Mouse ESC Differentiation to Nkx2.1+ Lung and Thyroid Progenitors

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[Abstract] The *de novo* derivation of lung progenitors from pluripotent stem cells provides the opportunity to model early lung development *in vitro* and allows easy access to cells for tissue engineering or basic cell biology studies. This detailed protocol allows the generation of lung and thyroid progenitors from mouse embryonic stem cell (ESC) or induced pluripotent stem cell (iPSC) lines. When used together with a published Nkx2.1-GFP knock-in ESC line, the protocol allows tracking and purification of lung and thyroid progenitors by sorting on the GFP reporter based on the induction of the earliest known marker of lung and thyroid cell fate, Nkx2.1. After sorting, a pure population of Nkx2.1+ cells can then be replated for further expansion, differentiation, and maturation in culture in serum-free conditions.

Materials and Reagents

1. Mouse ESCs or iPSCs carrying a GFP reporter knocked in to the Nkx2.1 locus (Nkx2.1-GFP ESCs) (Longmire *et al.*, 2012)
2. 1x 0.05% Trypsin-EDTA (Life Technologies, Gibco®, catalog number: 25300-054)
3. Defined Fetal Bovine Serum (Hyclone, catalog number: SH30070.03)
4. IMDM powder (Life Technologies, Invitrogen™, catalog number: 12200-036)
5. $NaHCO_3$ (Sigma-Aldrich, catalog number: S-5761)

6. Pen/Strep (Life Technologies, Invitrogen™, catalog number: 15140-148) (10,000 U Penicillin and 10 mg Streptomycin per ml)
7. Cellgro water (VWR, catalog number: 45000-672)
8. Ham's F-12 (Cellgro, catalog number: 10-080-CV)
9. B-27 supplement with RA (Life Technologies, Invitrogen™, catalog number: 17504-044)
10. N-2 supplement (Life Technologies, Invitrogen™, catalog number: 17502-048)
11. BSA Fraction V 7.5% in PBS (Life Technologies, Invitrogen™, catalog number: 15260-037)
12. 1-thioglycerol (MTG) (Sigma, M6145-25ml)
13. 200 mM L-Glutamine (Life Technologies, Invitrogen™, catalog number: 25030-081)
14. Ascorbic Acid (Sigma-Aldrich, catalog number: A4544-25G)
15. 1M HEPES (Gibco, 15630-080)
16. $CaCl_2$ (Sigma-Aldrich, catalog number: C4901)
17. BSA (Sigma-Aldrich, catalog number: A9418-10G)
18. 100x ITS supplement (BD Biosciences, catalog number: 354352)
19. mNoggin (R&D Systems, catalog number: 1967-NG-025)
20. SB431542 (Sigma-Aldrich, catalog number: S4317)
21. mWnt3a (R&D Systems, catalog number: 1324-WN-010)
22. hBMP4 (R&D Systems, catalog number: 314-BP-050)
23. hEGF (R&D Systems, catalog number: 236-EG-01M)
24. mFGF2 (R&D Systems, catalog number: catalog number: 3139-FB-025)
25. mFGF7 (R&D Systems, catalog number: 5028-KG-025)
26. hFGF10 (R&D Systems, catalog number: 345-FG-025)
27. Heparin sodium salt (Sigma-Aldrich, catalog number: H4784-250mg)
28. Dexamethasone (Sigma-Aldrich, catalog number: D4902)
29. 8 - Br - cAMP (Sigma-Aldrich, catalog number: B7880)
30. IBMX (Sigma-Aldrich, catalog number: I5879)

31. DMSO, Hybri-Max (Sigma-Aldrich, catalog number: D2650)
32. Ethanol (Sigma-Aldrich, catalog number: E7023)
33. Activin A (R&D Systems, catalog number: 338-AC)
34. PBS (Life Technologies, Gibco®, catalog number: 14190-250)
35. 0.1% Gelatin in ultrapure water (EMD Millipore, catalog number: ES-006-B)
36. Cxcr4 Antibody: APC Rat anti-mouse CD184 (Cxcr4) (BD-Pharmigen, catalog number: 558644)
37. cKit Antibody: PE Rat anti-mouse CD117 (cKit) (BD-Pharmigen, catalog number: 553355)
38. APC Isotype: APC Rat IgG_{2b}, κ (BD-Pharmigen, catalog number: 553991)
39. PE Isotype: PE Rat IgG_{2b}, κ (BD-Pharmigen, catalog number: 553989)
40. IMDM (see Recipes)
41. Serum free differentiation medium (SFD) (see Recipes)
42. Complete serum free differentiation medium (cSFDM) (see Recipes)
43. BASE medium for DCI+K (see Recipes)
44. Anteriorization medium (see Recipes)
45. Ventralization medium (see Recipes)
46. DCI+K medium (see Recipes)
47. Preparation of 10x cAMP+IBMX stock (see Recipes)

