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Mouse Lung Directed Differentiation into Primordial Progenitors – Kotton Laboratory

INTRO

This protocol describes the methods to derive, sort and plate primordial lung progenitors from mouse iPSCs/ESCs. This protocol was published in Cell Stem Cell as part of Herriges et al., 2023 (https://www.cell.com/cell-stem-cell/fulltext/S1934-5909(23)00283-7)

REAGENTS

A) cSFDM (complete serum free differentiation media):

Volume for 500 ml	Final concentration	Reference
375 ml IMDM	75%	Invitrogen 12440
125 ml Ham's F12	25%	Cellgro 10-080-CV
5 ml B-27 (with RA) supplement	1%	Invitrogen 17504-44
2.5 ml N-2 supplement	0.5%	Invitrogen 17502-048
3.3 ml BSA (7.5% stock)	0.05%	Invitrogen 15260-037
1 ml Primocin (100 µg/ml stock)	200 ng/ml	
5 ml Glutamax 100X	1X	
500 µl Ascorbic Acid (50 mg/ml stock)	50 µg/ml	Sigma A4544
19.5 ul MTG	$4.5x10^{-4}$M	Sigma M6145

B) Growth factors and chemical inhibitors:
Activin A, R&D 338-AC
rmNoggin, R&D 1967-NG
SB431542, Sigma S4317
rhBMP4 R&D 314-BP
rmWnt3a R&D 1324-WN
rhFGF2, R&D 233-FB
rhFGF10, R&D 345-FG
Rho-associated kinase (ROCK) inhibitor, Tocris Y-27632
Retinoic Acid (Sigma R2625)
rmFgf10, R&D biosystems6224-FG-025
Heparin Sodium Salt, Sigma H47840.05%
Trypsin-EDTA (1X), gibco 25300-054
ES-qualified FBS, HyClone SH30070.03
DMEM (1X), gibco 11995-065
Dispase
Collagenase Type IV
Matrigel (Corning 356231)

PROTOCOL (See section below for working with difficult lines)

1) MEF depletion, LIF withdrawal, and embryoid body (EB) formation (Day 0 to 2.5)[†#]
   A. Trypsinize 1 well of a 6-well plate containing undifferentiated mESCs growing on a layer of MEFs in mESC media supplemented with LIF:
      1. Wash the well with PBS x1 and add 1ml of pre-warmed 0.05% trypsin for 1-2 min (*Tip: checking under the microscope to assess if the cells have detached, can decrease the trypsin exposure time).*
      2. Pipet a few times and get close to a single cell suspension.
      3. Transfer the cell suspension to a 15ml conical tube containing 1ml ESC-qualified FBS and fill the tube with DMEM.

4. Centrifuge at 300*g* x 5 min.
5. Discard the supernatant and re-suspend the pellet in 10ml mESC media.
6. Transfer the suspension to a p100 tissue culture dish and incubate for 30 min; MEFs will attach to the dish while mESCs will mostly stay in the supernatant. Remove carefully the supernatant and transfer to a 15ml conical tube.
7. Collect the mESCs by centrifuging the supernatant at 300*g* x 5 min.
8. Discard the supernatant and re-suspend the cell pellet in 1ml of cSFDM.
9. Count the cells.

B. For starting the differentiation, add $5x10^5$ cells to a p100 Petri dish containing 10ml of cSFDM. Carefully shake the plate to homogenize suspension and place in the incubator for 60h to allow EB formation.

2) Definitive endoderm induction (Day 2.5 to 5):

A. Collect the EBs by transferring the content of the p100 Petri dish to a 15ml conical tube and either spin at 300*g* x 1 min or let the EBs settle by gravity for 3-4 min. The latter usually helps to get rid of debris sometimes observed at this stage. Discard the supernatant by careful aspiration.
B. Cells at this stage (EBs) are very sensitive to trypsin. Have cold 0.05% trypsin as well as cold ESC-qualified FBS ready (on ice). Add 1 ml of cold trypsin and re-suspend the EBs carefully but fast while keeping the conical tube on ice. Transfer to a water bath at 37°C for 1 min; shake the tube while in the water bath. Immediately, move the tube back on ice, pipet 2-3 times with a P1000 pipette, add 1ml of cold ESC-qualified FBS, and pipet 2-3 times to mix well. Working very fast at these steps is key to avoid toxicity due to overexposure to trypsin. Fill the conical tube with media (IMDM or cSFDM) to dilute the FBS and trypsin and centrifuge at 300*g* x 5 min; if cell/DNA clumps are observed due to overexposure to trypsin, use a cell strainer before centrifugation. Discard the supernatant, re-suspend cells in 1ml cSFDM and count them.
C. Transfer $1x10^6$ cells to a p100 Petri dish containing 10ml of cSFDM + Activin A at 50 ng/ml (1:200 dilution of a 10 µg/ml stock). Depending on the size of your experiment, you may elect to prepare more than one p100 Petri dishes at this stage. Shake the dish(es) to homogenize and place in the incubator for 60h.

