Video Article

Generation of Induced Pluripotent Stem Cells by Reprogramming Human Fibroblasts with the Stemgent Human TF Lentivirus Set

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Abstract

In 2006, Yamanaka and colleagues first demonstrated that retrovirus-mediated delivery and expression of Oct4, Sox2, c-Myc and Klf4 is capable of inducing the pluripotent state in mouse fibroblasts.[1] The same group also reported the successful reprogramming of human somatic cells into induced pluripotent stem (iPS) cells using human versions of the same transcription factors delivered by retroviral vectors.[2] Additionally, James Thomson *et al.* reported that the lentivirus-mediated co-expression of another set of factors (Oct4, Sox2, Nanog and Lin28) was capable of reprogramming human somatic cells into iPS cells.[3]

iPS cells are similar to ES cells in morphology, proliferation and the ability to differentiate into all tissue types of the body. Human iPS cells have a distinct advantage over ES cells as they exhibit key properties of ES cells without the ethical dilemma of embryo destruction. The generation of patient-specific iPS cells circumvents an important roadblock to personalized regenerative medicine therapies by eliminating the potential for immune rejection of non-autologous transplanted cells.

Here we demonstrate the protocol for reprogramming human fibroblast cells using the Stemgent Human TF Lentivirus Set. We also show that cells reprogrammed with this set begin to show iPS morphology four days post-transduction. Using the Stemolecule Y27632, we selected for iPS cells and observed correct morphology after three sequential rounds of colony picking and passaging. We also demonstrate that after reprogramming cells displayed the pluripotency marker AP, surface markers TRA-1-81, TRA-1-60, SSEA-4, and SSEA-3, and nuclear markers Oct4, Sox2 and Nanog.

Video Link

The video component of this article can be found at https://www.jove.com/video/1553/

Protocol

1. Reprogramming

1. Seed BJ cells at a density of 1 x 10^5 cells per well of a 6-well plate. Culture the cells in 2 ml of growth medium (450 ml EMEM supplemented with 50 ml ES qualified FBS, 5 ml 10 mM non-essential amino acids, 5 ml penicillin/streptomycin, 5 ml 200 mM L-glutamine and 0.9 ml 55 mM β-mercaptoethanol) overnight at 37°C and 5% CO_2.
2. On the same day, begin preparing MEF feeder plates by adding 0.1% gelatin diluted in water to a 6 well plate and incubating overnight at 37°C and 5% CO_2.
3. After incubation, remove medium from the BJ cells and add 2 ml growth medium supplemented with 6 µg/ml of polybrene, 500 µl hOct4-lentivirus, 50 µl hSox2-lentivirus, 50 µl hNanog-lentivirus and 50 µl hLin28-lentivirus to each well. Gently rock the plate to ensure an even coating of the medium. Incubate overnight at 37°C and 5% CO_2.
4. On the same day, remove 1 vial of CF-1 MEF cells from liquid nitrogen and thaw. Remove liquid from the gelatin coated wells (see step 1.2) and add MEF feeder cells at a density of 0.2 x 10^5 cells/well. The total volume per well should be 2ml of cells in MEF growth medium (450 ml DMEM supplemented with 50 ml FBS and 5 ml non-essential amino acids).
5. The next day, detach BJ cells with 0.05% trypsin/EDTA and centrifuge at 200 x g for 5 minutes. Aspirate the medium and resuspend in growth medium. Remove medium from the MEF feeder plate (see step 1.4) and add 2 ml/well of the BJ cell suspension. The concentration of cells should be approximately 5 x 10^4 cells/well. Incubate overnight at 37°C and 5% CO_2.
6. Replace medium 24 hours after re-seeding with human ES/iPS cell culture medium (400 ml DMEM/F12 supplemented with 100 ml Knockout Serum Replacement, 5 ml 10 mM non-essential amino acids, 5 ml 200 mM L-glutamine, 0.9 ml 55 mM β-mercaptoethanol and 20 ng/ml human recombinant bFGF). Change medium every 24 hours for 7 days.
7. After seven days, change medium to MEF conditioned medium (see section 2).

