

## Human Alveolosphere Directed Differentiation Protocol – Kotton Laboratory

### INTRO

This protocol describes an approach to establish three-dimensional (3D) culture of PSC-derived SFTPC+ alveolar cells as published in: Jacob et al. Cell Stem Cell. 2017. This protocol can also be found in Jacob et al. Nature Protocols, 2019.

For differentiation and purification of NKX2-1+ cells from PSCs, see Lung Progenitor Differentiation protocol.

### REAGENTS

#### A) cSFDM (complete serum free differentiation media):

| Volume for 500 ml                     | Final concentration    | Reference              |
|---------------------------------------|------------------------|------------------------|
| 375 ml IMDM                           | 75%                    | ThermoFisher 12440053  |
| 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 (50 mg/ml stock)        | 100 µg/ml              | Invivogen NC9141851    |
| 5 ml Glutamax 100X                    | 1X                     | ThermoFisher 35050-061 |
| 500 µl Ascorbic Acid (50 mg/ml stock) | 50 µg/ml               | Sigma A4544            |
| 1.5 ml MTG (from 26 µl in 2 ml IMDM)  | 4.5x10 <sup>-4</sup> M | Sigma M6145            |

#### B) CK+DCI Media

cSFDM Base

3µM CHIR99021 (Tocris 4423)

10ng/ml rhKGF (R&D 251-KG-010)

50nM Dexamethasone (Sigma D4902)

0.1mM 8BrcAMP (Sigma B7880)

0.1 mM IBMX (Sigma I5879)

#### C) Other Reagents

Rho-associated kinase inhibitor (Y-27632, Tocris 1254)

Growth Factor-Reduced Matrigel (Corning 356231)

Dispase (ThermoFisher 354235)

### PROTOCOL

**Begin this protocol after generating hPSC-derived NKX2-1+ lung progenitors and purifying by flow cytometry (related protocols: Human Lung Progenitor Differentiation, CD47/CD26 sorting of lung progenitors)**

#### A) Replating NKX2-1+ Lung Progenitors and Expansion in 3D Matrigel as Alveolospheres (Day 15 to 20+)

1. Thaw 3D Matrigel on ice (Corning 356231) and prepare CK+DCI media during NKX2-1 or CD47/CD26 lung progenitor sort.
2. Spin down sorted lung progenitor cells at 300g x 5min.
3. Resuspend 200-500 cells/ul in undiluted Matrigel. Plate in droplets ranging from 25-100uls per well of a 12 well plate (we typically do 400cells/ul in 50ul droplets).
4. Place in 37C incubator for 15-20 minutes *without* adding media to allow the Matrigel to solidify.
5. Add warm CK+DCI media + 10 µM Y-27632 to wells once Matrigel is solidified.
6. After 72 hours, change media to CK+DCI *without* 10 µM Y-27632

7. Change media every 2 days, or as necessary. Be careful when aspirating to avoid dislodging the Matrigel drop. At later timepoints, change media more frequently as needed.
8. If using a reporter line, you should begin to see SFTPC<sup>tdTomato+</sup> cells within 2-5 days.
  - a. Passage alveolospheres when they become confluent within the Matrigel drop (usually 12-14 days), otherwise efficiency will decrease.

## B) Alveolosphere dissociation and passaging method

1. Aspirate media from Matrigel drop.
2. Add 1 ml dispase (2mg/ml) per well to break apart Matrigel drop, leave at 37C for 1 hour, pipetting up and down 1-2 times after ~30 mins.
3. Transfer 1ml dissociated organoids in dispase into a 15ml conical, add 10ml DMEM or empty media to wash.
4. Centrifuge 200g x 4min, aspirate supernatant
  - a. *If a clear haze is seen above the pellet, the Matrigel has not totally dissolved and more dispase can be added to the pellet for another 20 minutes at 37C.*
5. Aspirate supernatant, aspirate leftovers with a P200 tip, leaving as little supernatant as possible.
  - a. *It is important to remove all dispase since it will dissolve Matrigel in culture as well.*
6. At this point you have 3 options:
  - a. **Option 1: Replate single cells**
    - i. Resuspend cells in 1ml trypsin/well, incubate at 37C for 10-12 minutes, until pipetting only 3-5 times results in single cell suspension
 

