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Reprogramming mouse embryonic fibroblasts using different systems

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Abstract

This protocol describes 4 methods to reprogram MEFs into iPSCs: the pMXs-based retroviral 4-factor, the polycistronic lentiviral 4-factor, the doxycycline inducible 4-factor secondary, and the iCD1 medium based Sox2+Oct4 2-factor systems.

Subject terms: Cell biology

Keywords: Somatic reprogramming mouse embryonic fibroblast induced pluripotent stem cells.

Reagents

Medium composition:
Plat-E/ HEK293T medium
500 ml DMEM high glucose (HyClone #SH30022.01B)
55 ml FBS (PAA #A15-101)

MEF medium
500 ml DMEM high glucose (HyClone #SH30022.01B)
55 ml FBS (PAA #A15-101)
6 ml Glutamax (GIBCO #35050079)
6 ml non-essential amino acids (GIBCO #11140076)

Serum-based ESC medium
500 ml DMEM high glucose (HyClone #SH30022.01B)
90 ml FBS (GIBCO #10099141)
6 ml Glutamax (GIBCO #35050079)
6 ml sodium pyruvate (GIBCO #11360070)
600 µl β-mercaptoethanol (GIBCO #21985023)

6 ml non-essential amino acids (GIBCO #11140076)
60 µl LIF (1000 U/ml final) (Enzo Life Science #ALX-201-242)

iCD1 medium
Please refer to Reference 4 for details.

Procedure

pMXs-based retroviral 4-factor system
Day 1
Plate Plat-E cells in Plat-E medium at 8×10^6 cells per 10-cm dish. Distribute the cells evenly over the dish.

Day 2
In the evening, transfect Plat-E cells from day 1 (cells should be 70-80% confluent and evenly distributed) using the modified calcium phosphate transfection method[1] as follows:
Replace the medium of the 10-cm dish with 5 ml fresh Plat-E medium.
For each factor use one 15 ml tube, add:
1. 24 µg plasmid
2. 1043 µl ddH2O
3. 156.25 µl 2M CaCl2
4. 1.25 ml 2×HBS
Mix vigorously immediately after adding 2×HBS, incubate at room temperature for 3-5 minutes.
5. add 2.5 ml Plat-E medium
Mix gently and transfer the 5 ml transfection mixture into the dish with 5 ml fresh Plat-E medium.
Revive OG2 MEFs in MEF medium.
Note: The condition of the MEFs and passage number are critical factors for successful reprogramming. For this reason, they are plated just before the start of the reprogramming experiment to keep the passage number as low as possible and the proliferation rate high.

Day 3
In the early morning, 8-12 hours after transfection, replace the medium of the transfected Plat-E cells with 10 ml Plat-E medium/10 cm dish.

Day 4
In the morning, split OG2 MEFs at 16,000-20,000 cells/well of P12 plate. Distribute the cells evenly over the well.
In the evening, start the first infection as follow:
Harvest the supernatant of the transfected Plat-E plates using a syringe and filter through a 0.45 µm filter. This is the first viral supernatant. Add fresh Plat-E medium (10 ml/10-cm dish) to the

transfected Plat-E cells for a second harvest of viral supernatant 24 hours later.
Mix the 4 supernatants of each factor with equal volume, add one volume of fresh Plat-E medium (at 1/4 of the total supernatant volume, for example: add 2.5 ml fresh medium for 10 ml supernatant), add polybrene to a final concentration of 4-8 μg/ml.
Replace the medium of OG2 MEFs with the infection mixture. Use 3 ml infection mixture per well.

Day 5
Repeat the infection a second time as in day 4 using the second round of viral supernatant.
Discard the Plat-E cell dishes.

Day 6
Change the medium of the infected OG2 MEFs to FBS-based ESC medium. Change new ESC medium every other day in the first 3-5 days before cells get confluent and every day afterwards.

Day 22
18 days post infection count iPSC colonies that are GFP positive.

Polycistronic lentiviral 4-factor system
The following protocol is based on the publication[2], with minor modifications.
Day 1
Plate HEK293T cells in HEK293T medium (the same with Plat-E medium) at 8×10^6 cells/10-cm dish. Distribute the cells evenly over the dish.

