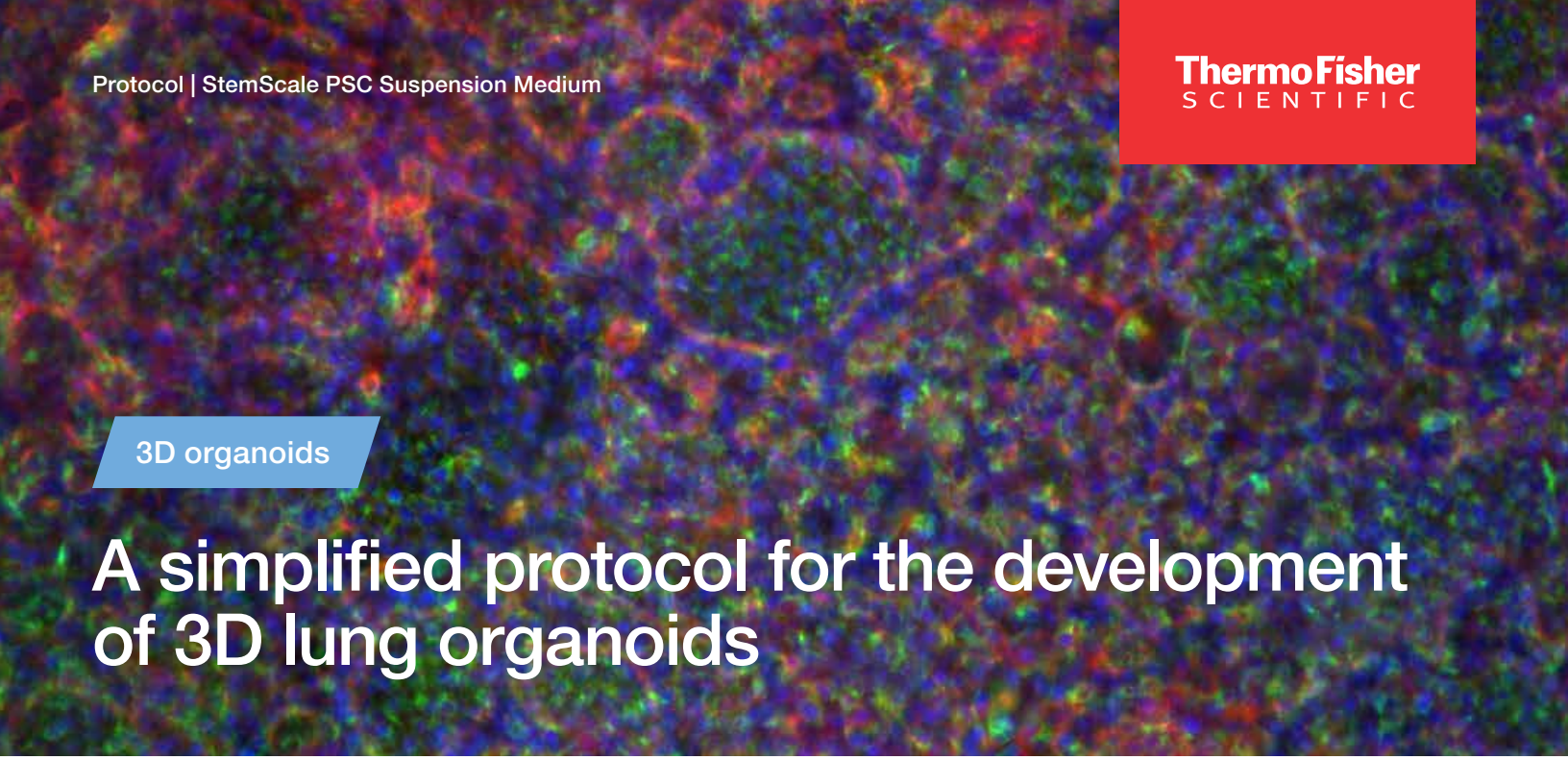

3D organoids

A simplified protocol for the development of 3D lung organoids

Introduction

Respiratory diseases constitute major sources of mortality worldwide, with lower respiratory infections and chronic obstructive pulmonary disease (COPD) representing, respectively, the fourth and sixth leading causes of death globally in 2019 [1]. Since 2019, respiratory infections from SARS-CoV-2 have also risen to be a leading cause of mortality and illness [2]. Organoid models of the human lung airway and alveoli, derived from pluripotent stem cells (PSCs), are utilized to study lung development, disease, and homeostasis *in vitro*. Increasingly, these models are used for pharmaceutical development and toxicity testing. Lung organoid models have been engrafted into animal models successfully, and efforts are being directed toward utilizing these models in the future for regenerative medicine [3,4]. Thus, reliable methods for production, scale-up, and biobanking of lung organoids are needed to support these efforts. This protocol includes steps to differentiate, scale up, and cryopreserve lung organoids from PSCs grown in suspension, enabling the creation of ready-to-use banks of these cells. Finally, methods are given for modeling viral infection and for culture of lung organoid cells at the air–liquid interface. Representative results produced by following these protocols are presented.

Contents

Media preparation

Differentiation medium (DM), 530 mL

1. Combine 375 mL of Gibco™ Iscove's Modified Dulbecco's Medium (IMDM) and 125 mL of Gibco™ Ham's F-12 Nutrient Mix.
2. To the combined medium, add:
   - 5 mL of 100X Gibco™ GlutaMAX™ Supplement
   - 5 mL of 100X Gibco™ N-2 Supplement
   - 10 mL of 50X Gibco™ B-27™ Supplement
   - 500 µL of 50 mg/mL ascorbic acid
   - 3.34 mL of 7.5% Gibco™ Bovine Albumin Fraction V
   - 5 mL of 100X Gibco™ Antibiotic-Antimycotic
3. Prepare monothioglycerol by diluting 26 µL of the stock from the bottle into 2 mL of Gibco™ IMDM. Add 1.5 mL of the diluted stock to the combined medium.
4. Mark the date and store at 4°C. Use for up to 1 month.

Anterior foregut endoderm medium, 50 mL

1. To 50 mL of the DM stock prepared above, add:
   - 50 µL of 10 mM SB431542 (10 µM final)
2. Mark the date and store at 4°C. Use for up to 1 week.

Lung progenitor medium, 50 mL

1. To 50 mL of the DM stock, add:
   - 15 µL of 10 mM CHIR99021 (3 µM final)
   - 50 µL of 10 µg/mL Gibco™ Human BMP-4 Recombinant Protein (10 ng/mL final)
2. Mark the date and store at 4°C. Use for up to 1 week.
3. Add retinoic acid (RA) to the medium:
   - RA is very light sensitive. A stock of 100 µM (1,000X) RA in dimethyl sulfoxide (DMSO) can be prepared in advance and stored at –20°C, protected from light.
   - Add RA fresh to the lung progenitor medium to a final concentration of 100 nM.
     - Example: Transfer 10 mL DM with CHIR99021 and BMP-4 to a tube. Add 10 µL of 100 µM RA (100 nM final).
   - Protect from light. Do not store the RA-supplemented lung progenitor medium.

Maturation medium, 50 mL

1. To 50 mL of stock DM, add:
   - 15 µL of 10 mM stock CHIR99021 (3 µM final)
   - 50 µL of 10 µg/mL stock Gibco™ Human FGF-7 Recombinant Protein (10 ng/mL final)
   - 25 µL of 0.1 mM stock dexamethasone (50 nM final)
   - 50 µL of 0.1 M stock 8-bromo-cAMP (0.1 mM final)
   - 50 µL of 0.1 M stock 3-isobutyl-1-methylxanthine (IBMX, 0.1 mM final)
2. Mark the date and store at 4°C. Use for up to 1 week.

Generation of lung organoids from PSCs

Note: Volumes are written for one well of a 6-well plate. Scale accordingly for other sizes, based on surface area.

PSC culture (day 0)

1. Passage PSCs in Gibco™ StemScale™ PSC Suspension Medium per **product protocol**.
2. Re-seed the desired number of wells of a non–tissue culture (TC)-treated 6-well plate with 2 mL per well of a 1.5 x 10^6 cells/mL suspension in StemScale medium supplemented with 10 µM Y-27632.
3. Place on a shaker (70 rpm) in an incubator at 37°C, 5% CO_2.
4. After 48 hours, perform a 50% medium replacement without Y-27632.
5. Place on a shaker (70 rpm) in an incubator at 37°C, 5% CO_2, for 24 hours.