3) Anterior foregut endoderm (Day 5 to 6):

A. Transfer the content of the p100 Petri dish(es) to a 15ml conical tube. Transfer 1-2ml to a separate tube to be used for evaluation of CKIT/CXCR4 co-expression to assess sufficient definitive endoderm induction (see CKIT/CXCR4 staining protocol).
B. Let the rest of the EBs settle or collect them by centrifugation at 300*g* x 1 min. Re-suspend the content of each conical tube in 10ml of "anteriorization media" composed of cSFDM + 100 ng/ml rmNoggin + 10 µM SB431542 (1:100 dilution of a 10 µg/ml mNoggin stock and 1:1000 dilution of a 10 mM SB431542 stock). Incubate for 24h in p100 Petri dish(es).

4) Nkx2-1 induction/specification (Day 6 to Day 13-14)

A. Prepare specification media composed of cSFDM supplemented with 10 ng/ml rhBMP4 (1:1000 dilution of a 10 µg/ml stock) and 100 ng/ml rmWnt3a (1:1000 dilution of a 100 µg/ml stock). Supplementation of specification media with ROCK inhibitor (10 µM Y-27632) and 100nM RA (1:1,000 dilution from 100uM stock) for the first 48 hours of specification greatly increases survival after single cell suspension. If these cells are going on to a distal differentiation protocol add 50 ng/ml rmFGF10 (1:1,000 dilution of 50ug/ml stock) from Day 8 until sorting.
B. Thaw Matrigel (356231) on ice at this time. While Matrigel is thawing place a 6-well plate at 4°C to prechill and make spreading the Matrigel easier.
C. Prepare a 6 well plate by adding 100µl of Matrigel to each well and using a p100 pipette tip to spread the Matrigel and ensure the base of the well is fully covered by Matrigel. Allow Matrigel to solidify at 37°C for 20 minutes.
D. Trypsinize the whole preparation of EBs (as described previously) and plate $2x10^6$ cells per well of a 6-well plate. These cells should be counted on a hemocytometer to confirm count accuracy.
E. 2ml media per well of a 6-well plate is usually enough. Specification media is changed every day due to high density of cells. If media turns yellow in less than 24h, consider adding more than 2ml of media per well.

5) Sorting Nkx2-1+ lung progenitors and expansion in 3D Matrigel (Day 13-14)