Day 2
In the evening, transfect HEK293T cells from day 1 (cells should be 80-90% confluent and evenly distributed) as follows:
Replace the medium of the 10-cm dish with 5 ml fresh HEK293T medium.
In a 15 ml tube, add:
1. 20 μg 4F-IRES-dTomato + 15 μg psPAX2 + 6 μg pMD2.G
2. 1043 μl ddH2O
3. 156.25 μl 2 M CaCl2
4. 1.25 ml 2×HBS
Mix vigorously immediately after adding 2×HBS, incubate at room temperature for 3-5 minutes.
5. 2.5 ml HEK293T medium
Transfer the 5-ml transfection mixture into the dish.
Revive passage 0 MEFs in MEF medium.

Day 3
In the morning, 8-12 hours after transfection, change the medium of the transfected HEK293T

cells with 10 ml fresh HEK293T medium.

Day 4
In the evening, split MEFs at 16,000-20,000 cells/well of P12 plate. Distribute the MEFs evenly over the well.
Harvest the supernatant of the transfected HEK293T plates using a syringe and filter through a 0.45 μm filter. The virus supernatant can be stored at 4℃ for 3-5 days or in -80℃ for longer time. Discard the HEK293T dishes.

Day 5
In the morning, start the first infection as follow:
Add a volume of fresh HEK293T medium at 1:1 ratio of the supernatant volume (such as: 10 ml fresh medium for 10 ml supernatant), add the polybrene to a final concentration of 4-8 μg/ml.
Replace the medium of MEFs with the virus mixture. Use 1 ml infection mixture per P12 well.
8 hours after infection, change the medium of the infected MEFs to ESC medium. Change new ESC medium every other day in the first 3-5 days before cells get confluent and every day afterwards.

Day 17
12 days post infection, wash the cells twice using DPBS, fix the cells using 4% paraformaldehyde in room temperature for 15 minutes, stain the colonies using SSEA1 primary antibody (ab#16825, Abcam, 1/100 dilution) and green fluorescence secondary antibody Alexa Fluor® 488 Goat Anti-Mouse IgG (#A-11029, Invitrogen, 1/200 dilution), then count the SSEA1+/dTomato- colonies using a fluorescence microscope.

Doxycycline inducible secondary 4-factor system
Day 1
Revive the OG2 secondary MEFs (with the doxycycline (DOX)-inducible 4F transgenes inserted into Col1a locus[3]) in MEF medium.

Day 2
In the evening, split OG2 secondary MEFs at 16,000-20,000 cells/well of P12 plate.

Day 3
In the early morning, the medium was changed into mouse ESC medium containing 2 μg/ml doxycycline and 50 μg/ml vitamin C. Change new ESC medium every other day in the first 3-5 days before cells get confluent and every day afterwards.

Day 15

12 days post infection count GFP positive iPSC colonies.

iCD1 medium based Sox2+Oct4 2-factor system
Day 1 to 5, the same with pMXs-based 4-factor reprogramming system above. Transfect Sox2 and Oct4 only.

Day 6
Change the medium of the infected OG2 MEFs to iCD1+BMP4 medium[4]. Change new iCD1+BMP4 medium every other day in the first 3-5 days before cells get confluent and every day afterwards.

Day 16
12 days post infection count GFP positive iPSC colonies.

Timing

22 days for the pMXs-based retroviral 4F system
17 days for the polycistronic lentiviral 4F system
15 days for the doxycycline inducible secondary 4F system
16 days for the iCD1 medium based Sox2+Oct4 2F system.

Anticipated Results

For all the 4 methods, at 3-4 days after infection there will be obvious mesenchymal to epithelial transition observed. Cells should proliferate very fast in the early 4F reprogramming, with certain degree of apoptosis because of c-Myc expression. For the pMXs-based retroviral 4F system, the doxycycline inducible secondary 4F system, and the iCD1 medium based Sox2+Oct4 2F system, there should be GFP+ colonies appearing at the indicated time. For the polycistronic lentiviral 4F system, after staining SSEA1 with green fluorescence secondary antibody, the SSEA1+ and dTomato- colonies represent the iPSCs.

References

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4. Chen, J. et al. BMPs functionally replace Klf4 and support efficient reprogramming of mouse fibroblasts by Oct4 alone. Cell Res. 21, 205-212 (2011).

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Associated Publications

This protocol is related to the following articles:

- Autophagy and mTORC1 regulate the stochastic phase of somatic cell reprogramming

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Competing financial interests

The authors declare no competing financial interests.

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Readers' Comments

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