Cell Stem Cell
Article

Extensive Nuclear Reprogramming Underlies Lineage Conversion into Functional Trophoblast Stem-like Cells

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Generation of induced trophoblast stem cells (iTSCs)

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) only.
3. The cells must express high levels of the dox trans-activator M2rtTA.
4. The lentiviral vector backbone used to generate these cells is FUW-tetO.
5. **Before running these experiments make sure you know how to culture blastocyst-derived TSCs.**

Day 1- Transfection

1. For transfection of Gata3, Eomes, Tfap2c and Myc (GETM) or Gata3, Eomes and Tfap2c (GET), 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: 3µg of FUW-tetO-Gata3, 3µg FUW-tetO-Eomes, 3µg FUW-tetO-Tfap2c and 1µg FUW-tetO-Myc.

   2nd tube: 3.3µg of FUW-tetO-Gata3, 3.3µg FUW-tetO-Eomes and 3.3µg FUW-tetO-Tfap2c.

   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% CO2 overnight.
10. Thaw freshly isolated MEFs/TTFs, at passage 0, into two 10cm plates (the cells should be around 50-60% confluency in each plate).

1 Day post transfection:

1. Remove the media from the HEK293T plates and replace it with 10ml 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 about 90-100% confluency at the end of the infections).

2 Days post transfection:

1. Collect the Virus-supernatant from each HEK293T plates into two separate tubes.
2. Add Polybrene at a concentration of 8µg/ml.
3. Filter the supernatant through a 0.45µM filter (this is a very important step in order to get rid of any floating HEK293T cells).
4. Add new 10ml 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 (each plate receive 10ml of filtered medium).
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 each HEK293T plates into two separate tubes.

2. Add Polybrene at a concentration of 8µg/ml.
3. Filter the supernatant through a 0.45µM filter.
5. Remove the current medium from the MEFs/TTFs, and replace it with the filtered Virus-supernatant (each plate receive 10ml of filtered medium).
6. Wait at least 8 hours, and then remove the Virus-supernatant and add fresh 10% FBS medium to each plate. Incubate the cells at 37ºC and 5% CO2 overnight.

- **In total, the starting cells should be infected with fresh viruses 3 times.**

4 Days post transfection:

1. Replace the medium of the infected MEFs/TTFs to TSC reprogramming medium containing dox (2µg/ml, see list of media below). The cells should be around 90% confluency. Because the reprogramming process involves cell death do not split the cells before adding dox.
2. Change medium **every second day** to allow proper reprogramming process.

Three weeks later:

Watch the cells over the next 3 weeks.

The vast majority of the cells should exhibit robust morphological change as the cells undergo MET and rapid proliferation (especially in the GETM reprogramming cocktail). You should expect to see many colonies with different morphologies (some of the colonies even look like iPSCs but these **ARE NOT** iPSCs). It is hard to observe a clear morphology of iTSC colonies when the cells are still on dox.

1. Following 3 weeks, remove the TSC reprogramming medium containing dox and replace it with TSC culturing medium (without dox). Wait 10 days and scan thoroughly the plate for iTSC colonies. You should expect to see in the plate many differentiated regions that contain also clear iTSC colonies or a cluster of cells that contains iTSCs (it is easy to notice the iTSCs if your parental MEFs/TTFs contain a TSC fluorescent reporter such as Sox2),

Picking Colonies:

1. Mark the colonies under light microscope. Mark both clear iTSC colonies and differentiated regions containing cluster of cells.
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 regions under the Binocular using 10µl pippetor.
4. Transfer each colony/cells into 1 well of 96-well plate containing trypsin.

5. Incubate 5 minutes in the incubator.
6. Neutralize trypsin by adding 100µl of TSC culturing medium.
7. Pipette up and down and then transfer into one well of 6-well plate containing TSC culturing medium and feeder.
8. Culture the iTSC clones on feeder for at least 10 passages before weaning them from feeder. After 10 passages the cells should adopt the culture condition and should exhibit all the function exerted by blastocyst-derived TSCs. Notice that during the 10 passages, massive differentiation may occur but stable colonies will always emerge after few days.

TSC reprogramming medium

RPMI

20% FBS

1% L-glutamine

1% Pen/Strep

1% Non-essential amino acids

Fgf4 (25ng/ml)

Heparin (1µg/ml)

During reprogramming add 2 µg/ml doxycycline.

TSC culturing medium

70% of MEF-conditioned medium [TSC reprogramming medium (without supplements and dox) that was incubated for three days on MEF-feeder cells and filtered through 0.45µM filter].

30% TSC reprogramming medium (without dox).

Add fresh Supplements (Fgf4 and Heparin)!

DMEM 10%FBS

DMEM

10% FBS

1% L-glutamine

1% Pen/Strep

Troubleshooting

Massive cell death is seen after the infection:

- Your HEK293 cells produce too much of viruses, infect fewer times or seed the MEFs/TTFs at a higher confluency (preferable).

The conversion process is not initiated:

- Make sure your cells are on their log phase of proliferation and that they express the M2rtTA transactivator.
- Make sure your infection works on the parental cells.
- Infect four times instead of three times.

I do not get colonies:

- Examine the levels of the reprogramming factors. They should be highly expressed.
- Following three weeks of dox addition, one can trypsinize the entire plate and seed all the cells on a new plate containing feeder. This step aids in stabilizing any potential iTSC colonies.
- **VERY IMPORTANT- make sure your MEF-CM, Fgf4 and Heparin support the growth of blastocyst-derived TSCs before using them on iTSC culture.**

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