Definitive endoderm induction (day 3)

1. Tilt the culture plate containing the StemScale medium, and allow cell aggregates to settle to the bottom.
2. Collect aggregates using a serological pipette, wide-bore 1,000 µL pipette tip, or cut 1,000 µL pipette tip. Transfer aggregates to a 15 mL conical tube (for smaller well sizes, a 1.5 mL microcentrifuge tube can be used).
3. Allow the cell aggregates to settle by gravity (a few minutes).
4. Carefully remove the supernatant.
5. Gently resuspend the aggregates in 2 mL of Gibco™ PSC Definitive Endoderm Induction Medium A and transfer to a new, non–TC-treated 6-well plate.
6. Place on a shaker (70 rpm) in an incubator at 37°C, 5% CO_2.
7. After 24 hours, repeat steps 1–4.
8. Gently resuspend the aggregates in 2 mL Gibco™ PSC Definitive Endoderm Induction Medium B and transfer to a new, non–TC-treated 6-well plate.
9. Place on a shaker (70 rpm) in an incubator at 37°C, 5% CO_2, for 24 hours.

Anterior foregut endoderm induction (day 5)

1. Tilt the suspension culture plate and allow cell aggregates to settle to the bottom.
2. Collect aggregates using a serological pipette, wide-bore 1,000 µL pipette tip, or cut 1,000 µL pipette tip. Transfer aggregates to a 15 mL conical tube (for smaller well sizes, a 1.5 mL microcentrifuge tube can be used).

3. Allow the cell aggregates to settle by gravity (a few minutes).
4. Carefully remove the supernatant.
5. Gently resuspend the aggregates in 2 mL of anterior foregut endoderm medium (prepared in "Media preparation" section) and transfer to a new, non–TC-treated 6-well plate.
6. Place on a shaker (70 rpm) in an incubator at 37°C, 5% CO_2, for 48 hours.

Lung progenitor induction (day 7)

1. Tilt the suspension culture plate and allow cell aggregates to settle to the bottom.
2. Collect aggregates using a serological pipette, wide-bore 1,000 µL pipette tip, or cut 1,000 µL pipette tip. Transfer aggregates to a 15 mL conical tube (for smaller well sizes, a 1.5 mL microcentrifuge tube can be used).
3. Allow the cell aggregates to settle by gravity (a few minutes).
4. Carefully remove the supernatant.
5. Gently resuspend the aggregates in 2 mL of lung progenitor medium (prepared in "Media preparation" section) and transfer to a new, non–TC-treated 6-well plate.
6. Place on a shaker (70 rpm) in an incubator at 37°C, 5% CO_2, for 8 days, replacing the medium every 48 hours.
    - The incubation time can be shortened to 6–7 days if necessary.

Transition to dome culture (day 13–15)

1. Tilt the suspension culture plate and allow cell aggregates to settle to the bottom.
2. Collect aggregates using a serological pipette, wide-bore 1,000 µL pipette tip, or cut 1,000 µL pipette tip. Transfer aggregates to a 15 mL conical tube (for smaller well sizes, a 1.5 mL microcentrifuge tube can be used).
3. Allow the cell aggregates to settle by gravity (a few minutes).
4. Carefully remove the supernatant.
5. Gently resuspend the aggregates in 1 mL of Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent. Incubate the tube in a 37°C water bath for 10 minutes, agitating occasionally to resuspend cells.
6. Gently triturate the cell suspension 5 times with a 1,000 µL pipette tip to break the aggregates into single cells. Take a 10 µL sample for counting.
7. Add 4 mL of IMDM to the conical tube with the dissociated cells, and centrifuge at 200 x *g* for 4 minutes. During this step, determine the viable cell concentration from the 10 µL sample using the Invitrogen™ Countess™ Automated Cell Counter or another method of choice. Calculate the total number of viable cells remaining in the conical tube.
8. After centrifuging, remove the supernatant.
9. Resuspend the cells in an appropriate volume of maturation medium plus 10 µM Y-27632.
10. Embed 2,000 cells/µL in Gibco™ Geltrex™ matrix domes at a final Geltrex matrix concentration of 10 mg/mL (check the Certificate of Analysis for the exact protein concentration of the lot; typically ~2 parts Geltrex matrix to 1 part maturation medium). Handle the Geltrex matrix on ice to prevent gelling. Example procedure:
    - To a new 15 mL conical tube, transfer the volume of cell suspension containing the number of cells required for the desired number and volume of domes. Typically, use 50 µL domes with 100,000 cells, but scale accordingly.
    - If the transferred volume of cell suspension is larger than required to achieve a final Geltrex matrix concentration of 10 mg/mL, centrifuge the tube at 400 x *g* for 4 minutes, remove the medium from the supernatant until the remainder is of the desired volume. Gently triturate 10 times to resuspend the cells. Place the tube on ice.
    - Add ice-cold Geltrex matrix to reach the desired final concentration of 10 mg/mL. Triturate 20 times with a 1,000 µL pipette tip.
    - Use a 1,000 µL pipette tip to pipet domes onto a non–TC-treated plate or Petri dish (e.g., 3–4 domes per well of a 6-well plate, or 10–12 domes per well of a 60 mm dish).
11. Once domes have been pipetted onto a plate or dish, carefully invert the vessel and place it in a 37°C incubator for 20 minutes to solidify.
12. Turn the plate right side up, and add the appropriate volume of maturation medium supplemented with 10 µM Y-27632 to each well. Use 2–3 mL per well of a 6-well plate, or about 6 mL per 60 mm dish.
13. Place in the incubator at 37°C, 5% CO_2, for 3 days.

Passage embedded cultures (day 16–18)

- 2,000 cells/µL is a high seeding density used to maximize the number of organoid-forming cells in culture. For optimal culture health, domes should be diluted out after 3 days.

1. Break up domes by pipetting medium with a 1,000 µL pipette tip, and transfer to a 15 mL tube.
2. Triturate 20 times with a 1,000 µL pipette tip, and then 20 times with a 200 µL pipette tip.
3. Increase the volume to 12 mL by adding basal medium (e.g., IMDM or F-12). Mix by triturating twice with a serological pipette.
4. Spin at 400 x *g* for 4 minutes.
5. Remove the supernatant. Leave the cell pellet and Geltrex matrix layer (usually ~0.5 mL depending on the number of domes being processed), as many organoids are likely still trapped. The Geltrex matrix layer typically appears as a semitransparent gel above the cell pellet.

6. Add 1 mL of basal medium, and triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.
7. Increase the volume to 12 mL by adding basal medium. Mix by triturating twice with a serological pipette.
8. Spin at 400 x *g* for 4 minutes.
9. Remove the supernatant and Geltrex matrix carefully (the last bit can be removed with a 1,000 µL or 200 µL pipette tip).
10. Resuspend the cell pellet in maturation medium for the desired split ratio (recommend starting at 1:3–1:4) and Geltrex matrix concentration. Place the tube on ice.
    - Typically, 1 part maturation medium to 2 parts Geltrex matrix for final Geltrex matrix concentration of ~10 mg/mL.
11. Add Geltrex matrix and triturate to mix.
12. Use a 1,000 µL pipette tip to pipet domes onto a non–TC-treated plate or Petri dish (e.g., 3–4 domes per well of a 6-well plate, or 10–12 domes per well of a 60 mm dish).
13. Carefully invert vessel and transfer to a 37°C incubator for 20 minutes.
14. Turn the plate right side up, and add an appropriate volume of maturation medium per well. Use 2–3 mL per well of a 6-well plate, or about 6 mL per 60 mm dish.
15. Place in the incubator at 37°C, 5% CO_2, for 2–3 days.
16. Replace the maturation medium every 2–3 days (typically every Monday, Wednesday, and Friday).
17. Monitor the culture until the organoids are overcrowded, or their average diameter reaches ~250 µm.
18. Passage organoids.
    - Depending on the cell line, cultures can perform better with mechanical (cluster) passaging or single-cell passaging. Protocols for both are included in the "Passaging of lung organoids" section. Single-cell passaging is preferred for homogenizing organoid size. However, some cultures are sensitive to singularization and will not regrow. It is recommended to initially maintain cultures with both methods in parallel.

Maturation

1. Continue to maintain lung organoid cultures, passaging when they become overcrowded, or average diameter reaches ~250 µm.
2. Replace the maturation medium every 48–72 hours (typically every Monday, Wednesday, and Friday).
3. Cultures should adopt a more homogeneous cystic morphology as they mature. This typically happens over the course of a few passages between day 35 and 50, which can vary by cell line and experiment. Morphology is driven by differentiation to epithelial lineages that form the more organized cystic organoids.

