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

Hongmei Mou
Rajagopal Lab
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Step I: Protocol to induce definitive endoderm (DE) from human ESCs and iPSCs

1. Grow human ESCs and iPSCs with complete mTeSR1 medium on Geltrex-coated plates. Feed the cells every day and passage the cells at 65-80% confluence (~ every 3-4 days) at 1:4 to 1:10, depending on the confluence of the ESCs/iPSCs. On the day before splitting, thaw a Geltrex aliquot on ice, coat appropriate amount of plates for the next day (see below). To split ESCs / iPSCs, aspirate medium and wash cells with PBS. Aspirate PBS and apply the Accutase solution (0.8 ml per 6-well plate; 2.5 ml per 10-cm dish; 5 ml per 15-cm dish). Incubate cells at room temperature and observe colonies under the microscope. When the cells start to lose shape and detach, agitate the dish or plate at the corner to facilitate the detachment. When a good part of the colonies loose cell-cell and cell-dish attachment and the cells look round and shiny white, add PBS and pipette up and down several times gently to dissociate the cells from the dish and also make a single-cell suspension. Transfer cell suspension into a 50 ml falcon tube. Spin at 1000 RPM for 5 minutes. Aspirate supernatant and resuspend the cells in an appropriate volume of complete mTeSR1. Aspirate Geltrex coating solution from the new Geltrex-coated plates. Transfer cell solution to fresh-coated plates.

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

<u>Human iPSC maintenance medium</u>
500ml mTeSR1
50 ml of 10x supplement

<u>Geltrex coating</u>
For 6-well plate:
9 ml of ice cold RPMI-1640 medium
150 miroliters of Geltrex
Mix well
Add 1.5 ml to each well of 6-well plate
Incubate at 37oC for 2-16 hours.

2. Two days before the differentiation experiments, seed the cells at 35-40% confluency. Let the cells recover and grow robustly for 2 days (usually on the day of differentiation, the cell confluency will be at ~60-70%).

3. Aspirate the mTeSR1, rinse with warm RPMI-1640 two times to remove the residual growth factors in mTeSR1. Replace the medium with definitive endoderm differentiation medium (For 6-well plate, use 3 ml per well and include 10 uM Y-27632 to decrease cell death due to trypsinization). This is **Day 0**.

Definitive endoderm differentiation medium
RPMI-1640
2% B-27 (without RA)
0.1% Albumax II
Glutamax
1x non-essential amino acids (NEAA)
Penicillin/streptomycin
5 uM LY294002 (PI3K inhibitor)
100 ng/ml Activin A
[Add Albumax II, PI3K inhibitor and Activin A before use]

4. Change medium daily, differentiating the cells for 4 days (**Day 4**). To check the definitive endoderm efficiency, fix the cells and stain with Foxa2 and Sox17. Alternatively, the efficiency of definitive endoderm generation can be quantified using FACS analysis with cKit/CXCR4 and cKit/EpCAM combination

Note: In normal situation, we should only observe none or mild cell death during the DE differentiation. If dramatic cell death is observed, check the cell for the correct karyotype, expression of multipotent markers (SOX2, OCT3/4, NANOG, C-MYC) and mycoplasma contamination

Note: Different batches of LY294002 (PI3 kinase inhibitor) can affect cell death and differentiation efficiency. We routinely use LY294002 purchased from Invitrogen and Promega.

Note: 4 days of Activin A differentiation is enough to induce definitive endoderm with high efficiency in most human ESCs and iPSCs. However, the duration of Activin A exposure needs to be optimized when a new cell line is used, especially for patient-specific iPSC line. Some iPS cell lines need 5 days of Activin A differentiation to obtain maximal DE efficiency.

Note: RPMI-1640-based DE differentiation medium can be applied for most human ESCs and iPSC. However, we do find that some patient-derived iPSCs need different DE medium. For example, one of our iPS cell lines differentiates poorly in the above RMPI-1640-based DE medium, but differentiates much more efficiently in DMEM: F12 (2:1)-based DE medium.

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

5. On day 4, rinse the cells with warm PBS two times to remove residual Activin A and other factors. If the cells are not very confluent at this moment, you may directly

treat the cells with anteriorization buffer (see below). Alternatively, if the cells are too confluent, split the cells 1:3 and then continue the next differentiation step.

