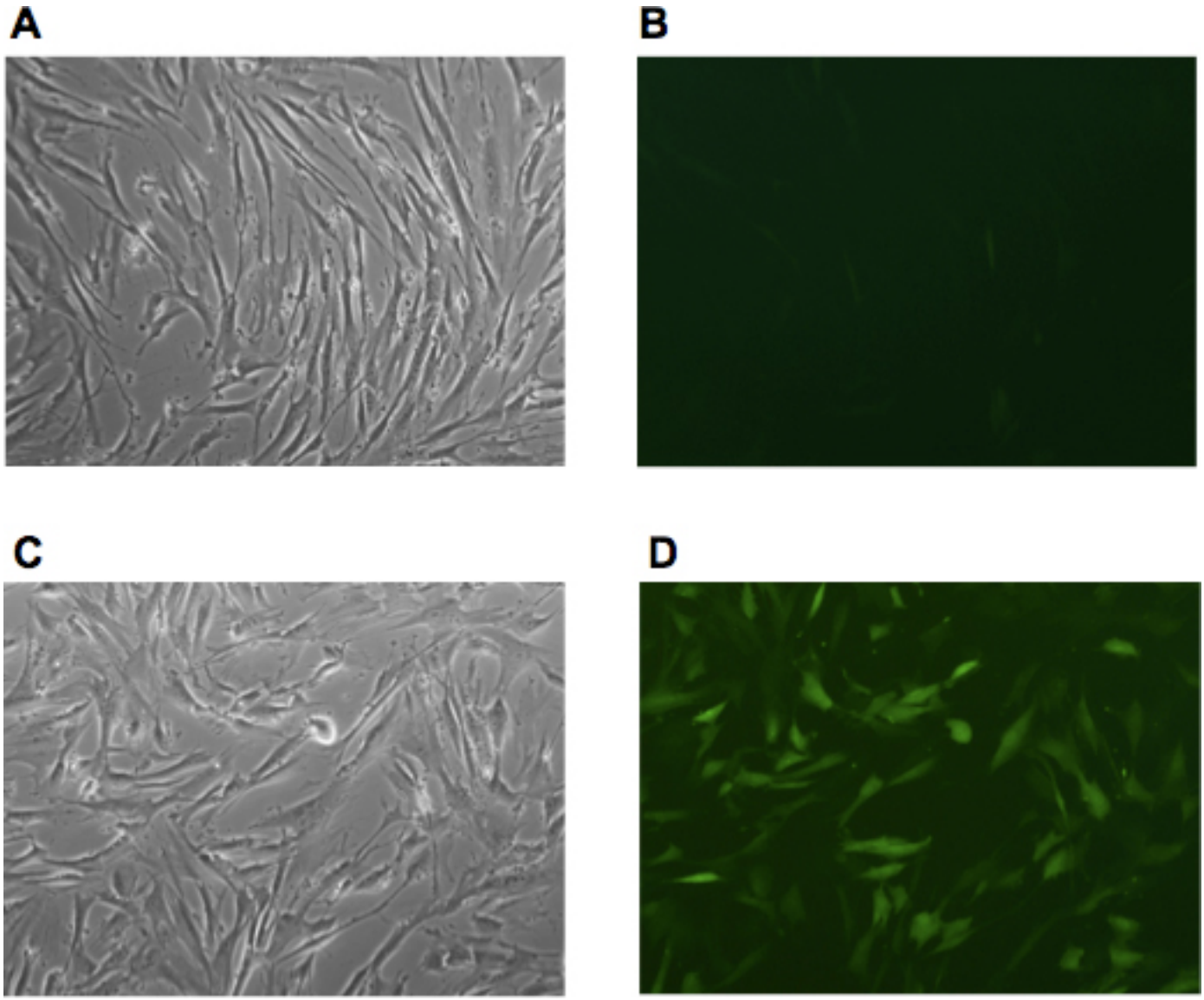

Figure 1. The efficiency of viral transduction determined using GFP expressing retrovirus. (A, B) Human adult fibroblasts derived from Friedreich's ataxia patient (GM03665, Coriell Repository) were visualized after two consecutive infections with GFP retroviral media. (C, D) Human fibroblasts were infected with the same batch of the GFP retroviral media followed by centrifugation of the cells directly on the 6-well plates for 1h at 1600g. Images were captured 48h after infection.

