Direct lineage reprogramming of fibroblasts to induced endoderm progenitors (iEPs)

1. Retrovirus Production

Materials

- Cells
  - 293T cells
- DNA
  - Retroviral expression plasmid
    - pGCDNsam-Hnf4α-t2a-Foxa1-IRES-GFP, *or*
    - pGCDNsam-Hnf4α-IRES-GFP, pGCDNsam-Foxa1-IRES-GFP
  - Retroviral packaging plasmid
    - pCL-Eco
- Reagents
  - DMEM
  - X-tremeGENE 9 Transfection Reagent (Roche)
- Media
  - MEF medium: DMEM with 10% FBS, 1% pen/strep, 1× 2-Mercaptoethanol, 0.22-µm filtered

Steps

Day -2

1. Thaw and plate 293T at 3×10^6 per 10-cm plate for next-day transfection
   Typically, one tube of frozen 293T comes from one confluent 10-cm plate and contains ~15×10^6 cells, which is enough for four to five 10-cm plates

Day -1: Transfection

2. Check that your cells are 80-90% confluent
   Cells should be 80-90% confluent after 24 hours; adjust seeding density accordingly to your schedule
3. Calculate DNA volumes; for each 10-cm plate, you'll need:
   - 5 µg pCL-Eco
   - 5 µg retroviral expression plasmid
4. Label two 1.5-mL microcentrifuge tubes for each 10-cm plate
5. In the first tube, combine:
   - 200 µL DMEM
   - 15 µL X-tremeGENE 9

   Pipet X-tremeGene 9 directly into DMEM without touching the plastic tube wall

6. In the second tube, combine:
    - 200 µL DMEM
    - x µL pCL-Eco (5 µg)
    - x µL retroviral expression plasmid (5 µg)
7. Add DMEM/DNA mix dropwise to DMEM/X-tremeGENE 9 mix, and incubate at room temperature for 20 minutes
8. Add the transfection mixture dropwise to cells
   Plan to do transfection in the late afternoon for overnight incubation with the transfection mix

Day 0

9. Remove the transfection mix by changing to fresh media

Day 1: Virus collection

10. Collect the virus-containing media with a 10-mL syringe; set syringe aside
11. Add fresh media to 293T
12. Filter the virus through a 0.45-µm filter and store at 4 degree
    Virus can be stored at 4 degree for about a week; plan to have MEFs ready for transduction around Day 1 of virus collection

Day 2: Virus collection

13. Collect the virus-containing media with a 10-mL syringe; set syringe aside
14. 293T cells can now be discarded
15. Filter the virus through a 0.45-µm filter and store at 4 degree
    Virus can be used fresh; alternatively, concentrated virus can be used or stored at -80. See "PEG virus concentration" for protocol

2. MEF Transduction

Materials

- Cells
  - E13.5 mouse embryonic fibroblasts
- Virus of reprogramming factors Hnf4α and Foxa1
- Reagents
  - TrypLE Express
  - Protamine sulfate (10 mg/mL stock, 500×)
  - 0.1% gelatin, 0.22-µm filtered
    - 47.5 mL PBS
    - 2.5 mL 2% gelatin
  - Collagen coating solution, 0.45-µm filtered
    - 10 mL PBS
    - 11.4 µL glacial acetic acid
    - 167 µL collagen stock (3 mg/mL)
- Media and supplements
  - MEF medium: DMEM with 10% FBS, 1% pen/strep, 1× 2-Mercaptoethanol, 0.22-µm filtered
  - HepBase: DMEM/F12 with 10% FBS, 1% pen/strep, 10 mM nicotinamide, 10^{-7} M dexamethasone, 1 µg/mL insulin, 1× 2-Mercaptoethanol, 0.22-µm filtered
    - Quick reference: for a 225-mL batch, combine
      - 200 mL DMEM/F12 glutamax-I
      - 22 mL FBS
      - 2.2 mL pen/strep
      - 1.1 mL nicotinamide stock (2 M)
      - 20 µL dexamethasone stock (1 mM)
      - 20 µL insulin stock (10 mg/mL)
      - 220 µL 2-Mercaptoethanol (1000×)
  - Epidermal growth factor (100 µg/mL stock)
    - Quick reference: use 2 µL per 10 mL media for a final concentration of 20 ng/mL
- HepBase stock recipes
  - Nicotinamide (2 M): resuspend 2.44 g in 10 mL DMEM/F12
  - Dexamethasone (1 mM): resuspend 25 mg in 25 mL of absolute ethanol, then add 38.7 mL DMEM/F12
  - EGF (100 µg/mL): resuspend 0.1 mg in 1 mL filtered PBS + 1% FBS

iEP Protocol
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Steps

Day -2

1. Coat dishes with 0.1% gelatin; incubate at room temperature for 30 minutes and wash 2× with PBS
2. Thaw and plate E13.5 MEFs at 200,000 cells per 6-cm dish
   MEFs seeding density can be adjusted accordingly to be lower for early passage MEF, or higher for late passage MEF, and also to your schedule

Days 1-6: Transduction

3. MEFs should be 20-30% confluent on Day 1
   You should have some virus ready at this point
4. For each 6-cm dish, aspirate media and add 3 mL of virus with 6 µL of 500× protamine sulfate stock
5. Repeat transduction for a total of 4-6 times
   Check for GFP expression after 24-48 hours
6. One day after the last transduction, change media to HepBase

Day 5-7: Replating should be done when reprogramming cells become confluent

7. Coat 6-well plates with collagen; incubate at room temperature for 30 minutes and wash 2× with PBS
8. Wash cells 1× with PBS, then treat with TrypLE Express for 5 minutes at 37 degree
9. Using a P1000, gently pipet cells up and down until most cells are dissociated
10. Neutralize with media and spin down at 300× g for 5 minutes
11. Count cells and plate at 150,000 cells per well of a 6-well plate in HepBase + EGF
    Change media every 3-4 days. Passage every week. iEP colonies should start emerging after 2 weeks!