If Split the cells before anteriorization:

6. If splitting of cells is necessary, dissociate the cells with trypsin gently, and split the cells 1:3. Resuspend the cells in anteriorization medium (3 ml medium per well of 6-well plate) with10 microM Y-27632 and seed on Geltrex-coated plates

Anteriorization medium
RPMI-1640
2% B-27 (without RA)
0.1% Albumax II
Glutamax
1x non-essential amino acids (NEAA)
Penicillin/streptomycin
0.5-2 uM TGFb antagonist A8301
100-500 nM WNT antagonist IWR-1

Add Albumax II, A8301 and IWR-1 before use

Note: The concentration of A8301 and IWR-1 varies between cell lines. For most human ESC and iPSC, 0.5-1 microM A8301 and 100-200 nM IWR-1 are enough to induce anteriorization efficiently.

Note: A8301 can be replaced with 5-10 microM of SB4315342

Note: Some protocols include BMP antagonist Noggin in this step. We found Noggin is not necessary for anteriorization.

7. Anteriorization usually takes 2-4 days depending on the cell line. Fix the cells and check expression of Foxa2/Sox2 to determine the anteriorization efficiency.

Note: Some human ES cell lines and iPS cell lines are resistant to turning on Sox2 expression. However, the cells can still generate Nkx2.1 positive cells in later differentiation steps.

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

8. After finishing anteriorization, wash the cells with PBS and add Nkx2.1 induction medium (4 ml per well of 6-well plate). Or, if the cells are too confluent, split the cells 1:2 or more depending on the cell density, and reseed the cells on Geltrex-coated plates. Add ROCK kinase inhibitor Y-27632 in medium to maintain viability after trypsinization.

Nkx2.1 induction medium

RPMI-1640
2% B-27 (supplement with RA)
0.1% Albumax II
Glutamax
1x non-essential amino acids (NEAA)
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.

9. Incubate the cells for 4-5 days or longer. Change the media daily.

10. Fix the cells and check for Nkx2.1 and other endodermal marker expression.

Note: For most of human ESCs and iPSCs, 4-5 days of induction is enough to induce Nkx2.1 expression. Nkx2.1 expression can be detected after 48 hour of differentiation. Longer time of differentiation than 5 days might induce slightly more Nkx2.1+ cells. However, it also increases the number Nkx2.1-negative cells.

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

11. Directly switch to proximalization differentiation medium. If the cells are too confluent, split the cells first and then do proximalization, the cells were not split in the previous several steps. If you split the cells, reseed the cells on the plates pre-coated with Geltrex.

<u>Proximalization differentiation medium</u>
RPMI-1640
2% B-27 (supplement with RA)
0.1% Albumax II
Glutamax
1x non-essential amino acids (NEAA)
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)

12. Incubate the cells for 4 days or longer depending on the cell line used. Change the media daily. Fix the cells and check expression of Nkx2.1 and other markers. Longer period of proximalization than 4-5 days might increase the percentage of Nkx2.1+Sox2+ cells out of the total number of Nkx2.1+ cells. However, it also increases the number of Nkx2.1- negative fast cycling cells.

Materials used for this protocol

Name	Vender	Stock Concentrations
mTeSR1	StemCell 05850	
Geltrex	GIBCO/Invitrogen 12760	
Accutase	Innovative Cell Technologies AT104	
Activin A	Peprotech E coli 120-14E	100 ug/mL in PBS + 0.1% BSA
LY294002 (PI3 kinase inhibitor)	Promega V1201 Gibco (Invitrogen) PHZ1144	5 mM in DMSO
RPMI-1640	Sigma R8758	
Glutamax	Invitrogen 35050061	
Penicillin-Streptomycin	Invitrogen 15070-063	
B27 (without RA)	GIBCO 12587010	
B27 (RA-supplement)	GIBCO 17504044	
NEAA Non-Essential Amino Acids Solution	Sigma M7145	
Trypsin 0.25% 1x	Hyclone SH30042.01	
Albumax II	Invitrogen 11021-029	10% solution in H2O or PBS
Y-27632	Tocris	10 mM in H2O or PBS
GSK3iXV	CalBiochem	1-5 mM in DMSO