Equipment

1. P100 Petri dish (100 mm x 15 mm Bacteriological Petri Dish, nontreated polystyrene, BD Falcon™, catalog number: 351029)
2. P150 Petri dish (150 mm x 15 mm Bacteriological Petri Dish, nontreated polystyrene, BD Falcon™, catalog number: 351058)
3. 12 x 75 mm, 5 ml polystyrene round bottom test tube with a cell strainer cap (BD, 352235)

4. 1.5-ml Eppendorf Snap-Cap microcentrifuge tubes (Thermo Fisher Scientific, catalog number: 05-402-25)
5. Centrifuges
6. LSRII flow cytometer
7. 0.22μm filter

Procedure

A. Timeline

1. Timepoint: 0 h
   a. Nkx2-1-GFP mouse ESCs or iPSCs are cultured in 2i_LIF (serum-free, feeder-free) conditions. For each experiment, a new vial of passage 23 is thawed and cells are used after two passages.
   b. Seed 500,000 Nkx2-1-GFP mouse ESCs or iPSCs (in suspension) per P100 Petri dish with 12.5 ml/dish cSFDM.
   c. During this period of time ESCs or iPSCs will form embryoid bodies (EBs) between 100-300 μm that will remain in suspension.
2. Timepoint: 60 h
   a. Collect EBs from one dish in a 50-ml conical, let EBs settle for 1-2 min and carefully remove most of the supernatant containing dead cells and debris.
   b. Spin 5 min, 300 *x g*, 4 °C.
   c. Aspirate off supernatant.
   d. Add 1 ml of 0.05% trypsin/EDTA per tube, incubate at 37 °C water bath for 1 minute while swirling the tube.
   e. Disaggregate EBs to form single cell suspension by gentle trituration.
   f. Add 1 ml serum to block the trypsin.
   g. Add 4 ml IMDM.

h. Spin 5 min, 300 *x g*, 4°C.

i. Resuspend the cells in 5 ml cSFDM.

j. Count cells.

k. Plate 0.5-1 x 10^6 cells (in suspension) per P100 or P150 Petri dish in 25 ml cSFDM supplemented with 50 ng/ml Activin A for definitive endoderm induction and EB formation.

3. Timepoint: 120 h (Day 5)

a. Perform staining for surrogate definitive endoderm markers (Cxcr4/cKit, see FACS protocol) to confirm efficient definitive endoderm induction (more than 40-50% of cells should be $Cxcr4^+/cKit^+$).

b. Collect EBs and wash in IMDM.

c. Spin 5 min, 300 *x g*, 4 °C.

d. Resuspend in 10-15 ml of Anteriorization media and plate in suspension in new P100 Petri dish.

4. Timepoint: 144 h (Day 6)

a. Collect EBs.

b. Wash in IMDM.

c. Spin 5 min, 300 *x g*, 4 °C.

d. Get cell count by trypsinizing a 1 ml aliquot of EBs to get single cells and calculating total cell number equivalent.

e. Resuspend in ventralization media.

f. Plate the EB equivalent of 50,000 cells/cm^2 on gelatin--coated plates or dishes (*e.g.* 100,000/well of a 24--well plate). For coating procedure see step 2 below.

g. Change media every other day.

h. GFP^+ will start emerging by Days 8-9.

5. Timepoint: Day 15

a. Remove ventralization media and add appropriate volume of trypsin (*e.g.* 1 ml per well of a 6-well plate or 3-4 ml per P100 dish).

b. Transfer cells from several wells in a 50-ml conical, create a single cell suspension by gentle trituration and inactivate with an equal volume of FBS.

c. Resuspend in PBS^+ (PBS+2% FBS).

d. Sort GFP^+ cells.

e. Spin 7 min, 500 *x g*, 4 °C (note the change in centrifuge settings).

f. Replate 25,000 cells/cm^2 (*e.g.* 50,000 cells/well of a 24-well plate) in cSFDM supplemented with FGF2 (250 ng/ml), FGF10 (100 ng/ml) and heparin salt (100 ng/ml).

g. Change media every other day for 7 days.

6. Timepoint: Day 22

a. Remove media and rinse with PBS.

b. Switch to DCI+K media.

c. Harvest cells on Day 25 (procedure same as on Day 15).

B. Gelatin coating

Apply 0.1% gelatin (dissolved in ultrapure water) for 25-30 min at room temperature (*e.g.* 1 ml in a well of a 6-well plate). Aspirate gelatin, rinse with PBS and aspirate again. The plate is now ready to use.