2. Preparation of MEF Conditioned Medium

1. Seed CF-1 MEF feeder cells at 2 x 10^5 cells/well on a 6 well plate in 2 ml MEF growth medium. Incubate overnight at 37°C and 5% CO_2.
2. Change medium to human ES/iPS cell culture medium. Incubate overnight at 37°C and 5% CO_2.
3. Collect supernatant every 24 hours for four days. Filter the supernatant through a 0.22 µm filter.
4. Supplement supernatant with 50 ng/ml of bFGF.

3. iPS Colony Selection and Passaging

1. Select an ES-like colony and re-seed in human ES/iPS culture medium supplemented with 10 µM of Stemolecule Y27632 on cell culture dishes pre-seeded with CF-1 MEF feeder cells. Incubate overnight at 37°C and 5% CO_2.
2. Change the culture medium every 24 hours for the first seven days (without supplementing with Stemolecule Y27632). After seven days, change medium to MEF conditioned medium (see section 2 for preparation). We continued passaging the cells until they showed typical human ES morphology.

4. Immunocytochemical Examination of Pluripotency Markers

1. Wash the cells gently three times with PBS.
2. Fix the cells with 500 µl of fixative for 20 minutes at room temperature.
3. Wash the cells gently three times with PBS.
4. Block non-specific binding with 500 µl blocking buffer for one hour at room temperature.
5. Incubate the cells with 250 µl of the specific primary antibody overnight at 4°C (we used TRA-1-81, TRA-1-60, SSEA-4, SSEA-3, Oct4, Sox2, Nanog and Lin28 at 1:100 dilutions).
6. Wash the cells gently three times with PBS.
7. Incubate the cells with 250 µl of secondary antibody for 1 hour at room temperature, keeping away from light (we used Goat anti-Mouse IgM Cy3 conjugate, Goat anti-Mouse IgG Cy3 conjugate, Goat anti-Rat IgM Cy3 conjugate and Goat anti-Rabbit Cy3 conjugate at 1:300 dilutions).
8. Wash the cells gently three times with PBS.
9. Add DAPI (final concentration 1 µg/ml) to the last wash and incubate 5 minutes to visualize nuclei.
10. Analyze the cells under magnification.

Part 5: Representative Results

1. Morphology Results:

Human foreskin fibroblast (BJ) cells were co-transduced with Oct4, Sox2, Nanog, and Lin28. Morphological changes were observed as early as day 4 post-transduction, and the cluster of cells became more tightly packed at day 17 (Figure 1). Colonies were manually picked at day 25 and cultured on CF-1 MEF feeder cells. To facilitate the iPS cell colony formation after reprogramming, we used Stemolecule Y27632, a ROCK inhibitor, for the initial overnight seeding during each passage. iPS cell colonies with good morphology were observed after three sequential rounds of colony picking and passaging.

2. Expression of Pluripotency Markers:

To further characterize the isolated iPS cell colonies, we looked for the presence of common pluripotency markers expressed in ES cells. The colonies exhibited strong alkaline phosphatase (AP) activity (Figure 2). Additionally, immunocytochemistry (ICC) was performed on the iPS cell colonies with a panel of pluripotency marker-specific antibodies, including surface markers TRA-1-81, TRA-1-60, SSEA-4 and SSEA-3 as well as nuclear markers Oct4, Sox2 and Nanog. The isolated iPS colonies were positive for all markers (Figure 3). The ICC results show that the iPS cells exhibited the appropriate pluripotency marker expression pattern, demonstrating that these iPS cells closely resemble undifferentiated human ES cells.

