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Article

Direct Reprogramming of Fibroblasts into Embryonic Sertoli-like Cells by Defined Factors

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Generation of induced embryonic Sertoli-like cells (ieSCs)

Important points

1. The starting cells must be in their log phase of proliferation. We are using only freshly isolated (Passage 0 or 1) cells.
2. This protocol is calibrated for mouse embryonic fibroblasts (MEFs) and tail tip fibroblasts (TTFs).
3. The cells must express high levels of the dox trans-activator M2rtTA.
4. The vectors used to generate these cells can be found in Addgene. https://www.addgene.org/browse/article/6005/

Day 1- Transfection

1. For transfection, prepare two 10cm plates of HEK293T cells at a density of 70%. It is advisable to seed the HEK293T cells on gelatin (0.2%). This will allow them to adhere better to the plate.
2. Warm "Mirus" reagent to room temp and vortex gently (you can use any transfection reagent that you like as long as it works well for HEK293T cells).
3. Take two sterile eppendorf tubes and place 1ml of RPMI-1640 in each tube.
4. Add 40µl "Mirus" reagent to each tube.
5. Pipet gently to mix completely.
6. Add the following dox-inducible lenti-viral vectors to the diluted "Mirus" reagent according to the below concentrations and ratios (each tube will eventually get a total of 20µg of DNA):

   1st tube: 5µg of FUW-tetO-Nr5a1 and 5µg FUW-tetO-Wt1

   2nd tube: 3.3µg of each FUW-tetO-Gata4, FUW-tetO-Sox9 and FUW-tetO-Dmrt1.

   To each tube add 10µg of virus packaging proteins: 7.5µg psPAX2 and 2.5µg pGDM.2. Pipette gently to mix completely.

7. Incubate at room temperature for 15-30 minutes. In the meantime, aspirate medium from the 10cm HEK293T plates, and **carefully** add new 9ml of 10% FBS DMEM medium.
8. Add "Mirus"/DNA mixture drop by drop into the HEK293T plates.
9. Place the plates in the incubator at 37ºC and 5% C02 overnight.
10. Thaw freshly isolated MEFs or TTFs, at passage 0 or 1, into one 10cm plate (the cells should be around 50-60% confluency).

1 Day post transfection:

1. Remove the media from the HEK293T plates and replace it with 7ml regular DMEM 10%FBS medium to each plate.
2. Monitor the MEFs/TTFs that you thawed yesterday to make sure they are at good confluency ~70% (in order to reach 90-100% confluency at the end of the infections).

2 Days post transfection:

1. Collect the Virus-supernatant from the two HEK293T plates into one tube (total 14ml).
2. Add Polybrene at a concentration of 8µg/ml.
3. Filter the supernatant through a 0.45um filter (this is a very important step in order to get rid of any floating HEK293T cells).
4. Add new 7ml of 10%FBS DMEM medium gently to the same plates of HEK293T cells and place back in the incubator.
5. Remove the current medium from the MEFs/TTFs, and replace it with the filtered Virus-supernatant.
6. Wait at least 8 hours, and then do the same procedure of infection. Incubate the cells at 37ºC and 5% CO2 overnight.

3 Days post transfection:

1. Collect the Virus-supernatant from the two HEK293T plates into one tube (total 14ml).
2. Add Polybrene at a concentration of 8µg/ml.
3. Filter the supernatant through a 0.45um filter.
4. Add new 7ml of 10%FBS DMEM medium gently to the same plates of HEK293T cells and place back in the incubator.
5. Remove the current medium from the MEFs/TTFs, and replace it with the filtered Virus- supernatant.
6. Wait at least 8 hours, and then do the same procedure of infection. Incubate the cells at 37ºC and 5% CO2 overnight.

- **In total, the starting cells should be infected with fresh viruses 4 times.**

4 Days post transfection:

Split the infected MEFs/TTFs into two 10cm plates whereby one of the plates will receive 70% of the infected cells and the other plate will get 30% of the infected cells. This is a very important step as the cells at this stage should be fully confluence and proliferation is essential for any reprogramming process. We split the plate in a ratio of 30:70 because in some cases strong infection will result in cell death and therefore we need the more dense plate (70%) and in other cases the infection will be relatively weak and the 70% plate will be crowded too early and then we will need the 30% plate.

5 Days post transfection:

1. Add Sertoli reprogramming medium to the plate (see medium list below).
2. Change medium **every second day** to allow proper reprogramming process.

One to three weeks later:

Watch the cells over the next 1-3 weeks.

The cells should acquire an epithelial morphology. This can also be tested by quantitative RT-PCR as EMT genes such as Twist1, Snai1, Snai2 (for more genes see the paper) should be downregulated and epithelial markers such as Cgn should be upregulated.

After three weeks other embryonic Sertoli markers such as Ptgds, Dhh, Gdnf, Erbb4, Amh (for more genes see the paper) should be strongly upregulated.

Three weeks after transfection

1. To polarize the cells prepare 6-well plate with matrigel (BD) (1:1) ration with reprogramming medium.
2. Seed reprogrammable cells (in different density) on top of the matrigel and monitor their morphology for two weeks. The cells should acquire a shiny morphology (see the paper). For the entire period the cells should be cultured in Sertoli reprogramming medium containing dox.
3. Collect individual colonies for further analyses.
4. The cells should grow on a plastic plate as a semi-epithelial colonies and should acquire aggregation capability (this can be seen after splitting; the cells cluster together and adhere to the plate as an aggregate- this phenomenon can also be seen sometime without the matrigel treatment).

Picking Colonies:

1. Mark the colonies under light microscope.
2. Take 96-well plate and add 100µl of trypsin to each well (the amount of wells is corresponding to the amount of colonies you would like to isolate).
3. Pick the marked colonies under the Binocular using 10µl pippetor.
4. Transfer each colony into 1 well of 96-well plate containing trypsin.
5. Incubate 5 minutes in the incubator.
6. Neutralize trypsin by adding 100µl of medium containing FBS.
7. Pipette up and down and then transfer into one well of 6-well plate containing Sertoli-reprogramming medium.

Sertoli-reprogramming medium

F12/DMEM

2% FBS

1% L-glutamine

1% Pen/Strep

1% Non-essential amino acids

LIF 1:10,000 (we are using homemade LIF, for the exact units see the manuscript)

2 µg/ml doxycycline

DMEM 10% FBS

DMEM

10% FBS

1% L-glutamine

1% Pen/Strep

Troubleshooting

Massive cell death is seen after the infection:

- Your cells got too much of viruses, infect three times instead of four times or seed the cells at a higher confluency.

The conversion process is not initiated:

- Make sure the cells contain the M2rtTA transactivator.
- Check the expression of your reprogramming factors 48h after dox addition.
- The cells are too dense, split the cells more sparsely.

Embryonic Sertoli markers are not upregulated after three weeks:

- Examine the levels of the reprogramming factors. In most cases the levels are not high enough to activate all the genes.

Please feel free to contact our lab for further information and help.