CReM CENTER FOR REGENERATIVE MEDICINE
BOSTON UNIVERSITY BOSTON MEDICAL
670 ALBANY STREET, 2ND FLOOR
BOSTON, MA 02118
TEL: 617-414-2969
HTTP://WWW.BUMC.BU.EDU/STEMCELLS/

Mouse Lung Mesenchyme Directed Differentiation and Epithelial-Mesenchymal Co-Cultures– Kotton Laboratory

INTRO

This protocol describes the methods to derive lung-specific mesenchyme from mouse iPSCs/ESCs and establish iPSC-derived lung epithelial-mesenchymal co-cultures. This protocol was published in Nature Communications as part of Alber et al., 2023 (https://www.nature.com/articles/s41467-023-39099-9). Initial steps of this protocol (lateral plate mesoderm differentiation) are based on Kwong et al., 2019 (https://www.cell.com/stem-cell-reports/fulltext/S2213-6711(19)30266-8?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS2213671119302668%3Fshowall%3Dtrue)

PART 1: Directed differentiation of mouse iPSCs into lung mesenchyme

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 (without RA) supplement	1%	Invitrogen 12587-010
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)
rhBMP4 (R&D, 314-BP)
rmWnt3a (R&D, 1324-WN)
Rho-associated kinase (ROCK) inhibitor (Tocris, Y-27632)
Retinoic Acid (Sigma, R2625)
Purmorphamine (Stemcell Technologies, 72202)
Trypsin-EDTA (1X) (Gibco, 25300-054)
ES-qualified FBS (HyClone, SH30070.03)
DMEM (1X) (Gibco, 11995-065)
Gelatin (Stemcell Technologies, 07903)
Doxycycline (Sigma, D3072)
Calcein Blue (Life Technologies, C1429)

PROTOCOL

1) MEF depletion, LIF withdrawal, and embryoid body (EB) formation (Day 0 to 2)
   A. Trypsinize required number of wells* of 6-well plate containing undifferentiated iPSCs† (use Tbx4-LER^{GFP} line if intending to sort based on reporter fluorescence) growing on a layer of MEFs in mESC media supplemented with LIF:
      1. Wash with 1ml PBS per well and add 1ml of pre-warmed 0.05% trypsin per well. Incubate for 3-4 min at 37°.

2. Pipet up and down a few times to get 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 $1.5x10^6$ cells to a p100 Petri dish containing 10ml of pre-warmed cSFDM. Carefully tilt the plate to homogenize suspension and place in the incubator for 48h to allow EB formation.
Note: Do not touch or move the plate after placing it in the incubator! Any movement will significantly decrease the yield.

2) Lateral plate mesoderm induction (Day 2 to 5):

A. Collect the EBs by transferring the content of the p100 Petri dish to a 15ml conical tube and let the EBs settle by gravity for 5-10 min. Discard the supernatant by careful aspiration.
B. Prepare a fresh p100 Petri dish with 10 ml of pre-warmed cSFDM + 2 ng/ml Activin A, 3 ng/ml rhBMP4 and 3 ng/ml rmWnt3a.
C. Use 1 ml of the freshly prepared medium to resuspend settled EBs. Pipet once and transfer to the fresh p100 Petri dish. Carefully tilt the plate to distribute EBs and place in the incubator for 72h.
Note: EBs are very sensitive at this stage. Minimize time cells spend outside of the indubator, avoid excessive pipetting and make sure culture medium is warmed up to 37°C.

3) Lung mesenchyme differentiation (Day 5 to 11):

A. Transfer the content of the p100 Petri dish(es) to a 15ml conical tube (If performing a KDR stain for lateral plate mesoderm induction evaluation set aside 1 ml for trypsinization and staining).
B. Let the rest of the EBs settle by gravity for 5-10 min. Discard the supernatant by careful aspiration.
C. Prepare cSFDM medium + 2 µM RA and 10 µM ROCK inhibitor warmed up to 37°C.
Note: RA is unstable and light sensitive. Protect RA stock solution from light and only add to the medium right before use. Do not store cSFDM with diluted RA for later use.
D. Coat a 12-well plate with 1 ml gelatin per well, let sit for 5 min, then wash with 1m PBS per well.
E. Resuspend EBs in cSFDM medium + 2 µM RA and 10 µM ROCK inhibitor, resuspend carefully by slowly inverting the 15ml conical tube multiple times and plate into appropriate number of wells in 12-well plate using a 5 ml transfer pipet (do not use P1000 pipet, since this can potentially disrupt EBs). For collected EBs from 1 p100 Petri dish use 6 ml of medium and plate into 6 wells of a 12-well plate. This corresponds to an approximate density of 40'000 cells/cm^2. Place in the incubator for 48h.
Note: since cells are plated as EBs without enzymatic digestion the actual plating density might vary between wells. In order to distribute as evenly as possible, make sure to invert the 15 ml conical tube containing the cells multiple times before plating and tilt plate after plating to distribute EBs in the well.
F. On day 7, aspirate culture media and replace with 1ml/well cSDFM + 2 µM RA and 0.5 µg/ml Purmorphamine. Place in the incubator for 48h.
G. On day 9, aspirate culture media and replace with 1ml/well cSDFM + 2 µM RA and 2 µg/ml doxycycline. Place in the incubator for 48h.

4) Tbx4-LER^{GFP} sort (Day 11)

A. Prepare FACS Buffer
    1. PBS or HBSS
    2. 2 % ESC-qualified FBS
B. Wash plates with 1ml/well PBS and add 0.5 ml/well 0.05% Trypsin.
C. Incubate at 37°C for 3 - 4 min. Pipet up and down to get a single cell suspension.
D. Transfer cell suspension to 15 ml conical tube containing equal volume of ESC-qualified FBS (combine cell suspension from up to 6 wells into 1 15 ml conical tube). Fill up to 15 ml with PBS.