A. Prepare FACS buffer:
    1. PBS or HBSS 1x
    2. 2% ESC-qualified FBS
B. Aspirate the old media (consider washing with serum-free media once prior to the next step).
C. Add 1 mL of 1mg/ml Dispase and 1mg/ml collagense. Pipet a few times to break up the gel. Let cells incubate for 1 hour at 37°C to digest Matrigel. Do not disturb the cells during this time.
D. Transfer the cells and liquid in each well to a 15ml conical tube. Add 9 ml FACS buffer to the tube and pipette up and down a few times.
E. Centrifuge at 300g x 5 min.
F. (Optional epithelial enrichment) At this stage the majority of epithelial cells are found in tight balls that won't be broken up by digestion. Aspirate off supernatant and disrupt pellet by flicking the bottom tube. Add 5 ml FACS buffer and pipette up and down 5 times to help break up non-epithelial clumps. Spin tube at 100g x 1 min.
G. (Optional epithelial enrichment) Repeat step F, but spin at 20g x 1 min. The resulting pellet should be largely made of epithelial spheres.
H. Aspirate liquid and add 1ml warm 0.05% trypsin to each tube. Incubate at 37°C for 5 min.
I. Pipet a few times to make a single cell suspension. If you do not get a single cell suspension, return to 37°C for a couple minutes before pipetting again. Move the 15ml conical tube to ice and add 1ml cold ESC-qualified FBS (1ml ESC-qualified FBS for each well you are harvesting). Then add 8ml of PBS and filter through a 40µm filter x 1
J. Centrifuge at 300*g* x 5 min.
K. Discard the supernatant and stain cells with a conjugated antibody targeting EpCAM (1:500). Cells should bere-suspend in FACS buffer supplemented with antibody. Approximately 0.25ml per harvested well gives a cell density appropriate for cell sorting on the MoFlo. Incubate cells at 4°C for at least 25 min.
L. Add 5 ml FACS buffer to sample. Centrifuge at 300g x 5 min.
M. Discard supernatant and resuspend cells in 250ul of FACS buffer supplemented with Propidium Iodide (500x), Calcein Blue (1000X), or DRAQ7 (100x). Transfer sample to a FACS tube.
N. Prepare collection tubes with FACS buffer supplemented with ROCK inhibitor (10 µM Y-27632).
O. For 3D expansion thaw Matrigel (356231) on ice at this time.
P. Sort out live Nkx2.1+/EpCAM+ cells.
Q. Spin collected samples at 300*g* x 5min in either Eppendorf or 15ml conical tubes depending on the volume.
R. Discard the supernatant and place Eppendorf or conical tube on ice.
S. For 3D expansion, re-suspend each cell pellet at 500 cells per µl of Matrigel using cold 200µl pipette tips. Take care to (a) avoid bubbles and (b) distribute the cells evenly in the Matrigel.
T. Slowly pipette the Matrigel + cells into the center of a 12-well plate (15-25ul/drops, typically 50µl/well) or 6-well plate (up to 150ul/well).
U. Place the 12-well plate in the incubator for 25-30 min.
V. Add "distal" (cSFDM supplemented with 250 ng/ml rhFGF2 + 100 ng/ml rhFGF10 + 200 ng/ml rmWnt3a + 100 ng/ml Heparin Salt) or "proximal" (cSFDM supplemented with 250 ng/ml rhFGF2 + 100 ng/ml rhFGF10 + 100 ng/ml Heparin Salt) media supplemented with ROCK inhibitor 10µM (Y-27632)* or proceed to alternative differentiation protocols.
W. Re-feed every 48 hours with respective media not containing ROCK inhibitor.

DEALING WITH DIFFICULT LINES (B6 CELLS)

- On D(-2) passage ESCs with a low passaging ratio to rest cells while still maintaining a large cell number.
- For D0-D6 make cSFDM on the day of use (also known as SFD+)
    - Make SFD ahead of time. This includes everything in cSFDM except ascorbic acid and MTG
    - On the day of use make diluted MTG (13ul MTG/1ml IMDM).
    - On the day of use make SFD+ (30ul diluted MTG and 10ul ascorbic acid to 10ml SFD) and use this instead of cSFDM for any medias.
- For D7-D14 fresh SFD+ seems to be less important and can be stored for a few days without notable impact on differentiation efficiency.

COMMENTS

†Times are slightly flexible. Usually, for days 0, 5, and 6 I will start around 8AM and for day 2.5 I will start around 4PM.

Some lines are more finicky than others and require more support. If a cell line does not grow well. Consider adding ROCK Inhibitor 10uM for D0-D2.5 and increasing to 1e6 cells/plate at D0 and D2.5.

* Cells expanded in "FGF2+FGF10" media express markers of proximal and distal lung cell types, but the requirement/role of each of these factors has not been extensively tested in this protocol. Adding rmWnt3a at 200 ng/ml to the "FGF2+FGF10" media increases detection of the distal marker *Sftpc* by qPCR and SFTPC+ cell numbers while lowering expression of a proximal marker *Scgb1a1*. While the growth on Matrigel increases the yield of Nkx2.1+/EpCAM+ cells, these cells have some difficulty in the FGF2/10, mWnt3a media. Ongoing tests are being done to see if there is a better condition for growing these cells.

VERSION HISTORY

2017-04-26	Drafted by Maria Serra.	MS
2017-05-10	Edited and transferred to CReM letterhead.	KDA
2017-12-04	Minor text edits.	KDA
2018-05-06	D6-D14 Changes for 3D Matrigel and Nkx2.1+/EpCAM+ sort	MJH
2018-08-23	Minor edits + Transferred to CReM letterhead	LI
2018-09-13	Added Epithelial enrichment spins	MJH
2021-03-04	Updated to reflect WBF protocol and reduced 3D Matrigel	MJH
2023-05-24	Added "Dealing with difficult lines" section	MJH