Passaging of lung organoids

Cluster passaging protocol

1. Break up domes by pipetting the medium with a 1,000 µL pipette tip, and transfer to a 15 mL tube.
2. Triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.
3. Increase the volume to 12 mL by adding basal medium (e.g., IMDM or F-12). Mix by triturating twice with a serological pipette.
4. Spin at 400 x *g* for 4 minutes.
5. Remove the supernatant. Leave a Geltrex matrix layer, as some organoids are likely still mixed with the disrupted matrix.
6. Add 1 mL of basal medium, and triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.
7. Increase the volume to 12 mL by adding basal medium. Mix by triturating twice with a serological pipette.
8. Spin at 400 x *g* for 4 minutes.
9. Remove the supernatant and Geltrex matrix carefully (last bit can be removed with a 1,000 µL or 200 µL pipette tip).
10. Resuspend in maturation medium plus 10 µM Y-27632 for the desired split ratio and Geltrex matrix concentration. Place the tube on ice.
    - Typically, 1 part maturation medium to 2 parts Geltrex matrix for a final matrix concentration of ~10 mg/mL.
11. Add Geltrex matrix and triturate to mix.
12. Use a 1,000 µL pipette tip to pipet domes onto a non–TC-treated plate or Petri dish (e.g., 3–4 domes per well of a 6-well plate, or 10–12 domes per well of a 60 mm dish).
    - The dome size and plate format can be altered.
13. Carefully invert the plate and transfer it to incubator for 20 minutes.
14. Turn the plate right side up, and add an appropriate volume of maturation medium supplemented with 10 µM Y-27632 per well. Use 2–3 mL per well of a 6-well plate or about 6 mL per 60 mm dish.
15. Place in the incubator at 37°C, 5% CO_2 for 2–3 days.

Single-cell passaging protocol

1. Break up domes by pipetting the medium with a 1,000 µL pipette tip, and transfer to a 15 mL tube.
2. Triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.
3. Increase the volume to 12 mL by adding basal medium (e.g., IMDM or F-12). Mix by triturating twice with a serological pipette.
4. Spin at 400 x *g* for 4 minutes.

5. Remove the supernatant. Leave a Geltrex matrix layer, as organoids are likely still trapped.
6. Add 1 mL PBS without calcium or magnesium, and triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.
7. Increase the volume to 12 mL by adding PBS without calcium or magnesium. Mix by triturating twice with a serological pipette.
8. Spin at 400 x *g* for 4 minutes.
9. Remove the supernatant and Geltrex matrix carefully (the last bit can be removed with a 1,000 µL or 200 µL pipette tip).
10. Resuspend the cells in 1 mL of 0.05% trypsin/EDTA.
11. Incubate at 37°C for 10 minutes.
12. Place a 100 µm strainer in a 50 mL conical tube.
13. After 10 minutes, remove the tube from the incubator and gently triturate the cells 10–20 times with a 1,000 µL pipette tip.
14. Use a serological pipette to add 4 mL of stop medium (IMDM or F-12 plus 5% FBS) to the cells. Collect the cells and pass them through the strainer into the 50 mL tube.
15. With a new serological pipette, collect the cells and transfer them to a new 15 mL conical tube.
16. Spin at 400 x *g* for 4 minutes.
17. Remove the supernatant carefully.
18. Resuspend the cells in 200–1,000 µL of maturation medium plus 10 µM Y-27632.
    - For fewer than five domes, use 200 µL.
19. Determine the viable cell concentration using the Countess Automated Cell Counter or another method of choice.
20. Transfer the appropriate volume of cell suspension for a density of 400 cells/µL (e.g., 2 x 10^4 cells per 50 µL dome) to a microcentrifuge tube.
    - If needed, spin down and resuspend in an appropriate volume of maturation medium plus 10 µM Y-27632 for seeding domes.
21. Place tube on ice.
22. Add Geltrex matrix and triturate to mix.
    - Typically, 1 part maturation medium to 2 parts Geltrex matrix for a final Geltrex matrix concentration of ~10 mg/mL.
23. Use a 1,000 µL pipette tip to pipet domes onto a non–TC-treated plate or Petri dish (e.g., 3–4 domes per well of a 6-well plate, or 10–12 domes per well of a 60 mm dish).
    - The dome size and plate format can be altered.
24. Carefully invert plate and transfer to incubator for 20 minutes.
25. Turn the plate right side up and add appropriate volume of maturation medium supplemented with 10 µM Y-27632 per well. Use 2–3 mL per well of a 6-well plate, or about 6 mL per 60 mm dish.
26. Place in the incubator at 37°C, 5% CO_2, for 2–3 days.

Scalable suspension culture of lung organoids

Passaging lung organoids from embedded to suspension culture (singularizing)—see Figure 4C for representative data

1. Break up domes by pipetting the medium with a 1,000 µL pipette tip, and transfer to a 15 mL tube.
2. Triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.
3. Increase the volume to 12 mL by adding basal medium (e.g., IMDM or F-12). Mix by triturating twice with a serological pipette.
4. Spin at 400 x *g* for 4 minutes.
5. Remove the supernatant. Leave the Geltrex matrix layer, as organoids are likely still trapped.
6. Add 1 mL of PBS without calcium or magnesium, and triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.
7. Increase the volume to 12 mL by adding PBS without calcium or magnesium. Mix by triturating twice with a serological pipette.
8. Spin at 400 x *g* for 4 minutes.
9. Remove the supernatant and Geltrex matrix carefully (the last bit can be removed with a 1,000 µL or 200 µL pipette tip).
10. Resuspend the cells in 1 mL of 0.05% trypsin/EDTA.
11. Incubate at 37°C for 10 minutes.
12. Place a 100 µm strainer in a 50 mL conical tube.
13. After the 10-minute incubation, gently triturate cells 10–20 times with a 1,000 µL pipette tip. Collect cells with a 1,000 µL pipette tip, and pass through the strainer into the 50 mL tube.
14. Use a serological pipette to add 4 mL of stop medium (IMDM or F-12 plus 5% FBS) to the filtered cells. Collect the cells and transfer them to a new 15 mL tube.
15. Spin at 400 x *g* for 4 minutes.
16. Remove the supernatant carefully.
17. Resuspend the cells in 200–1,000 µL of maturation medium plus 10 µM Y-27632.
18. Determine the viable cell concentration using the Countess Automated Cell Counter or another method of choice.

19. To a 15 mL conical tube, transfer the appropriate volume of cell suspension for plating 5.26 x 10^5 cells/cm^2 (e.g., 1 x 10^5 cells/well in a 24-well plate) for the desired number of wells.

    - If needed, spin down and resuspend in an appropriate volume of maturation medium plus 10 µM Y-27632 for seeding domes.

20. Resuspend the cells in maturation medium plus 10 µM Y-27632 in 95% of the desired total volume. Use typical cell culture volumes (e.g., 0.5 mL per well of a 24-well plate, or 2 mL per well of a 6-well plate).
21. Place the 15 mL tube with cells and medium on ice and allow it to cool for ~1 minute.
22. Add ice-cold Geltrex matrix to the cell suspension at 5% of the desired total volume.
23. Triturate the cell suspension 10 times with a 1,000 µL pipette tip.
24. Place the 15 mL tube with the lung organoids in maturation medium plus 5% Geltrex matrix back on ice.
25. Add an appropriate amount of the cell suspension in maturation medium plus 5% Geltrex matrix to a well of the plate format you are using (e.g., 500 µL for a 24-well format; use of non–TC-treated plates is recommended).
26. Ensure that the cell suspension in the maturation medium plus 5% Geltrex matrix coats the entire well, by gently rocking the plate if the entire well is not already coated after pipetting.
27. Place in the incubator at 37°C, 5% CO_2, for 2–3 days.

Passaging lung organoids from embedded to suspension (cluster)

1. Break up domes by pipetting medium with a 1,000 µL pipette tip, and transfer to a 15 mL tube.
2. Triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.
3. Increase the volume to 12 mL by adding basal medium (e.g., IMDM or F-12). Mix by triturating twice with a serological pipette.
4. Spin at 400 x *g* for 4 minutes.
5. Remove the supernatant. Leave the Geltrex matrix layer, as organoids are likely still trapped.
6. Add 1 mL of basal medium, and triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.
7. Increase the volume to 12 mL by adding basal medium. Mix by triturating twice with a serological pipette.
8. Spin at 400 x *g* for 4 minutes.
9. Remove the supernatant and Geltrex matrix carefully (last bit can be removed with a 1,000 µL or 200 µL pipette tip).
10. Resuspend cells in maturation medium based on the desired split ratio (the optimal ratio may be cell line–dependent, but 1:3 is recommended as a starting point). Transfer the desired fraction of cells to a new 15 mL conical tube.
11. Add maturation medium to 95% of the desired total volume.
12. Place the 15 mL tube with cells and medium on ice and allow it to cool for ~1 minute.
13. Add ice-cold Geltrex matrix to the cell suspension at 5% of the desired total volume.
14. Triturate the cell suspension 10 times with a 1,000 µL pipette tip.
15. Place the 15 mL tube with the lung organoids in maturation medium plus 5% Geltrex matrix back on ice.
16. Add an appropriate amount of the cell suspension in maturation medium plus 5% Geltrex matrix to a well of the plate format you are using (use of non–TC-treated plates is recommended).
17. Ensure that the cell suspension in the maturation medium plus 5% Geltrex matrix coats the entire well, by gently rocking the plate if the entire well is not already coated after pipetting.
18. Place in the incubator at 37°C, 5% CO_2 for 2–3 days.

