Generation of Nkx2.1+ lung endoderm and Nkx2.1+Sox2+ proximal airway progenitors from mouse embryonic stem cells

Hongmei Mou
Rajagopal Lab
March 2012

Step I: Protocol to induce definitive endoderm from mouse ES cells

1. Mouse embryonic stem cells (mESCs) are grown in "mES self-renewal medium" on the irradiated MEFs. MEF feeders are seeded at the density of 1-2x10^6 cells per 6-well plate a day before use. The plates used for both mESC maintenance and definitive endoderm differentiation are pre-coated with gelatin.

2. Feed the mESCs every day. Split the cells at 70-80% confluence (~every 3 days). Differentiation experiment can be performed at 50-80% confluence of mESCs. We usually feed ESCs with fresh medium in the morning before splitting for differentiation experiment in the afternoon.

<u>mES self-renewal medium</u>
500 ml KO DMEM
15% Hyclone FBS (75 ml)
1% Glutamax (5 ml)
1% NEAA (5 ml)
1:1,000 BME stock (0.5 ml)
1:10,000 ESGRO LIF (0.05ml)
(Note: No antibiotics are used in mES self-renewal medium)

<u>Gelatin-coating</u>
7 ml of gelatin per 15 cm dish
750 microliter of gelatin per each well of 6-well plate
30 microliter of gelatin per each well of 96-well plate
Incubate at 37oC for 30 min (I usually do over night coating beforehand)

Note: check the ESCs periodically for the karyotype, expression of multipotent markers (SOX2, OCT3/4, NANOG, C-MYC) and mycoplasma contamination.

3. Trypsinize ESCs at 37^{o}C (usually takes 2-5 min, do not over-trypsinize the cells), quench with DMEM + 10% FBS, spin, resuspend the cells in ES medium and plate onto gelatin-coated 15 cm dishes (one 15-cm dish per 1 well of a 6-well plate)

4. Let MEF depletion proceed for ~30 min.

5. Prepare gelatin coat appropriate differentiation plate (we usually use a 6-well plate for bulk experiments or 96-well plates for characterization) and also prepare D0 differentiation medium. Pre-warm at 37oC for use.

D0 differentiation medium
Advanced DMEM
1% N2 (optional, can be omitted)
2% B-27 (retinoic acid-free)
0.05% Albumax II
Glutamax
Penicillin/streptomycin
Add Albumax II before use

6. After 30 min MEF depletion, examine the dish under the microscope. Almost all MEF cells and a small fraction of ESCs should have already attached to the plate while majority of ESCs are in the suspension state. If MEF cells are not attached well, give them extra 10-15 min for further attachment; examine the cells every 5 min.

7. Gently collect the supernatant containing ESCs without perturbation of the attached MEFs, transfer the cells to centrifugal tubes, and spin at 500 g for 5 min.

8. Aspirate media and rinse the cells with D0 differentiation medium (Note: it is important to dilute out the ES media, as LIF potently inhibits endoderm differentiation).

9. Spin and aspirate media. Resuspend the cell pellet with 2 ml of D0 differentiation media + 10 microM ROCK kinase inhibitor Y-27632 (Y-27632 is critical to decrease cell death due to trypsinization). Count the cells.

10. Based on the cell numbers, adjust the medium to appropriate volume and plate the cells onto gelatin-coated plates. Cells should be plates at a density of 5-8*10^3 cells/cm^2 (eg 5-8*10^4/well of a 6-well plate).

Note: Cell density has significant effect on differentiation, so obtaining cells near this range of density is important
Note: This cell seeding density is based on p2A mESC. The cell seeding density for other mESCs can be adjusted based on their cell death and their proliferation rate during the differentiation.

11. Count the day of plating as **day 0**. In most cases, the cells do not need new medium on day 1. Differentiation medium, compounds and growth factors will first be added on day 2 (usually at 48 hours, although interval can be 42-50 hours).

12. On day 2, aspirate the medium and replace with D2 differentiation medium. For 6-well plate, use 1.5 ml of D2 medium per well on this day.

D2 differentiation medium
Advanced DMEM
2% FBS + Glutamax
5 nM GSK3iXV
50 ng/mL Activin A
Penicillin/streptomycin

13. On day 3 and day 4, feed the cells daily with D3 differentiation medium. For 6-well plate, use 2.5 ml of medium per well on D3 and 3-4 ml of medium on D4 due to increase in cell number.

D3 differentiation medium
Advanced DMEM
2% FBS
Glutamax
50 ng/mL ActivinA
2 uM Dorsomorphin
Penicillin/streptomycin
Note: on day 3, the cells start to change morphology from typical small undifferentiated ES cells into slightly bigger and flatter endodermal cells. By day 4, a significant percentage of cells are Sox17+Foxa2+.

14. On day 5, the cells are usually confluent. >80-95% of cells should be Sox17+Foxa2+ based on immunofluoresent staining. Alternatively, the efficiency of definitive endoderm generation can be quantified using FACS analysis with cKit/CXCR4 and cKit/EpCAM combination.

Step II: Protocol to anteriorize the definitive endoderm to foregut endoderm

15. On day 5, rinse the cells with warm PBS 2 times to remove residual Activin A and other compounds. If the cells are not very confluent at this moment, you may continue to treat the cells with anteriorization medium (see below). Alternatively, if the cells are too confluent, split the cells at 1:3 ratio and then continue to the next differentiation step.

Split the cells before anteriorization:

16. If splitting of cells is necessary, dissociate the cells with trypsin gently, and split the cells 1:3 (i.e. 1 well into 3 well, you don't need to count the cells). Resuspend the cells in anteriorization medium (3 ml of medium per well of 6-well plate)

containing10 uM Y-27632. The plates are pre-coated with 804G-conditioned medium.

