Video Article

Generating iPS Cells from MEFS through Forced Expression of Sox-2, Oct-4, c-Myc, and Klf4

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Abstract

Pluripotency can be induced in differentiated murine by viral transduction of Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006; Wernig, et al., 2007; Okita, et al., 2007; Maherali, et al., 2007). We have devised a reprogramming strategy in which these four transcription factors are expressed from doxycycline (dox)-inducible lentiviral vectors (Brambrink et al., 2008). Using these inducible constructs, we can derive induced pluripotent stem (iPS) cells from mouse embryonic fibroblasts (MEFs). In this video, we demonstrate the procedure for the generation of inducible lentiviruses that express the four transcription factors and show how to infect MEFs with these viruses in order to produce iPS cells. By using inducible lentiviruses, the expression of the four factors in controlled by the addition of doxycyline to the culture medium. The advantage of this system over the traditional retroviral infection is the ability to turn the genes on and off so that the kinetics of reprogramming and gene expression requirements can be analyzed in detail.

Protocol

Step 1:

Thaw out 293 cells and plate for lentivirus production.

1. Prepare 9 ml of DMEM+ medium in a 15-ml tube.
2. Remove a vial of frozen 293 stocks from the liquid nitrogen tank and put the vial in a 37°C water bath until most (but not all) cells are thawed.
3. Remove cells from freezing vial and place into the 15ml tube from step 1.
4. Centrifuge at 180g for 5 min, and then discard the supernatant.
5. Resuspend the cells with 10 ml of DMEM+ medium, and transfer to a gelatin-coated 100-mm dish. Incubate the cells in a 37°C, 5% CO_2 incubator until they are 80–90% confluent.
6. Passage of the 293 cells: wash cells with PBS, aspirate the PBS, and add 4 ml per 10-cm dish of 0.05% trypsin/0.53 mM EDTA, and incubate for 1 min at 37°C.
7. Detach cells from dishes by tapping, resuspend with 10 ml DMEM+ medium, and transfer to a 15-ml tube. Centrifuge at 180g for 5 min, and aspirate the supernatant.
8. Add appropriate volume of DMEM+ medium, and break up the cells into a single cell suspension by pipetting up and down several times.
9. Count the number of cells and adjust the concentration to $8x10^5$ cells per ml with FP medium.
10. Seed cells at $8x10^6$ cells (10 ml) per 100-mm culture dish, and incubate overnight at 37°C, 5% CO_2.

Step 2:

Transfect 293 cells with lentivirus plasmids and harvest virus

1. For each 10-cm plate, use 10 µg of FUW-tetO-cDNA (Oct4, Sox2, Klf4 or c-Myc) lentivirus backbone with 2.5ug of pMD-G and 7.5 µg of pPax2.
2. While aliquoting the three plasmids into a tube, deliver 30µl of Fugene 6 transfection reagent into a second tube containing 500µl of DMEM without serum and mix gently by finger tapping and incubate for 5 min at room temperature.
3. Add the DNA mix drop-by-drop into the Fugene 6/DMEM-containing tube, mix gently by finger tapping and incubate for 20 min.
4. Add the DNA/Fugene 6 complex dropwise into the dish of 293's, and incubate overnight at 37°C, 5% CO_2.
5. Next morning: Aspirate the transfection reagent–containing medium, add 5 ml of fresh ES cell medium, and return the cells to the incubator.
6. At 48 hours and 72 hours after transfection, collect the medium from the 293's by using a 10-ml sterile disposable syringe, filtering it through a 0.45- µm pore size cellulose acetate filter, and transferring into a 15-ml tube.
7. Concentrate the virus supernatant by ultracentrifugation. Resuspend the virus pellet in desired volume and make amixture of equal parts of the medium containing Oct-3/4-, Sox2-, Klf4- and c-Myc-lentiviruses.
8. While making the virus, prepare Oct4-neo/rtTA or Oct4-GFP/rtTA MEFs (passage <3) to 90% confluency in 10-cm dishes ($2x10^6$ cells per dish)
9. Aspirate the culture medium and wash with 10 ml of PBS.
10. Discard PBS, add 1 ml per dish of 0.05% trypsin/0.53 mM EDTA, and incubate at 37°C for 10 min.
11. Add 9 ml of the culture medium, suspend the cells to a single cell, and transfer to a 15-ml tube.
12. Count the number of cells, and adjust the concentration to $8x10^4$ cells per ml. Transfer 10 ml of cell suspension ($8x10^5$ cells) to a 10-cm dish coated with gelatin. Incubate the dish overnight at 37°C, 5% CO_2.
13. Aspirate the medium from a fibroblast dish, and add 10 ml of the virus-containing medium. Incubate the cells from 4 h to overnight at 37°C, 5% CO_2.
14. After 24 aspirate the medium from a fibroblast dish, and add 10 ml of fresh ES cell medium.
15. Replace regular ES cell medium with medium containing Doxycycline to initiate the expression of the four genes; in other words, initiate reprogramming.
16. If using an Oct4-neo reporter, then initiate drug section anytime between 6 and 9 days.