Feeding lung organoids in suspension

- Suspension cultures should be fed every 2–3 days.

1. Collect suspended organoids and medium using a serological pipette, wide-bore 1,000 µL pipette tip, or cut 1,000 µL pipette tip. Transfer to a 15 mL conical tube.
2. (Optional) if cells are stuck to the bottom of the non-TC plate, rinse with several mL of basal medium and collect in the same 15 mL conical tube.
3. Spin at 400 x *g* for 4 minutes.
4. Remove the supernatant carefully.
5. Resuspend the cells to the original culture volume in fresh maturation medium supplemented with 10 µM Y-27632.
6. Add ice-cold Geltrex matrix to the cell suspension at 2.5% of the total volume (this replenishes Geltrex matrix lost during medium replacement).
7. Transfer suspended organoids back to the cell culture vessel.
8. Place in the incubator at 37°C, 5% CO_2 for 2–3 days.

Biobanking of lung organoids

Cryopreservation protocol—see Figure 4D for representative data

1. Break up domes by pipetting medium with a 1,000 µL pipette tip, and transfer to a 15 mL tube.
2. Triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.

3. Increase the volume to 12 mL by adding basal medium (e.g., IMDM or F-12). Mix by triturating twice with a serological pipette.
4. Spin at 400 x *g* for 4 minutes.
5. Remove the supernatant. Leave the Geltrex matrix layer, as organoids are likely still trapped.
6. Add 1 mL of basal medium and triturate 20 times with a 1,000 µL pipette tip and then 20 times with a 200 µL pipette tip.
7. Increase the volume to 12 mL by adding basal medium. Mix by triturating twice with a serological pipette.
8. Spin at 400 x *g* for 4 minutes.
9. Remove the supernatant and Geltrex matrix carefully (the last bit can be removed with a 1,000 µL or 200 µL pipette tip).
10. Resuspend in 90% FBS plus 10% DMSO and 10 µM Y-27632 for the desired number of cryovials.
    - Cryopreservation based on a split ratio is recommended. For example, cryopreserve 3 domes in 1 cryovial and re-seed those into ~9 domes upon thawing.
11. Transfer the cells to cryovials.
12. Transfer the cryovials to a Thermo Scientific™ Mr. Frosty™ Freezing Container and transfer to –80°C overnight.
13. On the next day, transfer the cells to liquid nitrogen for storage.

Modeling of the physiological environment

Air–liquid interface culture protocol—see Figure 6 for representative data

1. Coat the apical side of cell culture inserts with 1–2% Geltrex matrix diluted in basal medium (e.g., DMEM/F-12), and incubate at 37°C for 1–2 hours.
2. Follow the protocol on page 4 for single-cell passaging of lung organoids.
3. Carefully remove the Geltrex coating solution from the cell culture inserts (from step 1) using a 1,000 µL pipette tip.
4. Seed cells on the apical side of the inserts in maturation medium plus 10 µM Y-27632 at a density of 4.5 x 10^5/cm^2. Typical volumes are 300 µL for Thermo Scientific™ Nunc™ 24-well cell culture inserts or 100 µL for Corning™ 24-well cell culture inserts.
    - Removal of CHIR99021 from the medium and addition of the γ-secretase/Notch inhibitor DAPT (10 µM) may increase the proportion of ciliated cells in air–liquid interface cultures [6,18].
5. Add 500 µL of maturation medium plus 10 µM Y-27632 to the basal chamber.
6. Place the plates in the cell culture incubator.
7. After 48–72 hours, check under a microscope to see that the cells have formed a monolayer. If they have not, change the apical and basal medium (without Y-27632) and incubate cells for a few more days. If they have, remove the apical medium and change the basal medium (without Y-27632).
    - Be careful not to disturb the cells when removing the apical medium.
8. Continue to feed the cells every 48–72 hours.
9. Motile cilia are expected to form 4 weeks after air-lifting the apical medium.

Results

Characterization of lung organoids

This protocol describes how to generate lung organoids from PSCs grown in suspension. Figure 1A shows an overview of the directed differentiation, which was adapted from published methods for adherent cells [5-7]. The protocol begins with nucleation of single PSCs in StemScale PSC Suspension Medium (Cat. No. A4965001) to PSC spheroids. Gentle agitation by an orbital shaker prevents the merging of spheroids after formation. Spheroids are differentiated in suspension to definitive endoderm (DE) cells using the PSC Definitive Endoderm Induction Kit (Cat. No. A3062601). DE spheroids are transferred to differentiation medium containing the TGF-β inhibitor SB431542 to induce anterior foregut endoderm (AFE) differentiation [7]. AFE spheroids are induced to ventralized anterior foregut endoderm (VAFE), also known as lung progenitor cells, by exposure to BMP4, retinoic acid, and the GSK-3β inhibitor CHIR99021, which is also a potent activator of Wnt signaling.

During these steps, the spheroids grow larger and darker and begin to form nonspherical morphology during the AFE and VAFE stages (Figure 1B). Gene expression changes observed when performing differentiation in this 3D system are comparable to those observed when performing the protocol in 2D adherent cells (Figure 1C–E) and do not require careful management of 2D cell confluency. The pluripotency marker *SOX2* is downregulated when PSCs are differentiated to DE, but its expression recovers after induction of AFE. *FOXA2* expression is induced as PSCs are differentiated to DE and AFE, consistent with reports of $SOX2^+$/$FOXA2^+$ cells as markers of AFE [7]. By day 15 of the protocol, upregulation of a key transcription factor gene for lung development, *NKX2-1*, is observed in VAFE spheroids. Limited testing has identified this expression in as little as 9 days into the protocol, indicating potential to optimize the timing of its induction, and some flexibility in the timing of the next step (e.g., day 13–15), with day 15 being the recommended optimum (though this may vary by cell line).