Note: For the following steps, we use a plate coated beforehand with 804G-conditioned medium with laminin-enriched matrix (1 ml per well of 6-well plate, coat plates at least 2-16 hours at 37oC). Coating with gelatin can also work with decreased cell viability and differentiation efficiency.

Anteriorization medium

Advanced DMEM
2% B-27 (RA-free)
1% Glutamax
0.5% FBS
Penicillin/streptomycin
1-2 uM TGFb antagonist A8301
100-500 nM WNT antagonist IWR-1
(Add A8301 and IWR-1 fresh before use)

Note: The concentration of A8301 and IWR-1 varies between cell lines. For p2A mES cell line, we use 1 microM A8301 and 100 nM IWR-1. Optimize the dose of TGFb and WNT antagonists for any new cell line used.

Note: A8301 can be replaced with 5-10 microM of SB4315342 (another TGFb antagonist). We find that A8301 works better than SB431542 in p2A cells.

17. Anteriorization usually needs 24-48 hours. Fix the cells and check expression of Foxa2/Sox2 to determine the anteriorization efficiency.

Note: For p2A mES cells, >70% of cells can be anteriorized based on Foxa2/Sox2 expression

Note: Timing of anteriorization depends on the cell line used. For p2A cells, one day (24-30 hours) of TGFb and WNT inhibition is enough to pattern the endoderm into anterior foregut cells. Timing and antagonist concentration need to be optimized for each new cell line used.

Note: Some cell lines (such as V6.5 mESC) are resistant to turn on Sox2 expression. However, the cells can still generate Nkx2.1-positive cells in later steps. Despite low or no Sox2 expression in some cell line, anteriorization is necessary for Nkx2.1-positive cell differentiation in the next step.

Step III. Generation of Nkx2.1 positive endoderm from anterior foregut cells

18. After 24-48 hours of TGFb inhibition + WNT inhibition (time of treatment and dose of compounds are varied between cell lines), wash the cells with PBS, add trypsin until

the cells detach, quench with DMEM + FBS, collect cells, spin and aspirate media. [*This step might be necessary especially when the cell splitting is omitted before Step II since the cells might be confluent. If the cells have been already split before Step II, in most cases Nkx2.1 induction can be continued without cell dissociation*]

19. Resuspend pellet in a desired volume of Nkx2.1 induction medium with 10 microM Y-27632 (For 96-well plate, ~150 ul of differentiation medium per well; for 6-well plate, 3-4 ml of differentiation medium for well). Again, addition of Y-27632 is vital for maintaining viability after trypsinization.

Nkx2.1 induction medium
Advanced DMEM
2% B-27 (supplement with RA)
1% Glutamax
0.5% FBS
Penicillin/streptomycin
20-100 nM BMP4
20-100 ng/ml FGF2
5-50 nM GSK3iXV (or 100-1000 nM CHIR-99021).
Note: The doses of BMP4, FGF2, GSK3iXV or CHIR-99021 need to be optimized for each cell line used. Usually, Nkx2.1 production efficiency is affected by BMP4 and FGF2 in a dose-dependent manner. Optimizing the concentration of the WNT agonist is important because low concentration may not be enough to induce Nkx2.1 expression effectively, while a high concentration will drive the cell to the hindgut fate.

20. Incubate the cells for 2-4 days, changing the media every 24 hours. Fix the cells and check for Nkx2.1 expression and other endodermal markers. Again, the timing of induction is varied between cell lines. For p2A cell, Nkx2.1 expression can be detected after 24 hour of differentiation. After 48 hours, Nkx2.1 efficiency reaches a plateau. Longer time of differentiation can induce more Nkx2.1+ cells, but the number of Nkx2.1-negative cells will increase as well.

Step IV. Generation of Nkx2.1+Sox2+ positive endoderm from lung endoderm cells

21. To generate Nkx2.1+Sox2+ proximal progenitor cells, switch the cells to the Proximalization differentiation medium. [If you find the cells are too confluent, you can always split the cells first and then do proximalization. When you split the cells, re-seed the cells on the plates pre-coated with 804G-conditioned medium pre-coated for 2 -16 hours at 37oC].

Proximalization differentiation medium
Advanced DMEM
2% B-27 (RA-supplemented)

1% Glutamax
0.5% FBS
Penicillin/streptomycin
50 ng/ml BMP7
50 ng/ml FGF7
50-100 ng/ml IWR-1 (WNT antagonist)
1-2 uM PD98059 (MAPKK/ERK antagonist)

22. Incubate the cells for 2-4 days or longer. Change the media every 24 hours. Fix the cells. Check Nkx2.1, Sox2, p63 and other necessary markers.

Note: Longer time of differentiation can increase the percentage of Nkx2.1+Sox2+ cells. However, it also increases the total cell number of Nkx2.1-negative cells.

Materials used for this protocol

Name	Vender	Stock Concentration
KO DMEM		GIBCO 10829
Advanced DMEM		GIBCO 12491
FBS		Hyclone SH30070.03 (much more expensive than regular FBS so only use for ES cells)
ESGRO LIF		Millipore/Fisher
Glutamax		Invitrogen 35050061
NEAA		Sigma M7145
BME (1000x)		Invitrogen 21985-023
Irradiated MEF		Globalstem GSC-6202G
0.1% Gelatin		Millipore ES-006-B
Trypsin 0.25% 1x		Hyclone SH30042.01
N-2		GIBCO 17502-048
B-27 (without RA)		GIBCO 12587-010
B-27 (RA-supplement)		GIBCO 17504044