17. Change the medium every day until the colonies become big enough to be picked up. Colonies should first become visible approximately a week after the initiation of reprogramming. They should become large enough to be picked up around day 20.

Step 3:

Picking up the iPS colonies

The Day before Picking:

1. Seed the required number of gelatin-coated 24-well plates with ?-irradiated DR4 MEFs

Picking Clones on Day 1:

1. Feed the colonies on the plates 1-2 hours before picking.
2. Add 15 µl of PBS to the wells of a V-bottomed 96-well dish.
3. Wash the dish containing the colonies to be picked once with PBS and add 10ml of PBS to the dish.
4. Pick the colonies with the small 5µl pipet tips (set the P20 pipetman on 4 µl). Transfer colonies to a well in the 96-well dish.
5. Add 20 µl of trypsin to each well using the multi-channel (12) pipetor. Pipet up and down 3 times. Incubate at 37°C for 4 minutes.
6. Add 100 µl of ES medium to the trypsinized colonies. Pipet up and down 10 times. Transfer 6 clones at a time from the 96-well dish to a 24-well dish using a tip at every other position on the 12 place pipetor.
7. Grow the cells on the 24-well plate in a 37°C, 5% CO_2 incubator until the cells reach 80–90% confluency, feeding daily with ES cell medium. Cells will probably be ready to expand in 3-7 days.

Step 4

1. Expansion of iPS cells
    1. Aspirate the medium, and wash the cells with 1 ml of PBS.
    2. Remove PBS completely, add 0.1 ml of 0.25% trypsin/1 mM EDTA and incubate at 37°C for 10 min.
    3. Add 0.4 ml of the ES medium and suspend the cells by pipetting up and down to single cell suspension.
    4. Transfer the cell suspension to a well of 6-well plate, add 1.5 ml ES cell medium, and incubate in a 37°C, 5% CO_2 incubator until cells reach 80–90% confluency in 6-well plates. At this point, prepare frozen stock of the cells, as follows.

2. Preparation of freeze stock
    1. Aspirate the medium, and wash the cells with 2 ml of PBS.
    2. Remove PBS completely, add 0.5 ml of 0.25% trypsin/1 mM EDTA and incubate at 37°C for 10 min.
    3. Add 2 ml of the ES medium and suspend the cells by pipetting up and down to single cell suspension.
    4. Transfer the cell suspension to a 15-ml tube, count the number of cells and spin down the cells.
    5. Discard the supernatant, resuspend the cells with ES medium to the concentration at $2x10^6$ cells per ml.
    6. Prepare 2 ES cell freezing medium (20% DMSO in ES medium) and aliquot it at 0.5 ml per vial.
    7. Transfer 0.5 ml of the cell suspension to freeze vials and mix gently.
    8. Put the vials in a cell-freezing container and keep it at -80°C overnight; transfer to liquid nitrogen the next day for long-term storage.

Discussion

In this video, we demonstrate how to use an inducible, lentiviral system to generate GFP-positive, pluripotent iPS cells from MEFs derived from Oct4-GFP/R26-M2rtTA and Nanog-GFP/R26-M2rtTA mice. This procedure is a useful method for generating iPS cells because it allows for the control of transgene expression during the reprogramming process. Although the use of viruses in the generation of iPS cells impedes the use of these cells in a clinical setting, this procedure does enable us to answer some of the major biological questions that underline the yet defined reprogramming process.

References

1. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663-676 (2006)

2. Wernig, M. et al. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448, 318-324 (2007).

3. Okita, K., Ichisaka, T. & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. Nature 448 , 313-317 (2007).

4. Maherali, N. et al. Global epigenetic remodeling in directly reprogrammed fibroblasts. Cell Stem Cell 1, 55-70 (200).

5. Brambrink, T et al. Sequential expression of pluripotency markers during direct reprogramming of somatic cells, Cell Stem Cell 2 (2): 151-9 (2008).