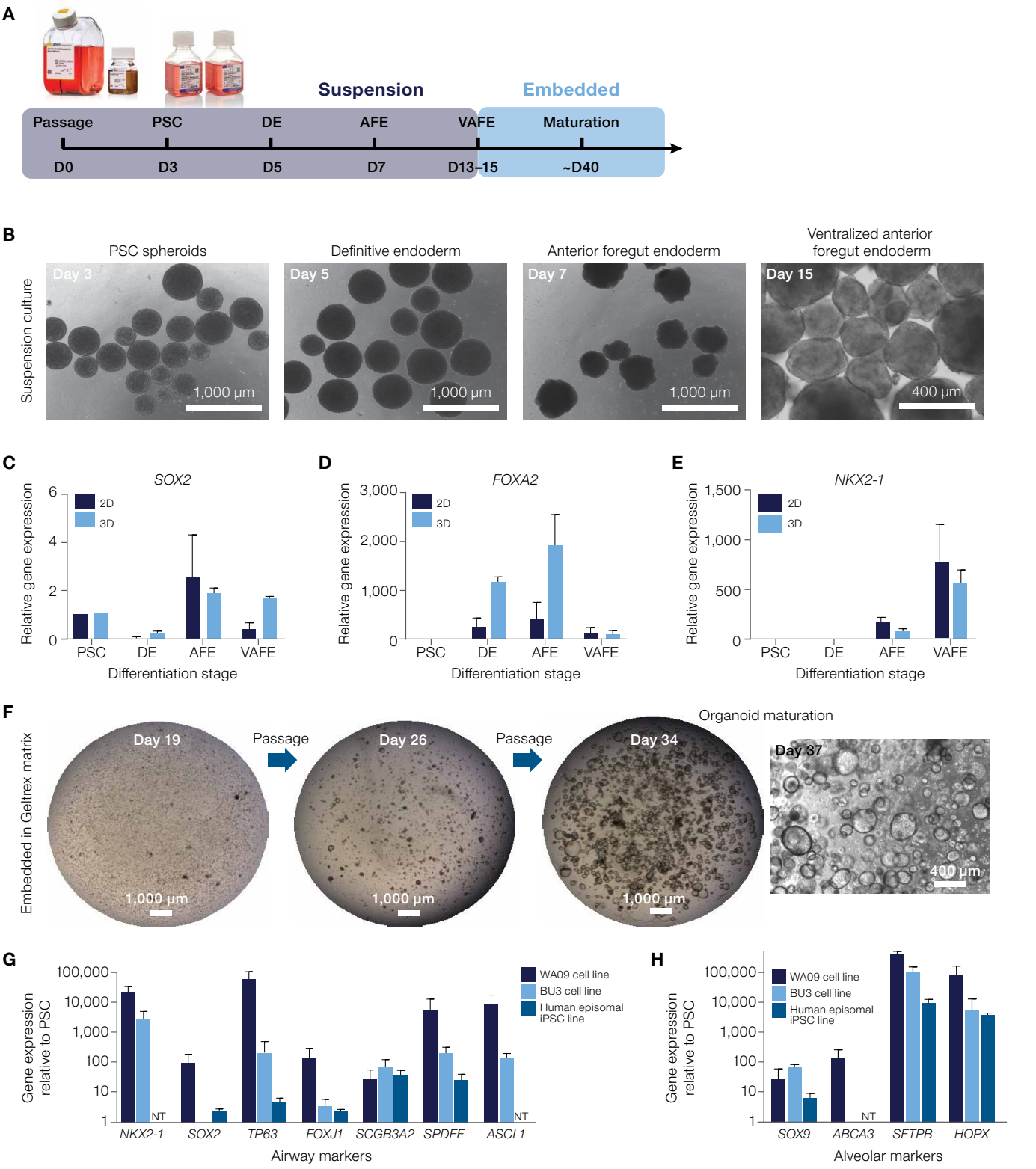

Figure 1. Timeline and gene expression changes during lung organoid differentiation. (A) Timeline for differentiation of PSCs from passaging suspension cultures, expanding PSC spheroids to sufficient size, differentiation to definitive endoderm (DE), anterior foregut endoderm (AFE), ventralized anterior foregut endoderm (VAFE, also known as lung progenitor cells), and maturation to lung epithelial cells. After reaching VAFE in suspension, spheroids are dissociated to single cells and embedded in Geltrex matrix. **(B)** Representative phase-contrast images of spheroids undergoing differentiation in suspension. **(C)** Gene expression measured by qPCR, normalized to GAPDH, and plotted relative to PSC expression of *SOX2*, **(D)** *FOXA2*, and **(E)** *NKX2-1* in cells undergoing differentiation in suspension (3D) compared to performing the protocol on 2D adherent cultures. **(F)** Representative photos of dissociated cells embedded in Geltrex matrix as they form spheroids and differentiate into lung organoids with a cystic, ring-like morphology. Embedded cultures are passaged when the average organoid diameter reaches 250 µm. **(G)** Gene expression analysis of differentiated lung organoids indicates enrichment for airway epithelial cell lineages, including basal (*TP63*), ciliated (*FOXJ1*), secretory (*SCGB3A2*), goblet (*SPDEF*), and pulmonary neuroendocrine (*ASCL1*). NT: not tested. **(H)** Gene expression analysis of differentiated lung organoids indicates enrichment for alveolar epithelial cell lineages (*SOX9*), including alveolar type II (*ABCA3*, *SFTPB*) and alveolar type I (*HOPX*).

On day 13–15, lung progenitor spheroids are enzymatically dissociated to single cells and embedded in Geltrex matrix at 2,000 cells/µL. This high density is used to maximize the number of organoid-forming cells in culture. Initially, cells will cluster into opaque spheroids. As the progenitor cells undergo further differentiation towards epithelial lineages, they will organize into cystic organoids, which appear like rings when viewed from above (Figure 1F). These early spheroids and the mature organoids should be passaged when they reach high density or an average diameter of around 250 µm. Mechanical disruption with a pipette is typically tolerated better by the cells than enzymatic dissociation and is recommended unless singularization is required for downstream assays. After several passages, the lung organoid cultures are expected to be predominately cystic in morphology, which typically occurs between days 35 and 50. Mature lung organoids express lineage markers for the major cellular constituents of the airway (basal, ciliated, secretory, goblet, and pulmonary neuroendocrine cells) (Figure 1G) and alveoli (alveolar type I and type II cells) (Figure 1H) of the lung. Representative phase-contrast images, as well as immunostaining for NKX2-1 and surfactant protein B (SFTPB), are shown in Figure 2A–D. An iPSC line endogenously labeled with a reporter for surfactant protein C (SFTPC), a marker specific for the alveolar type II cells, was differentiated using this protocol and demonstrates the induction of this cell type in the PSC-derived lung organoids (Figure 2E, F).

To examine global transcriptome changes induced by this differentiation, bulk RNA sequencing was used to compare undifferentiated PSCs to the induced lung organoids. Principle component analysis demonstrates that the lung organoid transcriptomes are distinct from the undifferentiated PSCs, with experimental replicates from the same cell line clustering slightly closer together, possibly indicating cell line–dependent differences in the differentiation (Figure 3A).

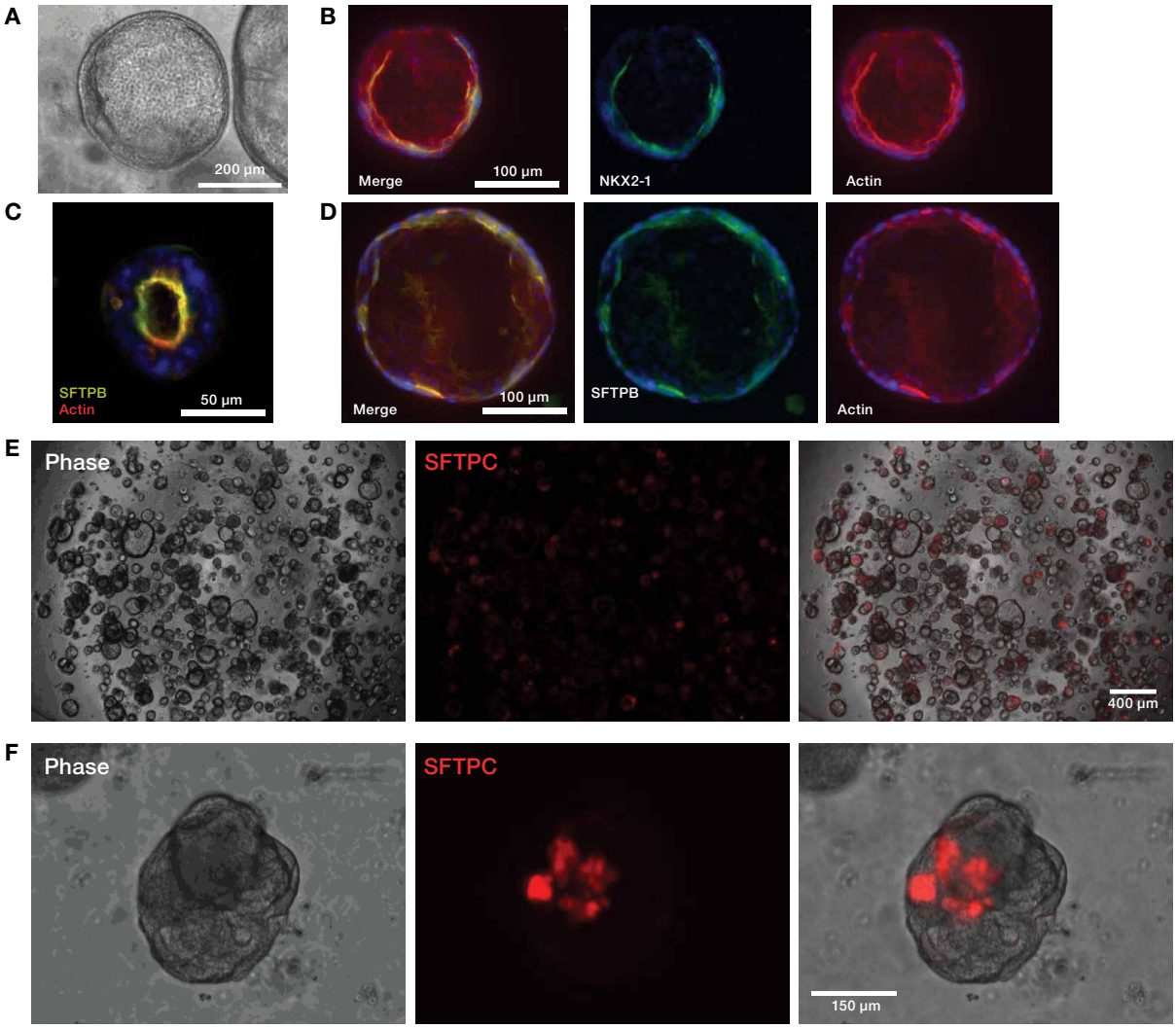

Figure 2. Protein detection of lung markers in organoids. (A) Representative phase-contrast image of cystic organoid morphology. **(B)** Immunofluorescent detection of NKX2-1 (green) in embedded lung organoids. Actin is stained with phalloidin (red). Nuclei are counterstained with Hoechst™ 33342 dye (blue). **(C, D)** Embedded lung organoids stained for SFTPB (green). Actin is stained with phalloidin (red). Nuclei are counterstained with Hoechst 33342 dye (blue). **(E, F)** iPSC cells engineered with an endogenous fluorescent reporter for SFTPC (red) were differentiated to lung organoids and indicate the presence of alveolar type II cells expressing this marker as well as other cell types.