C. FACS for Cxcr4/cKit

1. Cell count

a. Remove 1 ml of EBs, spin down and trypsinize (0.5 ml trypsin), count cells.

b. Based on previous cell count, remove a culture volume that corresponds to 2 - 2.5 x 10^6 cells.

c. Repeat procedure for undifferentiated ES cells.

2. Preparation of cells for staining (all steps on ice)
   a. Spin down EBs, resuspend in 1 ml trypsin (60 sec at 37 °C), monodisperse with a P1000 pipette.
   b. Inactivate with 1 ml serum, spin down (5 min, 300 *x g*, 4 °C) and wash once with 5 ml IMDM.
   c. Resuspend in 500 µl PBS^+ (PBS+2% FBS), cell concentration should be 0.4 - 0.5 x 10^6 cells/100 µl.
   d. Prepare 2 x 5 Eppendorf tubes (unstained, isotypes, Cxcr4, cKit, Cxcr4/cKit (double)), mark each tube series (D5 endoderm or undifferentiated ES cells) and transfer 100 µl of each culture to each tube.
3. Cell staining
   a. Add the appropriate antibodies per tube (*e.g.* no antibodies in the "unstained" tube, both antibodies in the "double" tube).

      Antibodies used for this protocol as of 08/15/12:

      Isotype: APC Rat IgG_{2b}, κ

      Isotype: PE Rat IgC_{2b}, κ

      Cxcr4: APC Rat α-mouse CD184

      cKit: PE Rat α-mouse CD117
   b. Vortex briefly and transfer on ice for 30 min (cover with aluminum foil, vortex once again at 15 min).
   c. Add 1 ml PBS^+ per tube, spin at 300 *x g* for 5 min in a tabletop centrifuge, carefully aspirate supernatant.
   d. Resuspend pellet in 500 µl PBS^+.
   e. Transfer to FACS polystyrene tubes with the cell strainer cap.
   f. Take cells to LSRII for analysis.

Recipes

1. IMDM

   1 packet IMDM powder

   3.02 g $NaHCO_3$

   10 ml Pen/Strep

   1 L Cellgro water

   Check pH (acceptable range 6.9≤ pH ≤7.3)

2. Serum free differentiation medium (SFD)

   IMDM-375 ml

   Ham's F-1-125 ml

   B-27 supplement with RA-5 ml

   N-2 supplement-2.5 ml

   BSA 7.5% in PBS-3.3 ml

3. Complete serum free differentiation medium (cSFDM)

   SFD -100 ml

   MTG 300 µl of stock (Stock: 26 µl MTG to 2 ml IMDM)

   200 mM L-Glut -1 ml

   Ascorbic Acid -1 ml of stock (Stock: 5 mg/ml distilled water, prepare fresh!)

4. BASE medium for DCI+K

   Ham's F-12-243.7 ml

   1.0 M HEPES (pH 7.4)-3.75 ml

   1.0 M $CaCl_2$-200 µl

   BSA-0.625 g

   100x ITS supplement-2.5 ml

5. Anteriorization medium

   cSFDM-10 ml

mNoggin -100 ng ml (Stock: 10 µg/ml)

SB431542 -10 µM (Stock: 10 mM in DMSO)

6. Ventralization medium

   cSFDM – 10 ml

   mWnt3a - 100 ng/ml (Stock: 100 µg/ml)

   hBMP4 - 10 ng/ml (Stock: 10 µg/ml)

   hEGF - 20 ng/ml (Stock: 20 µg/ml)

   mFGF2 - 250 ng/ml (Stock: 100 µg/ml)

   mFGF7 – 10 ng/ml (Stock: 10 µg/ml)

   hFGF10 - 10 ng/ml (Stock: 10 µg/ml)

   Heparin sodium salt - 100 ng/ml (Stock: 1 mg/ml)

7. DCI+K medium

   BASE media - 25 ml

   Dexamethasone - 50 nM (Stock: 250 µM in ethanol)

   KGF (FGF7) - 10 ng/ml (Stock: 10 µg/ml)

   cAMP+IBMX - 0.1 mM (Stock: 1 mM cAMP+1 mM IBMX)

8. Preparation of 10x cAMP+IBMX stock

   Dissolve 22.22 mg IBMX in 1 ml of DMSO (0.1 M IBMX stock, store at -20 °C)

   To prepare the 1 mM cAMP+1mM IBMX (10x) stock, dissolve 21.5 mg 8BrcAMP in 49.5 ml of BASE media and add 0.5 ml of IBMX stock

   0.22 µm filter

   Store at 4 °C for up to 4 weeks

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