*Dissociate cells in a tissue culture plate so that you can visualize them in a microscope. Cells are sensitive to over-pipetting at this stage, so leave them in trypsin long enough for most of the dissociation to be enzymatic. If alveolospheres have not dissociated into single cells by 12 minutes, spin down, add fresh trypsin, and leave another 5 minutes*
    - ii. Stop with FBS-containing media, spin at 300g x 5min, wash once with 10ml DMEM.
    - iii. Count and resuspend cells in 3D matrigel at desired concentration (400 cells/ul Matrigel, 50-100ul drops in 12-well plate)
    - iv. Place in 37C incubator for 15-20 minutes *without* adding media to allow the Matrigel to solidify.
    - v. Add 1ml CK+DCI + 10  $\mu$ M Y-27632 media over Matrigel drop.
    - vi. Refeed without Y-27632 after 72 hours, refeeding every 48 hours or as needed.
  - b. **Option 2: Sort alveolosphere cells**
    - i. Follow protocol in section 6a to dissociate to single cell.
    - ii. Resuspend in FACS buffer with 10  $\mu$ M Y-27632 and live/dead stain. Filter through 40um filter x2 and take for sorting (see lung progenitor sorting protocol).
    - iii. Sort live/NKX2-1+/SFTPC+ cells if using a PSC line containing knock-in reporters. If using a non-reporter PSC line, CPM or SLC34A2 can be used, see Anjali et al. Nature Protocols 2019 for antibody and staining details.
    - iv. Replate in Matrigel as in 6a.
  - c. **Option 3: Replate whole alveolospheres**
    - i. Resuspend whole spheres in 3D Matrigel at desired concentration (1:3 to 1:10 is reasonable)
 

*Note: Spheres often collapse when centrifuged/resuspended and do not grow out as well as those passaged at single cell – only use this method for specific purposes in which replating at single cell is not applicable.*
    - ii. Place in 37C incubator for 15-20 minutes *without* adding media to allow the Matrigel to solidify.

iii. Add 1ml CK+DCI+ 10  $\mu$ M Y-27632 media over solidified Matrigel drop

### C) CHIR Withdrawal and Addback (Day 30-40+)

1. Prepare alveolosphere cells for sorting as described above
2. Sort and replate NKX2-1<sup>GFP+</sup> cells in 3D matrigel culture if using reporter PSC line as described above (if not using a PSC line containing knock in reporters, CPM can be used as an extracellular marker).
3. After 24 hours in CK+DCI+10  $\mu$ M Y-27632, change media to K+DCI media (*NO CHIR99021*)
  - a. Consider adding 10  $\mu$ M Y-27632 to K+DCI for first 48 hours.
  - b. You should see that the cells do not grow as fast as they do in CK+DCI media.
4. After 4 days in K+DCI, change media back to CK+DCI media
  - a. You should see an increase in the size of the alveolospheres over this time and a SFTPC+ percent increase.

### D) Alveolosphere Freezing Method

1. Dissociate alveolospheres into single cell suspension
  - a. Aspirate media from Matrigel drop
  - b. Add 1 ml dispase (2mg/ml) per well to break apart Matrigel drop, leave at 37C for 1 hour, pipetting up and down once after ~30 mins.
  - c. Transfer 1ml dissociated organoids in dispase into a 15ml conical, add 10ml DMEM to wash.
  - d. Centrifuge 200g x 4min, aspirate supernatant, repeat once more
  - e. Resuspend cells in 1ml trypsin/ well, incubate at 37C for 5-10 minutes
  - f. Pipet up and down 5-10 times, stop with FBS-containing media
  - g. Spin at 300g x 5min, wash once with 10ml DMEM or other empty media.
2. Resuspend in 1ml Freeze Media/well (60%FBS, 30%CK+DCI, 10%DMSO) and freeze in a cryovial (first at -80C overnight, then long-term storage at -150C.)

### VERSION HISTORY

|            |                                                                                      |    |
|------------|--------------------------------------------------------------------------------------|----|
| 2017-07-10 | Drafted by Anjali Jacob.                                                             | AJ |
| 2017-08-09 | RA concentration changed from 50nM to 100nM                                          | LS |
| 2018-04-03 | Removed progenitor stage from protocol – see independent protocol to avoid confusion | FH |
| 2022-1-19  | Primocin final concentration 100 $\mu$ g/ml, separated from lung progenitor protocol | CB |
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