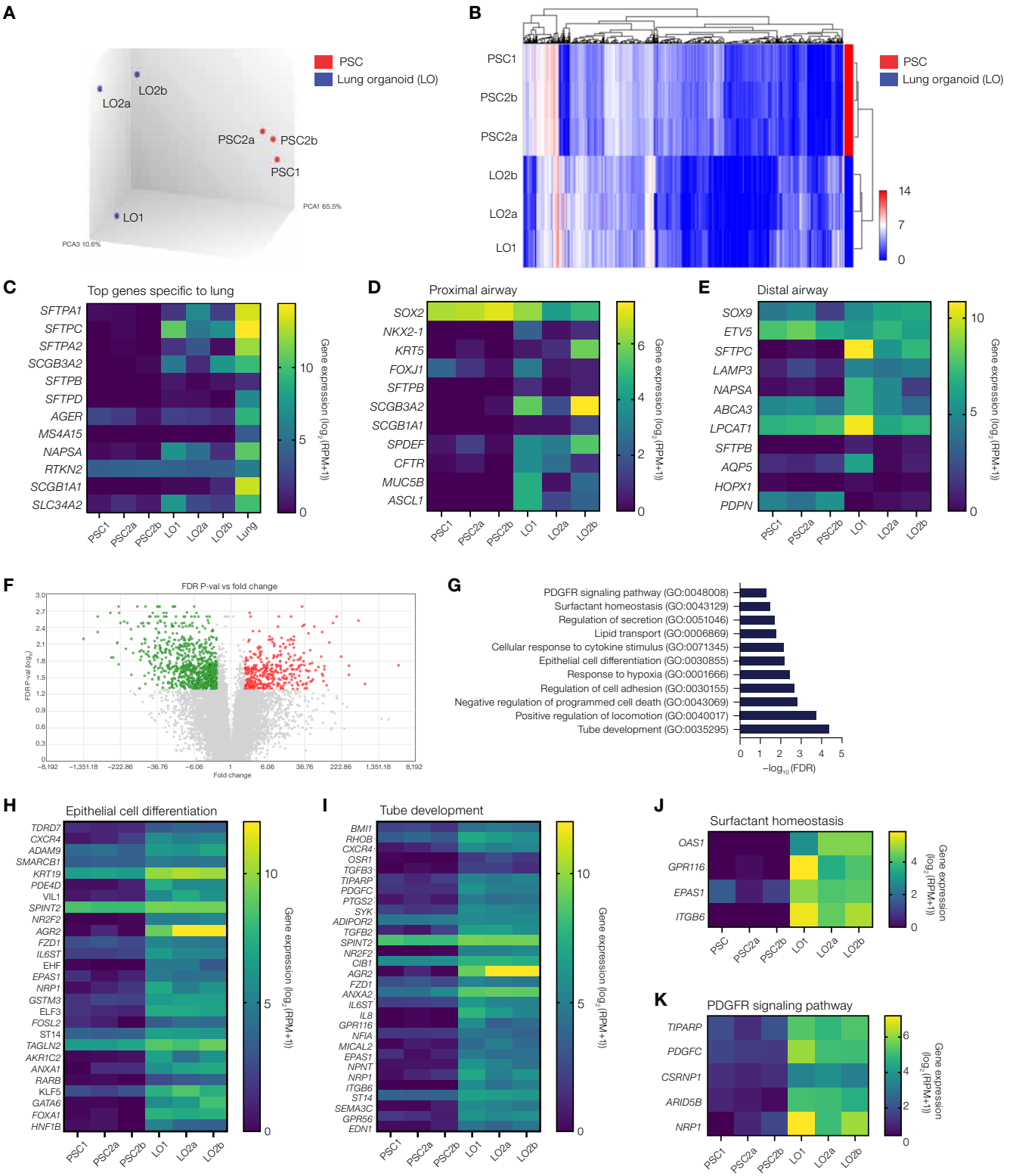

Figure 3. Transcriptome of lung organoids. Next-generation sequencing was used to measure the expression levels of over 20,000 human RefSeq genes in undifferentiated PSCs or in PSC-derived lung organoids. **(A)** Principal component analysis reveals the global transcriptome responses after differentiation. The plot indicates that undifferentiated PSCs from two different lines (one in experimental replicates a and b) are quite different than the lung organoids, which cluster on the other side of the first principal axis (PC1). **(B)** The transcriptome of lung organoids is highly altered from PSCs. The heat map indicates expression levels (log_2 (RPM+1)) of 4,861 genes with fold changes of >2 or <–2. RPM: reads per million. Supervised hierarchical cluster analysis shows that the samples cluster by differentiation status. **(C)** Gene expression of the 12 most enriched genes in human lung tissue [8-9] in PSCs, lung organoids, and primary human lung. **(E)** Gene expression of distal airway (alveolar) epithelial cell markers. **(F)** Volcano plot of all assayed genes illustrates the 365 upregulated genes in lung organoids (red) and 851 downregulated genes (green) at a fold-change cutoff of >2 and FDR <0.05. FDR: false discovery rate. **(G)** Gene ontology analysis of the 365 genes upregulated in lung organoids highlights enrichment for biological processes related to lung development and homeostasis. Analysis was performed with Panther v17.0 [19,20]. **(H–K)** Differentially expressed genes related to epithelial cell differentiation, tube development, surfactant homeostasis, and the platelet-derived growth factor receptor (PDGFR) signaling pathway.

Hierarchical clustering also groups these samples by differentiation status and reveals clusters of activated or inactivated genes in the lung organoids (Figure 3B). The 12 genes most enriched in the human lung were upregulated in lung organoid cells compared to undifferentiated PSCs (Figure 3C) [8,9]; the expression levels of these genes tend to be below that of primary lung cells (e.g., measured from Invitrogen™ Human Lung Total RNA, Cat. No. AM7968) suggesting that other niche factors may be required for complete maturation, which is consistent with reports in the literature [4,10]. Proximal airway and distal airway (alveoli) gene markers were also enriched compared to undifferentiated cells (Figure 3D–E). Differential gene expression analysis identified 365 upregulated and 851 downregulated genes (fold change >2, FDR <0.05) in the lung organoids (Figure 3F). Gene ontology analysis of the upregulated genes revealed that this list is enriched for biological processes related to lung epithelial homeostasis, including epithelial cell differentiation, tube development, surfactant homeostasis, and platelet-derived growth factor receptor (PDGFR) signaling (Figure 3G–K).

Scale-up and cryopreservation

The length and requirement for embedded culture during this protocol (Figures 1A, 4A–B) limit the ease of use and scalability. To improve scalability, a protocol was developed to culture the differentiated lung organoids in suspension, where dilute extracellular matrix (ECM) protein (5% Geltrex matrix volume) promotes cell nucleation and reformation of organoids after seeding as single cells or small clusters (Figure 4C). Overall, the suspension protocol is more economical, with time and cost savings, especially with reduced matrix consumption while expanding the lung organoids to larger cell numbers for high-throughput experiments or for cryopreservation. Lung organoids can be cryopreserved as small clusters of cells following mechanical passaging, which regrow into organoids upon thawing (Figure 4D). This enables an end-to-end workflow for biobanking where PSCs are differentiated to lung progenitors in suspension, induced to lung epithelial organoids in embedded culture, passaged into suspension culture for scale-up, and finally cryopreserved to create large working banks of frozen organoid cells.