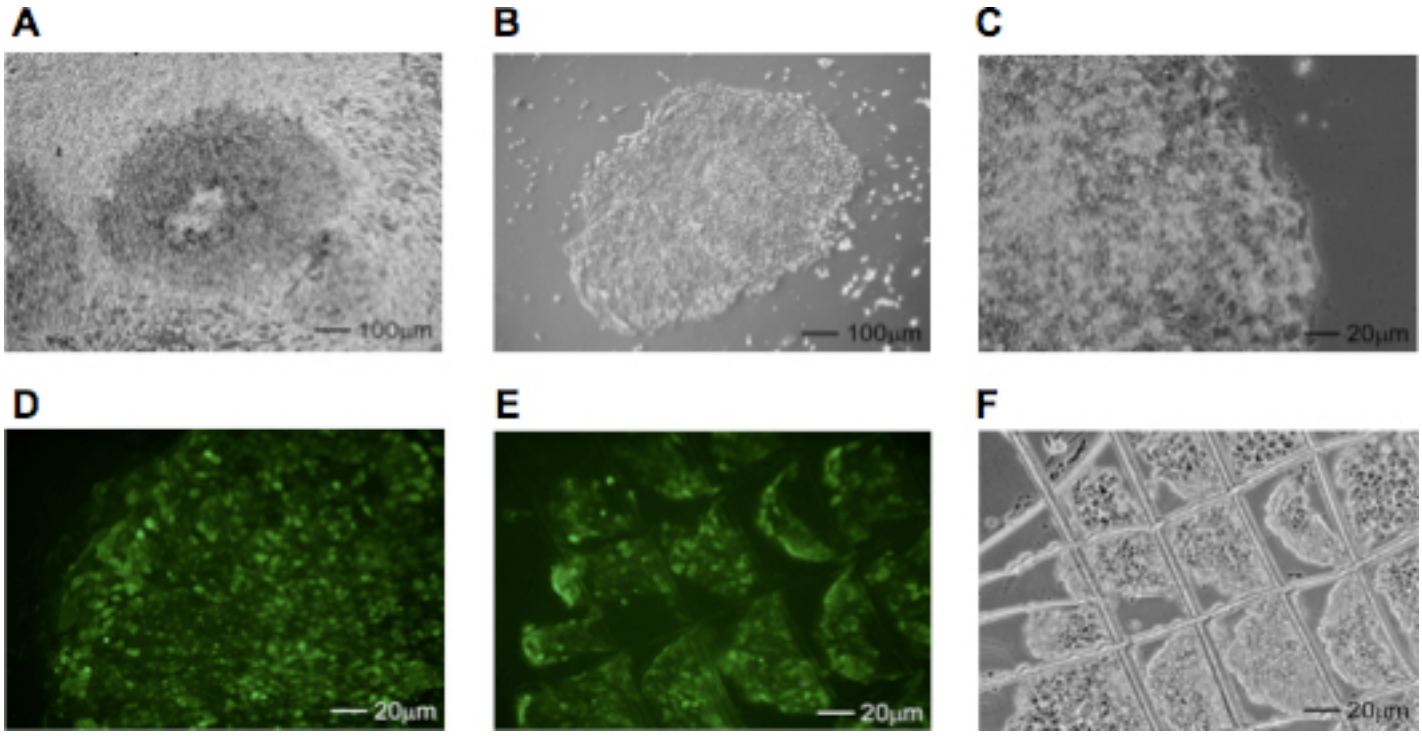

Figure 2. Identification and isolation of hiPSC colonies. (A) Phase contrast image of a plate containing hiPSCs surrounded by fibroblasts. The cells were cultured for 21 days on hES media. (B, C) The same hiPSC colony after removal of the surrounding fibroblast layer. (D) Correctly reprogrammed hiPSC colonies are identified by live staining with Tra-1-81 surface marker antibody, cut using a sterile needle (E, F) and transferred to separate wells of a 24-well plate.