E. Centrifuge at 300*g* x 5 min and aspirate supernatant.
F. Resuspend in FACS buffer with 1/1000 Calcein Blue. 1.5 ml FACS buffer for 6 wells of a 12-well plate gives appropriate density for sorting on the MoFlo.
G. Prepare collection tubes with FACS buffer supplemented with 10 µM ROCK inhibitor.
H. Sort for Tbx4-LER^{GFP}+ cells.

COMMENTS

*Optimal number of wells depends on iPSC line and required number of sorted cells for downstream experiments. For the Tbx4-LER^{GFP} iPSC line, 1-2 confluent wells of iPSCs is enough to collect 3x10^6 cells, which will suffice for 2 p100 petri dishes to start the differentiation, and typically yield approximately 200'000 – 300'000 sorted GFP+ cells at the end of the differentiation protocol.

†This protocol was optimized using the Tbx4-LER^{GFP} iPSC line, which allows for sorting GFP+ lung mesenchymal cells. If using other lines, certain steps of the protocol might have to be re-optimized. When using an ESC/iPSC line without fluorescent reporter keep in mind that the expected efficiency of this protocol is 10-25%, i.e. the majority of the cells will be non-lung mesenchyme.

PART 2: Co-culture of iPSC-derived lung mesenchyme and ESC-derived lung epithelial progenitors

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:
3D Matrigel (Corning, 356231)
rmWnt3a (R&D, 1324-WN)
rhFGF2 (R&D, 233-FB)
rhFGF10 (R&D,) 345-FG
Rho-associated kinase (ROCK) inhibitor (Tocris, Y-27632)
Heparin Sodium Salt (Sigma, H47840.05%)
Trypsin-EDTA (1X) (Gibco, 25300-054)
ES-qualified FBS (HyClone, SH30070.03)
Dispase (Gibco, 17105-041)
Epcam antibody (BD Biosciences, 563214)
DRAQ7 (Biolegend, 424001)

PROTOCOL

1) Plating and maintenance of co-cultures (Day 0-7)
A. Thaw 3D Matrigel on ice
B. Cover 48-well plate# with 150 µl 3D Matrigel per well. Let solidify at 37°C for 20 min.

C. Warm up required volume of distal (cSFDM +200 ng/ml rmWnt3a, 100 ng/ml rhFGF2, 100 ng/ml rhFGF10 and 10 µM ROCK inhibitor) or proximal (cSFDM +100 ng/ml rhFGF2, 100 ng/ml rhFGF10, 100 ng/ml Heparin Salt and 10 µM ROCK inhibitor) medium.
D. Combine 100'000 freshly sorted Tbx4-LER^{GFP}+ lung mesenchyme cells and 5'000 freshly sorted Nkx2-1^{cherry}+ day 14 lung epithelial cells (see Protocol "Mouse D14 Lung Progenitor Diff" for details on differentiation of Nkx2-1^{cherry} cells). Mix and centrifuge at 300*g* x 5 min. Remove supernatant.
E. Resuspend pellet in 500 µl of distal or proximal medium and plate on top of solidified 3D Matrigel in 48-well plate. Pipet slowly in order to not disrupt the 3D Matrigel. Place into incubator for 48 h.
Note: If plating Nkx2-1^{cherry} controls without mesenchyme, plate 25'000 cells per well in a 48-well plate.
F. On day 2, replace distal medium using cSFDM +200 ng/ml rmWnt3a, 100 ng/ml rhFGF2, and 100 ng/ml rhFGF10 (no ROCK inhibitor). Replace proximal medium with cSFDM +100 ng/ml rhFGF2, 100 ng/ml rhFGF10, 100 ng/ml Heparin Salt and 10 µM ROCK inhibitor.
G. Replace medium every 48h (No ROCK inhibitor in distal medium).

2) Harvesting co-cultures for FACS sorting (Day 7):

A. Prepare FACS buffer:
    1. PBS or HBSS 1x
    2. 2% ESC-qualified FBS
B. Aspirate the old media.
C. Add 0.5 mL of 2 mg/ml Dispase (warmed up to 37°C). Let cells incubate for 1 hour at 37°C to digest Matrigel. After 30 mins, pipet up and down several times to break up the Matrigel.
D. Pipet up and down multiple times again and transfer to 15 ml conical tube. Fill up to 10 ml with PBS.
E. Centrifuge at 300g x 5 min.
F. Remove supernatant.
Note: If there are clumps of cells/Matrigel floating or if pellet appears loose, remove as much supernatant as possible, pipet up and down multiple times, fill up with PBS and repeat centrifugation step.
G. Add 1 ml warm 0.05% trypsin to each tube and pipet up and down. Incubate at 37°C for 10 min. After 5 mins pipet up and down multiple times do dissociate cells.
H. Add 1 ml of ESC-qualified FBS. Fill up to 10 ml with PBS.
I. Centrifuge at 300g x 5 min and remove supernatant
J. Add 200 µl of FACS Buffer with 1/500 anti EpCAM antibody. Incubate on ice in the dark for 30 mins.
K. Add 1 ml of FACS buffer to dilute antibody and centrifuge at 300g x 5 min. Remove supernatant.
L. Resuspend in 300 µl of FACS buffer with 1/100 DRAQ7.
M. Prepare collection tubes with 400 µl of FACS buffer containing 10 µM ROCK inhibitor.
N. Sort on MoFlo: GFP+/EpCAM- cells to retrieve mesenchyme, Cherry+/EpCAM+ cells to retrieve epithelium.

COMMENTS

[#]Can be adapted to either 96-well or 24-well format.

VERSION HISTORY

2023-09-19	Drafted by Andrea Alber.	AA