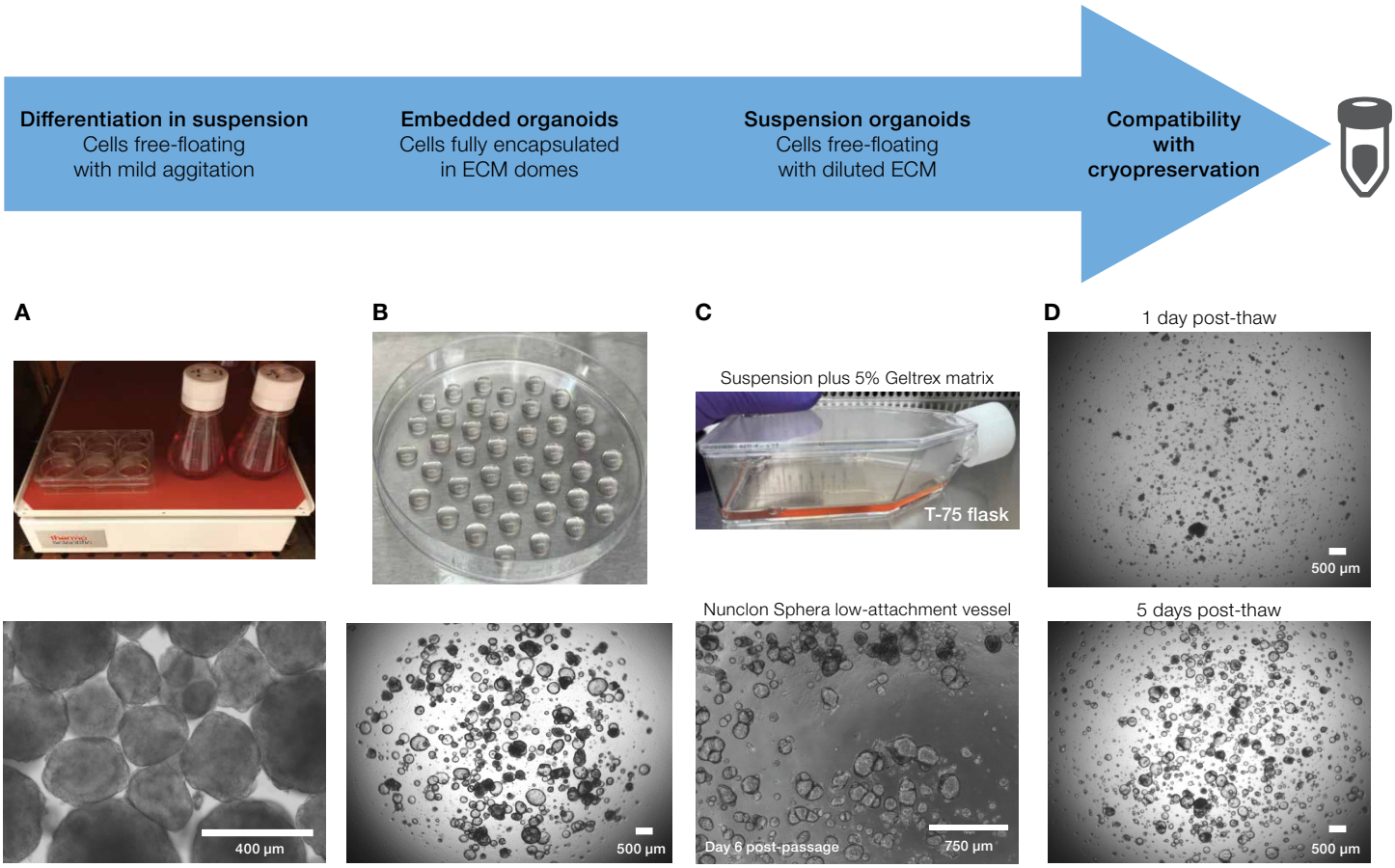

Figure 4. End-to-end workflow for differentiation and scale-up of large numbers of lung organoids for biobanking. (A) Representative suspension cultures (6-well plate and 125 mL shake flask) on an orbital shaker placed in a cell culture incubator. Lower: Phase-contrast image of ventralized anterior foregut endoderm spheroids grown in suspension. **(B)** 50 µL Geltrex matrix domes containing organoid cells plated in a 100 mm Petri dish. Lower: Phase-contrast image of embedded lung organoids. **(C)** T-75 flask containing suspension culture with dilute (5% by volume) Geltrex matrix. Lower: Lung organoids grown in suspension in the 5% Geltrex matrix. **(D)** Lung organoids recovered from cryopreservation and embedded in Geltrex matrix 1 day or 5 days post-thaw.

Applications of lung organoids

The lung organoid transcriptome includes expression of entry receptors for multiple respiratory viruses (Figure 5A) [11-13], indicating that these cells are candidates for modeling respiratory virus infection. As proof of principle, SARS-CoV-2 modeling was studied further. RNA expression levels of SARS-CoV-2 receptors from primary lung cells were accessed from a publicly available database [8, 14-17] and match closely to the expression levels in lung organoids (Figure 5B,C). These expression levels were also verified in the lung organoids via qPCR, demonstrating that ACE2 and TMPRSS2 are enriched in the lung organoids compared to undifferentiated PSCs, and that the organoid cells express high levels of both CTSB and CTSL (Figure 5D). These data suggest that the lung organoids should be permissive to SARS-CoV-2 infection. To test this, organoids were grown in 10 µL domes in black-walled 96-well plates (Figure 5E) and exposed to commercially available SARS-CoV-2 spike protein–pseudotyped lentivirus carrying a luciferase transfer gene (BPS Biosciences). Infected cells were detected by luminescence 3 and 5 days post-infection (Figure 5F). In a separate experiment, treatment with a SARS-CoV-2 spike protein neutralizing antibody, but not an isotype control, significantly reduced the signal from infected cells (Figure 5G). Together, these data demonstrate the *in vitro* utility for this type of model for viral infection research.

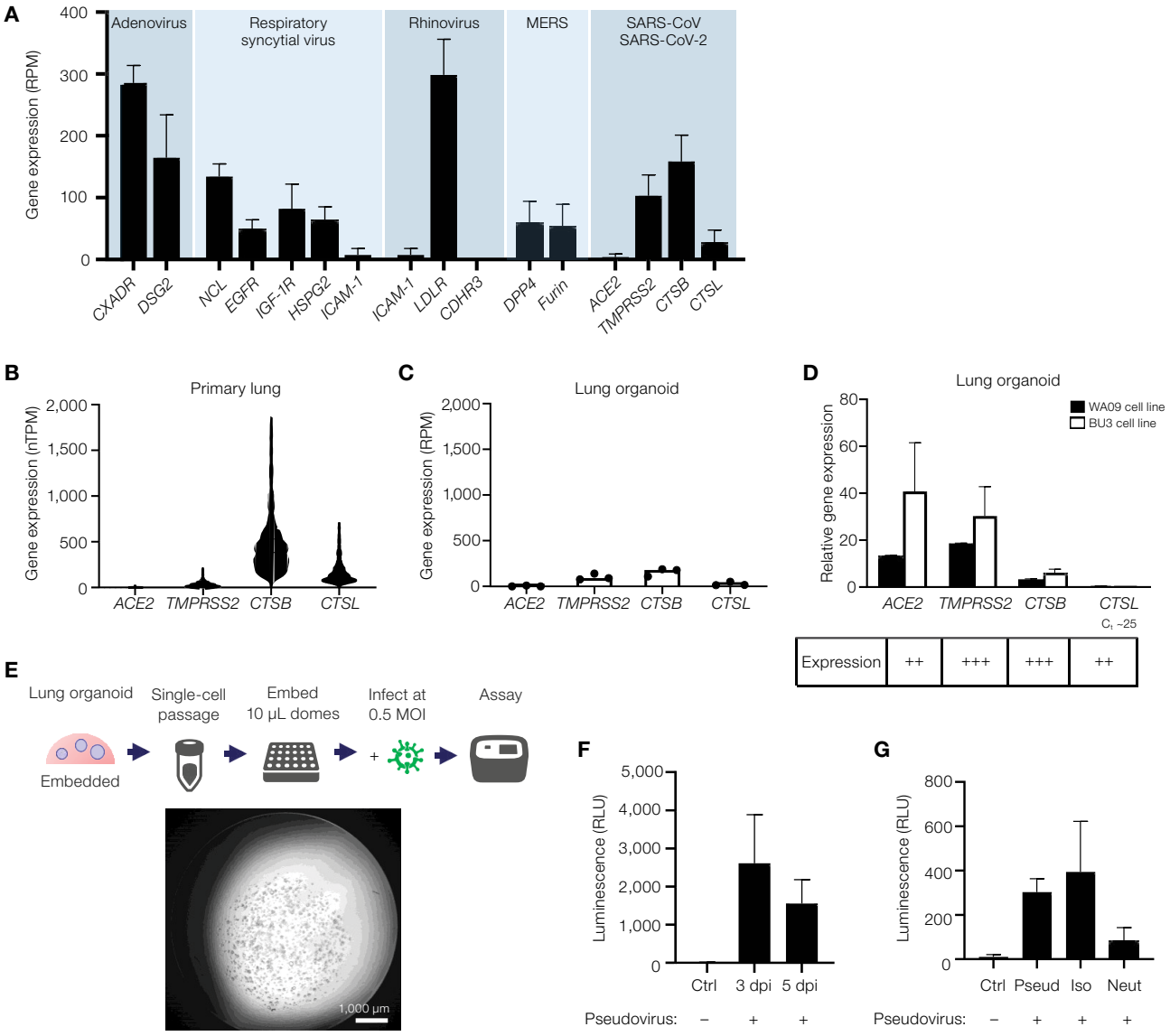

Figure 5. Modeling respiratory viruses with lung organoids. (A) Lung organoid gene expression of entry receptors for common respiratory viruses. RPM: reads per million. **(B)** Gene expression level in primary human lungs for SARS-CoV-2 entry receptors [8, 14-17]. nTPM: normalized transcripts per million. **(C)** Gene expression of SARS-CoV-2 entry receptors in lung organoids assessed by next-generation sequencing. **(D)** qPCR gene expression, relative to undifferentiated PSCs, of SARS-CoV-2 entry receptors in lung organoids. Overall expression levels based on threshold cycle (C_t) are indicated below the graph; e.g., CTSL is not enriched compared to PSCs but is still expressed in both cell types. **(E)** Lung organoid cultures were scaled down to 10 µL domes in black-walled 96-well plates and infected with SARS-CoV-2 spike protein–pseudotyped lentivirus carrying a luciferase transfer gene. **(F)** Luminescence of control or pseudovirus-treated lung organoid cultures 3 and 5 days post-infection (dpi). **(G)** Luminescence of control or pseudovirus-treated lung organoid cultures in the presence of a spike protein–neutralizing antibody (Neut) or an isotype control (Iso).

PSC-derived epithelial cells from lung organoids can be cultured at the air–liquid interface to model the physiological environment of these cells. Briefly, lung organoids are dissociated to single cells and the cells are seeded on the apical side of cell culture inserts (Figure 6A). Once the cells grow to confluence, the apical medium is removed. These monolayer cells will stratify into multiple layers and form tight junctions, as demonstrated by rising trans-epithelial electrical resistance (TEER) as the cultures are maintained (Figure 6B–D). Hematoxylin and eosin staining of a vertical cross-section of one of these cultures demonstrates the cellular organization (Figure 6E). Around 4 weeks after air-lift, motile cilia are visible at the apical surface. Immunostaining confirms the expression of the apical markers acetylated tubulin, surfactant protein C, and ACE2 (Figure 6F–H). Thus, PSC-derived lung organoid cells demonstrate mature features of lung cells when grown at the air–liquid interface.

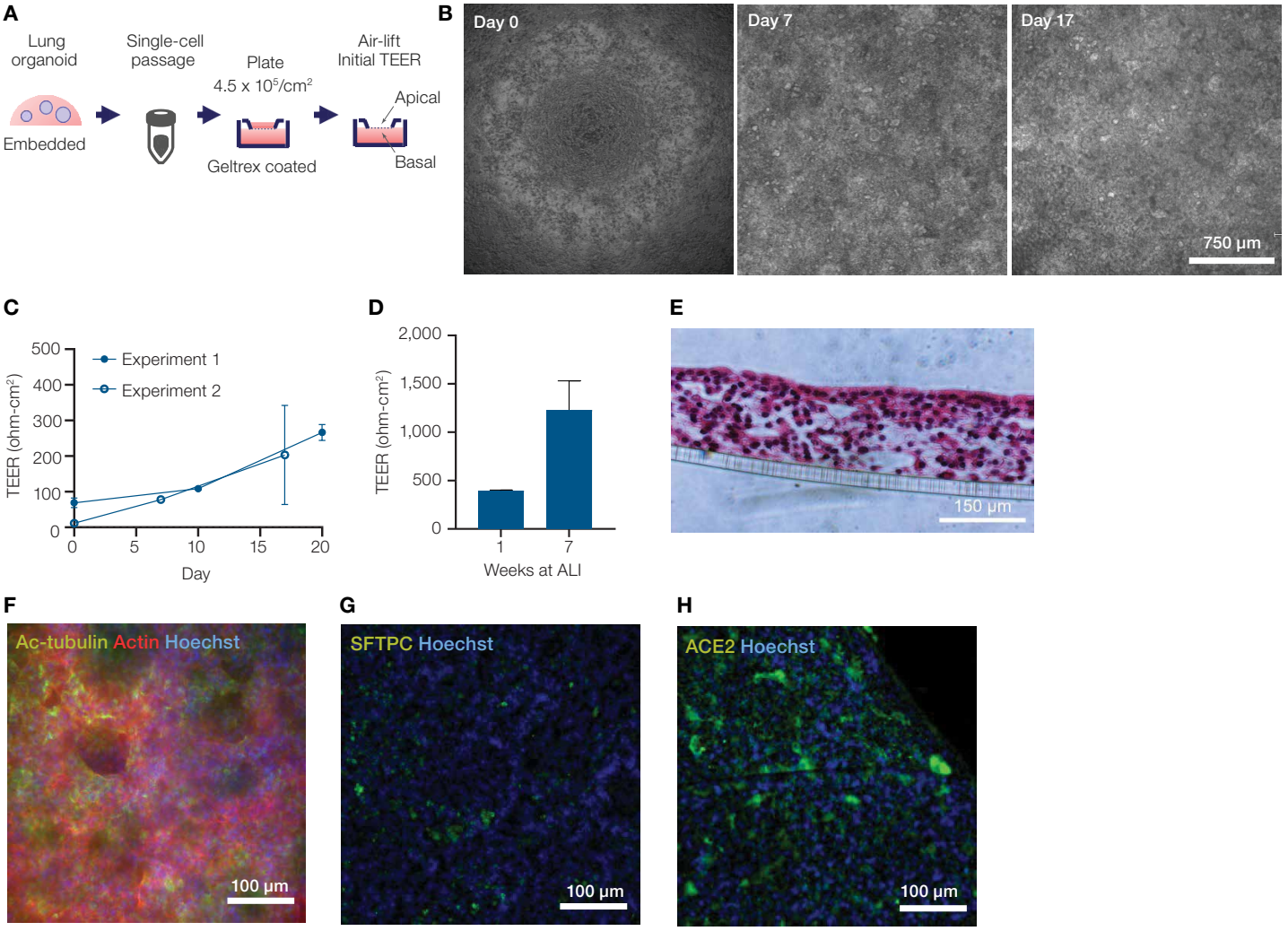

Figure 6. Air–liquid interface (ALI) culture of lung organoid–derived cells. (A) Protocol schematic showing that lung organoids were dissociated to single cells, plated on Geltrex matrix–coated cell culture inserts, grown to confluence in the presence of apical and basal medium, and then cultured with only basal medium and the apical surface exposed to air. **(B)** Phase-contrast images of cells on the day of seeding and 7 or 17 days after. **(C, D)** Trans-epithelial electrical resistance (TEER) measurements of lung organoid–derived cells grown at the ALI. Resistance increased with time in culture during separate experiments. **(E)** Hematoxylin and eosin (H&E) staining of a vertical cross-section of the cell culture insert. Cell nuclei appear purple and cell cytoplasm and extracellular matrix appear pink. The transparent membrane can be seen at the bottom of the culture. **(F)** Immunostaining of ALI cultures for acetylated tubulin (green). Actin is stained with phalloidin (red). Nuclei are stained with Hoechst 33342 dye (blue). **(G)** Immunostaining of ALI cultures for surfactant protein C (SFTPC, green). Nuclei are stained with Hoechst 33342 dye (blue). **(H)** Immunostaining of ALI cultures for ACE2 (green). Nuclei are stained with Hoechst 33342 dye (blue).

Conclusions

Human PSCs grown in StemScale medium can be differentiated in suspension to lung progenitor cells. Cells from these spheroids can be dissociated and embedded in ECM for further differentiation into lung organoids. The differentiated lung organoids can be cryopreserved for later use, and a suspension workflow has been optimized to significantly increase the ease of producing large numbers of these organoids for biobanking or high-throughput screening. PSC-derived lung organoids may be used for respiratory infection research or other disease modeling, and the cells can also be cultured at the air–liquid interface.

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Ordering information

Product	Manufacturer	Quantity	Cat. No.
StemScale PSC Suspension Medium	Thermo Fisher Scientific	1 L	A4965001
PSC Definitive Endoderm Induction Kit	Thermo Fisher Scientific	1 kit	A3062601
IMDM (Iscove's Modified Dulbecco's Medium)	Thermo Fisher Scientific	500 mL	12440053
Ham's F-12 Nutrient Mix	Thermo Fisher Scientific	500 mL	11765054
GlutaMAX Supplement	Thermo Fisher Scientific	100 mL	35050061
B-27 Supplement (50X), serum free	Thermo Fisher Scientific	10 mL	17504044
N-2 Supplement	Thermo Fisher Scientific	5 mL	17502048
Bovine Albumin Fraction V (7.5% solution)	Thermo Fisher Scientific	100 mL	15260037
Human BMP-4 Recombinant Protein	Thermo Fisher Scientific	10 µg	PHC9533
Human FGF-7 (KGF) Recombinant Protein	Thermo Fisher Scientific	10 µg	PHG0094
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	Thermo Fisher Scientific	5 mL	A1413202
Ascorbic Acid	MilliporeSigma	100 g	A4544
Monothioglycerol	MilliporeSigma	25 mL	M6145
Dexamethasone	MilliporeSigma	25 mg	D4902
8-Bromo-cAMP	MilliporeSigma	100 mg	B7880
3-Isobutyl-1-methylxanthine (IBMX)	MilliporeSigma	100 mg	I5879
SB431542	Selleck Chemicals	10 mg	S1067
Y-27632 2HCl	Selleck Chemicals	5 mg	S1049
CHIR99021	Tocris Bioscience	10 mg	4423
Retinoic Acid (ATRA)	Tocris Bioscience	50 mg	0695

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