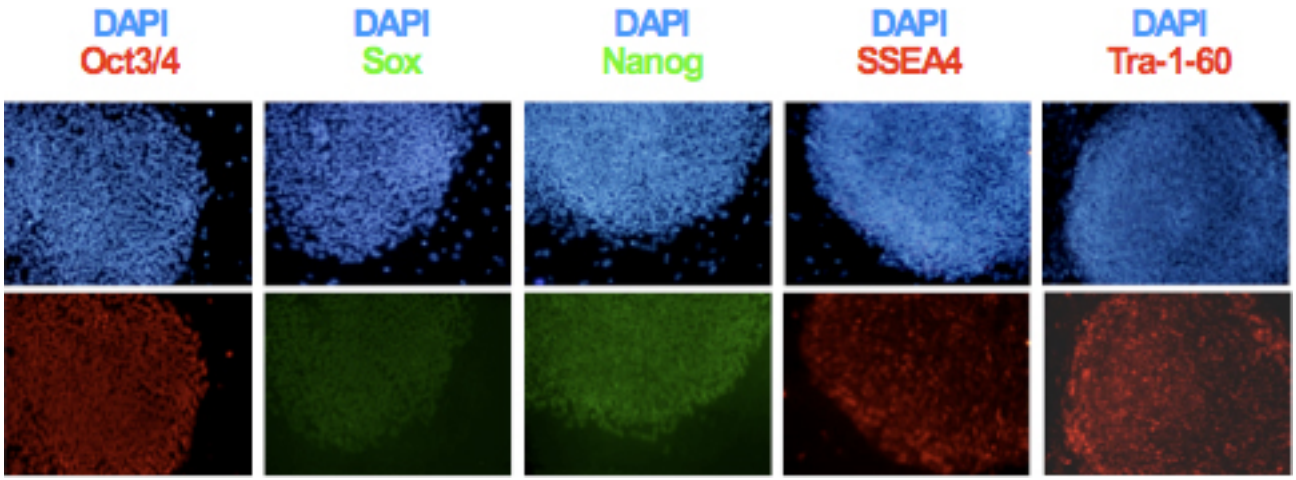

Figure 3. Expression of the pluripotency specific markers Oct3/4, Nanog, Sox2, SSEA4 and Tra-1-60 in hiPSCs was determined by immunocytochemistry.

Discussion

Studying human diseases, especially neurological and neurodegenerative, has been particularly challenging due to the inaccessibility of adequate human cellular models. The ability to reprogram easily obtainable somatic cells into induced pluripotent stem cells and the potential to differentiate them into diverse cell types opened a possibility to create cellular models of genetic diseases. In addition, iPSCs hold a great promise in the future of regenerative medicine. Therefore, it is essential to develop and optimize reliable, efficient and safe methods of reprogramming somatic cells to pluripotency.

From the perspective of therapeutic applications, it is crucial to develop safe approaches of iPSC generation lacking footprint mutations in the host genome. Methods of somatic cell reprogramming without introducing permanent alterations into the genome of reprogrammed cells such as transfection of episomal vectors, use of excisable transposons, adenoviral infection, and direct delivery of mRNA or proteins have already been established [11-15]. Thus far the reprogramming efficiency using these transgene-free methods is low and depends on the character, type and age of reprogrammed somatic cells. Therefore, these protocols are not suitable for reprogramming late passage, adult somatic cells frequently deposited in cell repositories. Additionally, to enable detailed characterization of multiple iPSC lines and to establish new models of human

diseases and to conduct high-throughput drug screens, reprogramming of somatic cells using viral delivery of transcription factors is an adequate strategy. To date more than 20 studies reported generation of patient-specific iPSCs to model human neurological and neuromuscular diseases. In all but one cases the iPSCs were generated using lentiviral or retroviral transduction [16].

Our data demonstrate that a simple protocol based on the retroviral delivery of OSKM transcription factors can be used to efficiently reprogram adult human fibroblasts, even of a high passage. The amount of virus obtained from a single transfection of Phoenix cells on 10 cm plate is sufficient for more than 10 reprogramming experiments. There are three critical steps in our reprogramming protocol of adult fibroblasts: (i) highly efficient viral transduction facilitated by an additional centrifugation step during transduction which dramatically increases the efficiency of transduction; (ii) seeding density of the transduced fibroblast onto the MEFs and (iii) use of live staining with the Tra-1-81 marker antibody to facilitate selection of potentially fully reprogrammed colonies which can be directly transferred to feeder-free cultures. The efficiency of reprogramming is significantly enhanced by using sodium butyrate during second week of the reprogramming. Moreover, we observed that a greater fraction of the iPSC colonies cultured in the presence of the drug can be expanded and established into fully reprogrammed iPSC lines.

Disclosures

The authors have nothing to disclose.

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