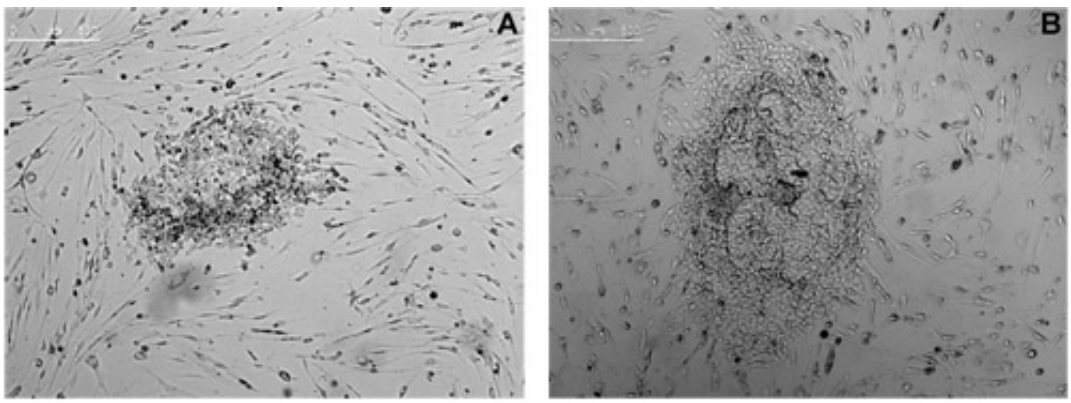

Figure 1: Morphological changes of transduced BJ cells. Bright field images of a typical iPS cell colony formed at (A) 4 days and (B) 17 days post-transduction.

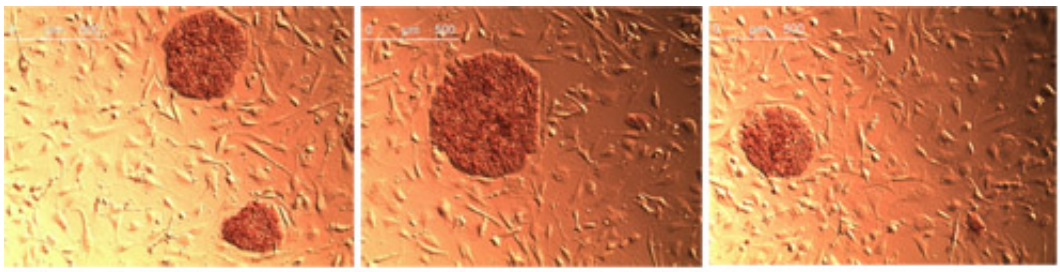

Figure 2: AP activity of reprogrammed BJ cells. Three different colonies stained with Stemgent Alkaline Phosphatase Staining Kit.

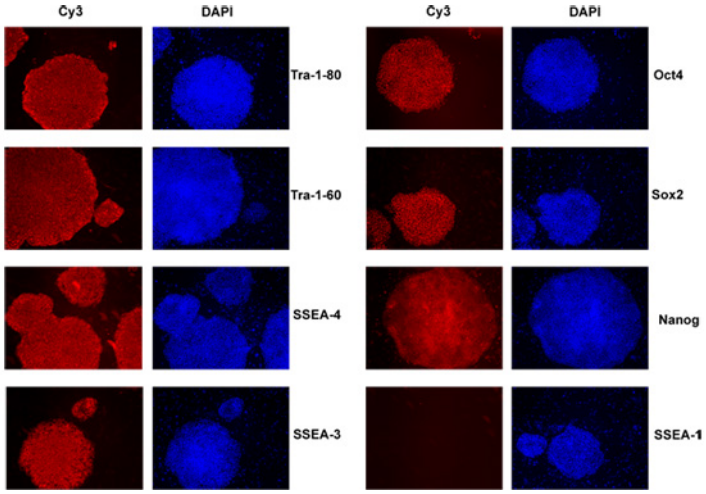

Figure 3: Human iPS cells express high levels of the following ES cell specific markers: surface markers TRA-1-81, TRA-1-60, SSEA-4 and SSEA-3, and nuclear markers Oct 4, Nanog and Sox2.

Discussion

These results demonstrate that the Stemgent Human TF Lentivirus Set can be used to efficiently generate iPS colonies by inducing the ectopic expression of transduced transcription factors in human fibroblasts. When designing reprogramming experiments, several variables should be considered to optimize the efficiency of reprogramming. First, the active virus-to-target ratio (multiplicity of infection M.O.I.) may need to be modified during the primary transduction step to achieve optimum transduction efficiency. Second, the growth condition of the target cells can impact reprogramming. Healthy and proliferative cells are more amenable to reprogramming. Third, when modifying the protocol for different cell numbers, it is recommended that target cell numbers be adjusted proportionally to the surface area of the culture dish. Lastly, applying ROCK inhibitors such as Y27632 should be considered to help ensure successful reprogramming as recent studies have demonstrated its utility in enhancing hES colony survival.[4,5]

Disclosures

The authors of this article are employed by Stemgent that produces reagents and instruments used in this article.

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