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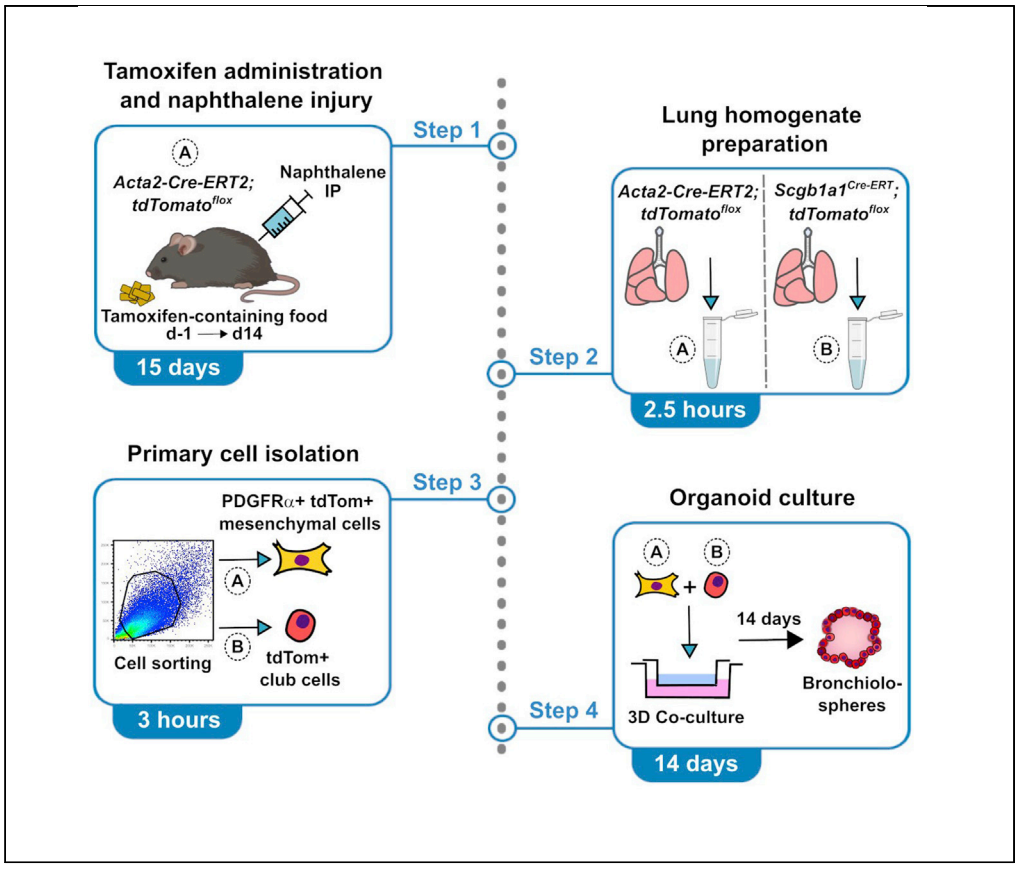
Protocol for the generation of murine bronchiolospheres



Organoid models have been shown to be valuable tools for studying epithelial-mesenchymal crosstalk during biological and pathological settings. Our data identified ACTA2+ PDGFRα+ repair-supportive mesenchymal cells (RSMCs) as an important component of the conducting airway niche. Here, we provide a detailed protocol for culturing airway organoids, or bronchiolospheres, which provide an assessment of the ability of mesenchymal cells to support club-cell growth.

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Highlights

Bronchiolospheres are a useful tool to model epithelial-mesenchymal interactions

Different types of mesenchymal cells can be used to support club cell growth

Cell differentiation within bronchiolospheres can be assessed after 16 days of culture

Bronchiolospheres can be passaged multiple times

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Protocol for the generation of murine bronchiolospheres

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SUMMARY

Organoid models have been shown to be valuable tools for studying epithelial-mesenchymal crosstalk during biological and pathological settings. Our data identified ACTA2+ PDGFRα+ repair-supportive mesenchymal cells as an important component of the conducting airway niche. Here, we provide a detailed protocol for culturing airway organoids, or bronchiolospheres, which provide an assessment of the ability of mesenchymal cells to support club-cell growth.

For complete details on the use and execution of this protocol, please refer to Moiseenko et al. (2020).

BEFORE YOU BEGIN

Timing: 15 days

Tamoxifen administration and naphthalene injury

Lung mesenchymal cells used for the co-culture with club cells are obtained from 8–12-week-old *Acta2-Cre-ERT2; tdTomato*^{flox} mice 14 days after naphthalene-induced injury. Provide tamoxifen-containing food at a concentration of 0.4 g of tamoxifen per Kg of food (Altromin, