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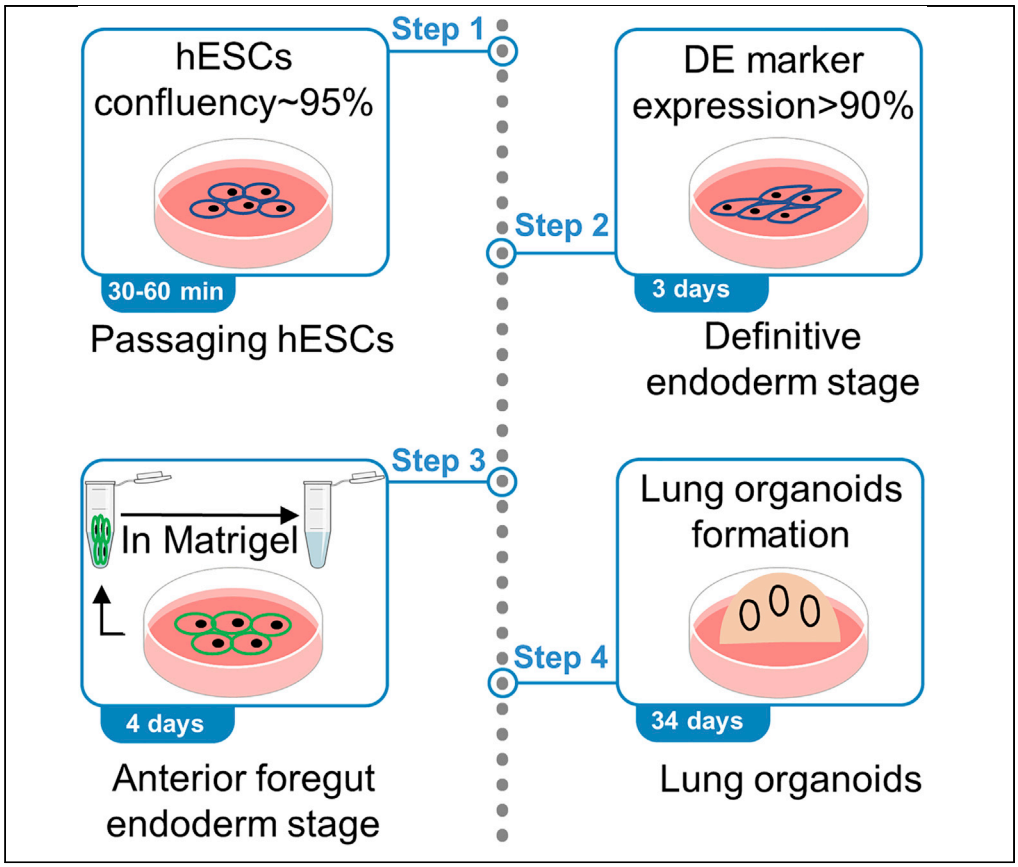
Protocol

Generation of human embryonic stem cell-derived lung organoids



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Highlights

hESC-derived lung organoids

A sequential 2D to 3D protocol

Uses less cytokines and small molecules

Model to study human lung development

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Generation of human embryonic stem cell-derived lung organoids

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SUMMARY

This protocol describes how to generate lung organoids from human embryonic stem cells. Lung organoids form by self-assembly in Matrigel and contain lung epithelial cell types. The protocol presented in this study is simple and only uses 6 cytokines or small molecules. This protocol provides a promising tool to study human lung development, drug screening, regeneration, and disease modeling *in vitro*.

For complete details on the use and execution of this protocol, please refer to Chen et al. (2018).

BEFORE YOU BEGIN

Planning lung organoids differentiation timings

Timing: 41 days

To date, the technology to generate human embryonic stem cell (hESC)-derived lung cells have changed from traditional two-dimensional (2D) monolayer cell-based differentiation to three-dimensional (3D) organoid differentiation (Chen et al., 2017, 2018; Dye et al., 2015; Kim et al., 2021; Li et al., 2021). Organoid models possess structural organization similar to the native organ and cell types from multiple germ layers, making them a physiologically complex model to investigate organ developmental processes, drug screening, regeneration and disease modeling *in vitro* (Chen et al., 2020, 2021; Clevers, 2016; Kim et al., 2021; Wang et al., 2021). However, current protocols use more cytokines/small molecules and require longer time to generate hESC-derived lung organoids (HLOs) (Chen et al., 2017; Dye et al., 2015, 2016; Strikoudis et al., 2019; Wang et al., 2020). The present study provides a simple protocol that uses less cytokines and small molecules to generate HLOs.

Preparation of matrigel-coated plates

Timing: 30–60 min

1. Thaw a 150 μL stock vial of Matrigel (hESC-qualified Matrix) on ice, overnight (12–16 h).

Note: Thaw stock solution of Matrigel (hESC-qualified Matrix, BD Bioscience, Cat#354277) on ice following the operation manual. Small aliquots (150 μL) can be stored at −80°C for 6 months.

2. Dilute the thawed Matrigel (150 μL) in ice-cold DMEM/F12 (12 mL). Diluted Matrigel can be stored at 4°C for 2 weeks.
3. For each well of a 6 well plate, add 1 mL of the diluted Matrigel.
4. Following Matrigel coating, incubate the plates in a 37°C/5% CO2 cell culture incubator for 30 min.

⚠ CRITICAL: Ensure that the diluted Matrigel solution covers the whole surface. Do not let the diluted Matrigel evaporate. To this purpose, we recommend preparing the Matrigel-coated plate soon before use (30 min – 4 h).

Preparation of differentiation media and growth factors

Timing: 2 h

Note: All procedures should be performed under sterile conditions and done in a Biosafety Level 2 (BSL2) tissue culture hood. All reconstituted media should be used within 2 weeks following preparation. If needed, reconstituted medium can be stored at −20°C for 6 months or at −80°C for long-term storage. Do not freeze again after thawing.

5. hESCs medium.
   a. Add 100 mL mTeSR1™ 5 × supplement to 400 mL mTeSR1™ basal medium.
6. hESCs-derived lung organoids differentiation medium.
   a. For definitive endoderm differentiation:
      i. Prepare definitive endoderm differentiation medium by adding 1× penicillin streptomycin (PS), 100 ng/ mL Activin A, 2 μM CHIR99021, 1× MEM Non-Essential Amino Acids Solution (NEAA), 1× GlutaMax, and 0.5% BSA to 100 mL RPMI 1640 medium.
   b. For anterior foregut endoderm differentiation:
      i. Prepare anterior foregut endoderm differentiation medium by mixing 100 mL Advanced-DMEM/F12, 200 ng/ mL noggin, 10 μM SB431542, 2 μM CHIR99021, 500 ng/ mL FGF4, 10 mM HEPES, 1× NEAA, 2× B27, 1× N2, 1× GlutaMAX, and 1× PS.
   c. For lung organoid differentiation:
      i. Prepare lung organoid differentiation medium by mixing 100 mL Advanced-DMEM/F12, 1% FBS, 1× NEAA, 1× GlutaMAX and 1× PS.
   d. For anterior foregut endoderm cell dissociation:
      i. Accutase with 10 μM Y-27632 was used for anterior foregut endoderm cell dissociation.
      ii. For each well of a 6 well plate, add 1 mL of the cell dissociation solution.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FOXA2 (1:50 dilution)	BD Pharmingen	Cat#561589
SOX17 (1:50 dilution)	BD Pharmingen	Cat#562205
NKX2.1 (1:250 dilution)	Abcam	Cat#ab76013
SOX9 (1:40 dilution)	R&D systems	Cat#AF3075
P63 (1:200 dilution)	Abcam	Cat#ab124762
Mature-SFTPC (1:800 dilution)	SEVEN HILLS	Cat#WRAB-76694
Acetylated Tubulin (ACE-TUB) (1:400 dilution)	Sigma-Aldrich	Cat#T7451

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Donkey anti-goat (Alexa 488) (1:500 dilution)	Jackson ImmunoResearch	Cat#705-545-147
Donkey anti-rabbit (Alexa 488) (1:500 dilution)	Jackson ImmunoResearch	Cat#A-21206
Donkey anti-mouse (Alexa 647) (1:500 dilution)	Invitrogen	Cat#A32787
Donkey anti-mouse (Alexa 568) (1:500 dilution)	Jackson ImmunoResearch	Cat#A10037
Chemicals, peptides, and recombinant proteins		
B27 supplement	Life Technologies	Cat#17504044
N2 supplement	Life Technologies	Cat#A1370701
Activin A	R&D Systems	Cat#338-AC-050
Noggin	R&D Systems	Cat#6057-NG-100
FGF4	Peprotech	Cat#100-31-1 MG
SB431542	Tocris Bioscience	Cat#1614-10 MG
CHIR-99021	Tocris Bioscience	Cat#4423-10 MG
DMSO	Sigma-Aldrich	Cat#D4540
Advanced DMEM/F12	Life Technologies	Cat#12634010
Matrigel™ Matrix	BD Biosciences	Cat#356237
Matrigel™ hESC-qualified Matrix	BD Biosciences	Cat#354277
mTeSR1™	STEMCELL Technologies	Cat#85850
Accutase	Life Technologies	Cat#A1110501
Y-27632	Selleck Chemicals	Cat#S1049
DMEM/F12	Life Technologies	Cat#10565018
DPBS	Life Technologies	Cat#14190144
GlutaMAX	Life Technologies	Cat#35050061
MEM Non-Essential Amino Acids Solution (NEAA)	Life Technologies	Cat#11140050
HEPES	Life Technologies	Cat#15630106
Penicillin streptomycin (PS)	Life Technologies	Cat#15140122
Experimental models: Cell lines		
Human embryonic stem cell line H9	WiCell	WA09
Other		
6 well plate	Life Technologies	Cat#140675
Serum pipette	Greiner Bio-one	Cat#612361

MATERIALS AND EQUIPMENT

Reagent	Final concentration	Amount needed (for 500 mL)
hPSC medium		
mTeSR1™ 5 × supplement	1×	100 mL
mTeSR1™ basal medium	N/A	400 mL
Definitive endoderm differentiation medium		
PS (100×)	1%	5 mL
BSA (5%)	0.5%	50 mL
CHIR99021 (2 mM)	2 μM	500 μL
Activin A (100 μg/ mL)	100 ng/ mL	500 μL
GlutaMAX (100×)	1%	5 mL
NEAA (100×)	1%	5 mL
RPMI 1640 medium	N/A	434 mL
Anterior foregut endoderm differentiation medium		
PS (100×)	1%	5 mL
B27 supplement (50×)	2%	10 mL
N2 supplement (100×)	1%	5 mL
GlutaMAX (100×)	1%	5 mL
NEAA (100×)	1%	5 mL
CHIR-99021 (4 mM)	2 μM	250 μL
SB431542 (20 mM)	10 μM	250 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount needed (for 500 mL)
HEPES (1 M)	10 mM	500 μL
Noggin (200 μg/ mL)	200 ng/ mL	500 μL
FGF4 (500 μg/ mL)	500 ng/ mL	500 μL
Advanced DMEM/F12 medium	N/A	468 mL
Lung organoids differentiation medium		
PS (100×)	1%	5 mL
GlutaMAX (100×)	1%	5 mL
NEAA (100×)	1%	5 mL
FBS	1%	5 mL
Advanced DMEM/F12 medium	N/A	480 mL
All reconstituted medium should be used within 2 weeks following preparation.		

Growth factor and other reagents preparation				
Reagent	Solvent	Stock concentration	Working concentration	Storage
Activin A	Sterile 0.2% BSA	100 μg/mL	100 ng/mL	−80°C in small aliquots; 6 months
FGF4	Sterile 0.2% BSA	500 μg/mL	500 ng/mL	−80°C in small aliquots; 6 months
Noggin	Sterile 0.2% BSA	250 μg/mL	100 ng/mL	−80°C in small aliquots; 6 months
CHIR-99021	Sterile DMSO	4 mM	2 μM	−80°C in small aliquots; 12 months
Y-27632	Sterile DMSO	10 mM	10 μM	−80°C in small aliquots; 6 months
SB431542	Sterile DMSO	20 mM	10 μM	−80°C in small aliquots; 12 months

STEP-BY-STEP METHOD DETAILS

Passaging hESCs for lung organoids differentiation

Timing: 30–60 min

Day – 1

This step describes how to prepare hESCs from colony to single-cell suspension which will be used for differentiation into definitive endoderm cells. This protocol requires a starting population of hESC colonies in a 6 well plate format to be in 75%–85% confluency. This protocol was adapted from previous reports (Chen et al., 2018; Meseguer-Ripolles et al., 2021).

1. Aspirate the medium from hESCs at 75%–85% confluency (Figures 1A and 1B).
2. Wash hESCs with 2 mL of 1 × room temperature DPBS (without $Ca2^{+}/Mg2^{+}$) per well of a 6 well plate.
3. Aspirate the DPBS from the well.
4. Add 1 mL of room temperature Accutase containing Y-27632 (10 μM) per well.
5. Leave the cultures in a 37°C/5% CO_2 cell culture incubator for 3–6 min to dissociate into single cells.

Note: Incubation time can vary depending on the extent of cell confluency or cell lines used (e.g., H1 hESCs). The use of Y-27632 (Rho-associated kinase inhibitor) enhances cell survival.

6. 3 min later, check the hESC colonies under the microscope, the cell-cell interactions should become loose and cells within the colonies should become more refringent (cells should not be floating) (Figure 1C).

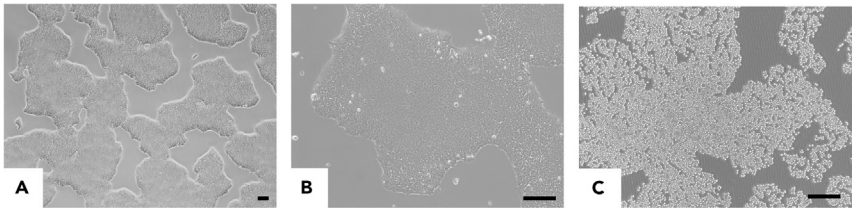

Figure 1. hESC colonies morphology

(A and B) Representative example of hESC colonies grown on Matrigel approaching 75%–85% confluency.

(C) Collection of hESCs by using Accutase incubation. Proper time for Accutase incubation (3–6 min) will not make cells float, check cells under the microscope every 2 min, the cell-cell interactions become loose and cells will be easily detached by pipetting medium over the cells. Scale bar, 100 μm.

7. Carefully aspirate the Accutase solution without disturbing the cells and immediately add 1 mL of pre-warmed mTeSR1™ with 10 μM Y-27632 per well into a 6 well plate.

   Note: Proper time of Accutase incubation (3–6 min) will not make cells float, check cells under the microscope every 2 min, the cell-cell interactions will become more relaxed and cells will be easily detached by pipetting medium over the cells (Figure 1C). A longer time of Accutase incubation (8–15 min) will make cells floating.

8. Use 1 mL tips to gently pipette up and down to detach the cells from the well into the medium.
9. Carefully collect the medium with cells into a 15 mL centrifuge tube.
10. Centrifuge cells at 200 g for 5 min at room temperature.
11. Aspirate the supernatant carefully without disturbing the cell pellet.
12. Carefully aspirate the Matrigel without damaging the Matrigel-coated surface.
13. Add 6–12 mL of pre-warmed mTeSR1™ with 10 μM Y-27632 to cells and gently pipette up and down to ensure a homogeneous single cell solution.
14. Count cells using an automatic cell counter using trypan blue to exclude dead cells.
15. Seed cells at a density of 1.0–2.0 × 10^5 cells/cm^2 into the Matrigel coated well using mTeSR1™ with 10 μM Y-27632 to a final volume of 2 mL per well in a 6 well plate.

   ⚠ CRITICAL: Gently move the plate several times back and forth and left to right to ensure an even distribution of the cell suspension.

16. Incubate the plate in a 37°C/5% CO_2 cell culture incubator overnight (12–16 h) to start differentiation into definitive endoderm cells the day after.

hESC-derived lung organoids

Timing: day 1, 41 days, 1 h daily

This step describes how to generate hESC-derived lung organoids by following embryonic lung developmental cues by temporarily introducing a variety of growth factors and small molecules to efficiently generate the definitive endoderm (DE) stage, anterior foregut endoderm (AFE) stage, lung progenitor stage and finally lung organoids (Figures 2A and 2B). This protocol generates lung organoids containing lung proximal and distal cell types, such as ciliated cells (ACE-TUB+), basal cells (P63+), club cells (SCGB1A1+), alveolar type I like cells (AT1, HOPX+) and alveolar type II like cells (AT2, SPC+/SOX9+). This protocol was adapted from a published paper (Chen et al., 2018).

17. After overnight growth when hESCs confluency reaches 95%, aspirate the medium from hESCs. Troubleshooting 1, Troubleshooting 2.

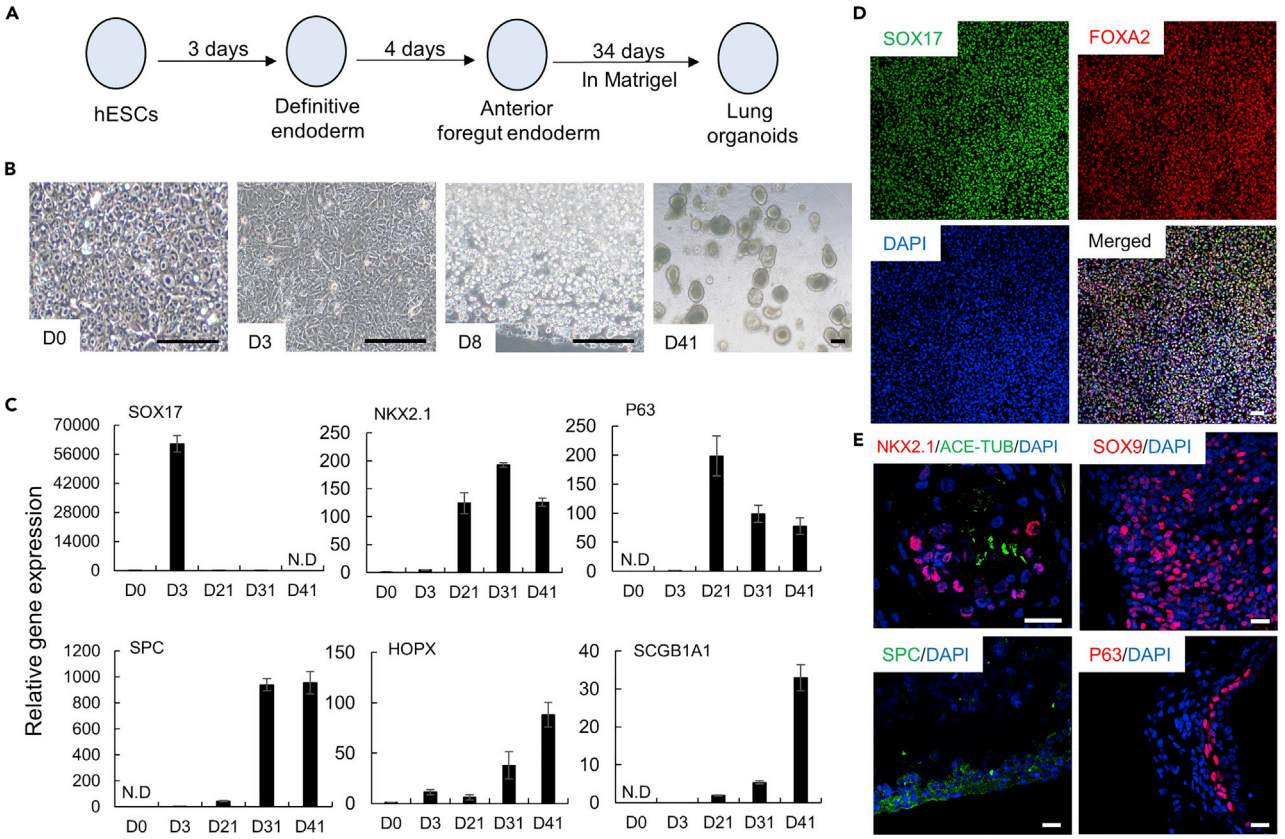

Figure 2. Generation of human lung organoids from hESCs

(A) Schematic of directed differentiation protocol to generate HLOs from hESCs.

(B) Representative brightfield images at the indicated differentiation stages. Scale bar, 100 μm.

(C) Relative genes expression during HLOs differentiation. qRT-PCR measurement of fold change (2^{-DDCt}) of mRNA expression (compared to day 0). Bars represent mean ± SD (n = 3 biological replicates of independent wells of a differentiation). N.D., none detectable. SOX17, definitive endoderm marker; NKX2.1, lung lineage marker; SCGB1A1, club cell marker; P63, basal cell marker; SPC, AT2 cell marker; HOPX, AT1 cell marker.

(D) Representative immunofluorescence stains of definitive endoderm markers at D3. Scale bar, 50 μm.

(E) Representative immunofluorescence stains of lung lineage markers at D41. Scale bar, 20 μm.

18. Wash hESCs with 2 mL of room temperature RPMI 1640 medium per well in a 6 well plate then aspirate the RPMI 1640 medium from the well.

⚠ CRITICAL: Remove the mTeSR1™ complete medium from hESCs and gently wash hESCs with room temperature RPMI 1640 medium.

19. Add 2 mL per well of definitive endoderm differentiation medium. Incubate cultures at 37°C/5% CO_2. Replenish the definitive endoderm differentiation medium every 24 h for 3 days.
20. On day 3, aspirate the medium from cultures, wash the cultures with 2 mL of room temperature RPMI 1640 medium per well in the 6 well plate then aspirate the RPMI 1640 medium from the well. Troubleshooting 3.

Note: Cultures can be collected to analyze marker of definitive endoderm (FOXA2+, SOX17+) (Figure 2D).

21. Add 2 mL per well of anterior foregut endoderm differentiation medium. Incubate cultures at 37°C/5% CO2. Replenish the anterior foregut endoderm differentiation medium every 24 h for 4 days.

22. On day 7, aspirate the medium from cultures, wash the cultures with 2 mL of 1 × room temperature DPBS (without Ca2+/Mg2+) per well in the 6 well plate.
    a. Aspirate DPBS from the well.
    b. Add 1 mL of room temperature Accutase containing Y-27632 (10 μM) per well and leave the cultures in 37°C/5% CO2 cell culture incubator for 2–4 min.
    c. Check the cells under the microscope after 2 min, the cell-cell interactions should become looser and cells should become more refringent (cells should not be floating).

Note: Proper time of Accutase incubation (2–4 min) will not make cells float, check cells under microscope every 2 min, cell-cell interactions will become more relaxed and cells will be easy detached by following pipetting medium over the cells. A longer time of Accutase incubation (8–15 min) will make cells float.

    d. Aspirate the Accutase carefully and immediately add 1 mL of pre-warmed lung organoids differentiation medium with 10 μM Y-27632 per well in the 6 well plate.
    e. Use a 1 mL tips to gently pipette up and down to detach the cells from the well into the medium.
    f. Carefully collect the medium with cells into a 1.5 mL sterile eppendorf tube. Count cells using an automatic cell counter using trypan blue to exclude death cells.
    g. Centrifuge cells at 200 g for 5 min at room temperature.
    h. Aspirate the supernatant carefully without disturbing the cell pellet.

Note: Use a 20 μL tip to further aspirate the remaining supernatant without disturbing the cell pellet (cells will be embedded in 3D Matrigel, so residual culture medium will affect the solidifying of the 3D Matrigel). Then, tap the cell pellet to ensure that there are no cell clumps.

    i. Put the eppendorf tube on ice for 1–3 min.
    j. Add 100–200 μL ice-cold 3D Matrigel (stock solution, 2.0–4.0 × 10^5 cells/25 μL ice-cold 3D Matrigel) to cells and gently pipette up and down several times to ensure an even distribution of the cell suspension.

Note: This procedure will be used to induce anterior foregut endoderm cells to lung progenitors in 3D Matrigel. To avoid 3D Matrigel evaporation, this procedure should be carried out on ice. Pipette with care to avoid making bubbles.

⚠ CRITICAL: 3D Matrigel should be stored at −80°C and thawed on ice overnight before use.

    k. Plate cells in 3D Matrigel (stock solution) using a 100 μL tip to estimate 20–30 μL of droplets (Figure 3A).

Note: Pre-cold tips are required. To estimate 20–30 μL of droplets into a 6 well plate (e.g., 6–10 droplets in a well of 6 well plate) (Figure 3A).

    l. Place the plate in tissue culture incubator for 15–30 min until Matrigel droplets have solidified (Figure 3A).
    m. Add 3–5 mL of pre-warmed lung organoids differentiation medium per well into a 6 well plate, ensuring that the medium completely covers the Matrigel droplet. Incubate cultures at 37°C/5% CO2. Replenish the lung organoids differentiation medium every 48–72 h for 34 days. Troubleshooting 4, Troubleshooting 5.
        i. Organoids should be re-embedded every 5–10 d, or sooner if organoids appear to grow out of the Matrigel or sink to the bottom of the Matrigel droplet.

Note: Check the cultures every 2–3 days under the microscope. Anterior foregut endoderm cells were embedded in 3D Matrigel to form organoids by self-assembly. Based on our

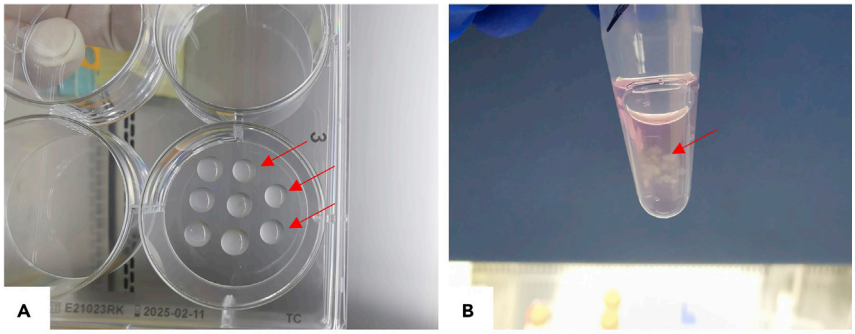

Figure 3. Representative image of the drops solidified

(A) Anterior foregut endoderm cells were embedded in 3D Matrigel. Red arrows indicate drops solidified.

(B) Entire droplet in a 1.5 mL sterile eppendorf tube.

experience, cells will first form small cell clumps 2–4 days later, and gradually form organoids by re-embedding every 5–10 days.

ii. Pick up the entire droplet with a serum pipette and move it to a 1.5 mL sterile eppendorf tube and remove all media using 200 μL or 1 mL tips (Figures 3B and 4).

Note: Centrifuge the cultures at 200 g for 5 min at room temperature will be easy to remove most of the media, then use a 20 μL tip to further aspirate residual culture medium (30–80 μL residual medium usually), residual culture medium will affect the solidification of 3D Matrigel (Figure 4).

iii. Gently pipette up and down to cut the organoids and the old Matrigel using 20 μL tips, then on ice for 1–2 min to make it cold before re-embedding into new 3D Matrigel (Figure 4).

Note: To make even distribution of the organoids in the re-embedding procedure, old 3D Matrigel droplets containing organoids were mechanically cut into small clumps before re-embedding with new 3D Matrigel by using a 20 μL tip to gently pipette up and down. Pipette with care to avoid making bubbles (Figure 4).

iv. Transfer 100–200 μL of ice-cold fresh 3D Matrigel to the eppendorf tube, mix well (Figure 4).

⚠ CRITICAL: To avoid 3D Matrigel evaporation, this procedure should be carried out on ice. Pipette with care to avoid making bubbles.

v. Plate re-embedded organoids in a 6 well plate (Figure 4).
vi. Place the plate in tissue culture incubator for 15–30 min until Matrigel droplets have solidified.
vii. Add 3–5 mL of pre-warmed lung organoids differentiation medium per well into a 6 well plate, ensuring that the medium completely covers the Matrigel droplet. Incubate cultures at 37°C/5% CO2. Replenish the lung organoids differentiation medium every 48–72 h.

⚠ CRITICAL: Keep the 3D Matrigel on ice throughout the procedure to avoid its polymerization inside the eppendorf tube.

Note: Pipette with care to avoid making bubbles. If there are bubbles, centrifuge the eppendorf tube at 200 g at 4°C for 5 min and carefully remove bubbles with 200 μL tips. Then, mix organoids and 3D Matrigel again to make an even distribution of organoids.

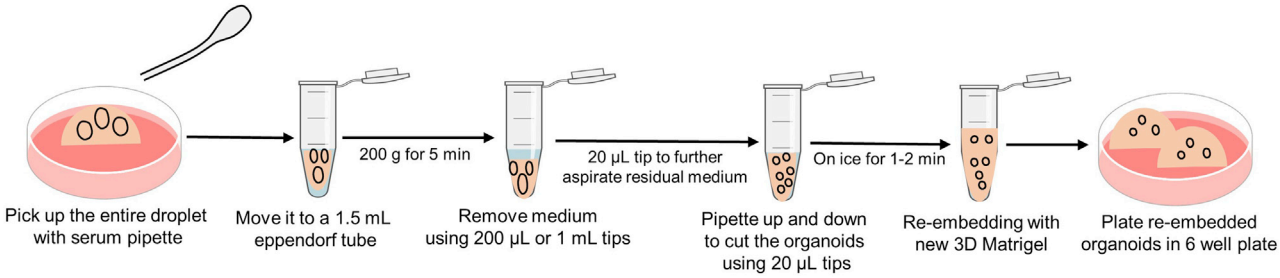

Figure 4. Schematic of organoid cutting and re-embedding with new 3D Matrigel

EXPECTED OUTCOMES

This protocol generates hESC-derived lung organoids by following embryonic lung developmental cues by temporarily introducing a variety of growth factors and small molecules to efficiently generate the definitive endoderm stage, anterior foregut endoderm stage, lung progenitor stage and finally lung organoids (Figure 2A). Anterior foregut endoderm cells are encapsulated in a 3D extracellular matrix to provide a 3D growth environment to generate lung organoids (Figures 2A and 2B). Using quantitative real-time PCR, we checked the expression of various lineage markers at day (D) 0, 3, 21, 31, and 41 along with differentiation. SOX17 is a DE marker, and it was highly expressed at D3. NKX2.1 and P63 are lung progenitor and stem cell markers and are highly expressed at D21 and D31. The markers for more differentiated cell types appeared later at D41, including SCGB1A1 (club cells), SPC (AT2 cells), and HOPX (AT1 cells) (Figure 2C). HLOs contain lung proximal and distal cell types, such as lung progenitor (NKX2.1+), ciliated cells (ACE-TUB+), basal cells (P63+), distal progenitor like cells (SOX9+) and alveolar type II like cells (AT2, SPC+) (Figures 2D and 2E). Long-term transplanted D21 HLOs possess AT2-like cells (SOX9+/SPC+) and BP (bipotent progenitor, PDPN+/SPC+/SOX9+) like cells (Figures 5A and 5B).

LIMITATIONS

If using different cell lines such as induced pluripotent stem cells (iPSCs), optimization of cell seeding, prior to differentiation, may be required. To optimize the cell density, try adding or reducing 1×10^5 cells/cm^2 at a time was recommended.

TROUBLESHOOTING

Problem 1

Single cell detachment is not achieved following the Accutase incubation time (step 17).

Potential solution

Ensure Accutase contains Y-27632 (10 µM), check if the cultures have been dissociated into single cells, observe under the microscope, and increase the incubation time if necessary (Figure 1C).

Problem 2

Following single cell seeding, if cell distribution is not homogeneous, it can lead to a heterogenous differentiation (expression of definitive endoderm markers less than 80% by FACS or immunofluorescence analysis) (step 17).

Potential solution

Undifferentiated hESCs are required for effective definitive endoderm differentiation, more information regarding the maintenance of hESCs with mTeSR™1 can be found at the STEMCELL Technologies website. To ensure that the single cell suspension is evenly dispersed across the well gently agitate the plate back and forth and side-to-side multiple times (> 10 times).

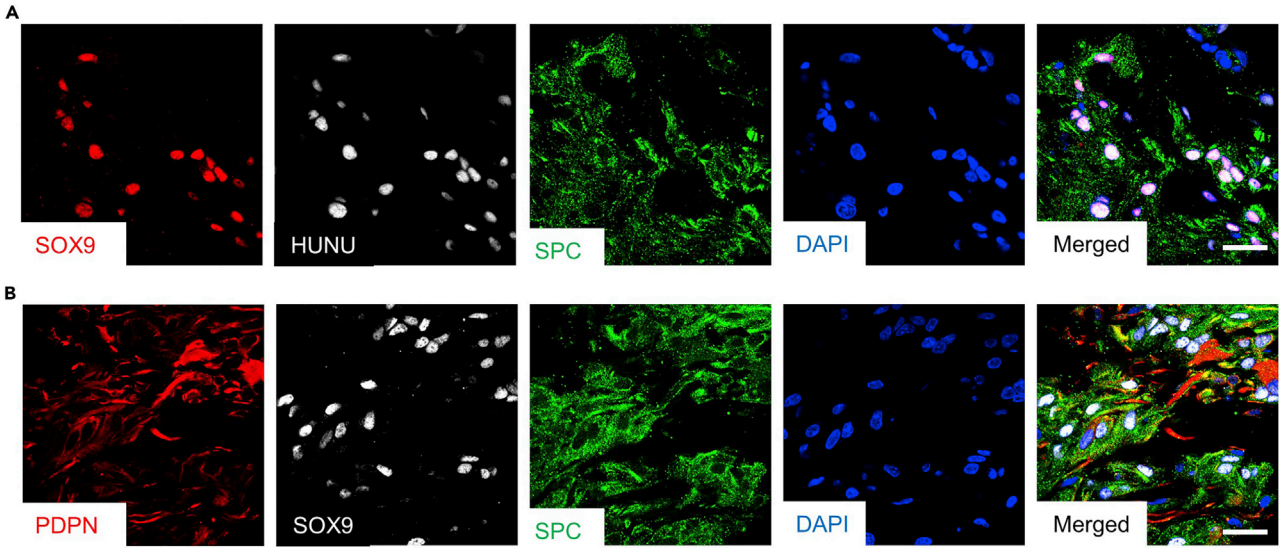

Figure 5. Application of HLOs for human lung development

(A and B) Long-term transplanted D21 HLOs possess AT2 like cells (SOX9+/SPC+) and BP (bipotent progenitor, PDPN+/SPC+/SOX9+) like cells. SPC, AT2 cell marker; SOX9, distal lung progenitor marker; HUNU, human-specific nuclear marker; PDPN, AT1 early maker; Scale bar, 10 μm.

Problem 3

No more than 50% cells left after definitive endoderm differentiation (step 20).

Potential solution

Adding 1 × 10^5 cells/cm^2 at a time to make the cell confluency reach 95% before definitive endoderm differentiation.

Problem 4

Cell death after seeding cells in 3D Matrigel (step 22-m).

Potential solution

Ensure Accutase contains Y-27632 (10 μM) when used for anterior foregut endoderm cell dissociation, and reduce the incubation time. Minimize the operation time of 3D culture as much as possible.

Problem 5

No organoids formation after seeding cells in 3D Matrigel (step 22-m).

Potential solution

Use proper volume of ice-cold 3D Matrigel (reduce 50 μL a time) and gently pipette up and down several times to ensure an even distribution of the cell suspension. Check cultures every 2–3 days under the microscope. Based on our experiences, cells will first form small cell clumps 2–4 days later, and gradually form organoids after re-embedding every 5–10 days.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yong Chen, gene_cell@hotmail.com.

Materials availability

This study did not generate new unique regents.

Data and code availability

This study did not generate datasets/code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

L.H. and S.S.Z conceived, planned, and performed the experiments. Y.C. conceived, planned, and performed the experiments and wrote the manuscript; F.X.Y. planned the experiments and wrote the manuscript. Y.L. and Z.L.R. conceived and planned the experiments. All authors provided critical feedback and helped shape the research, analysis, and the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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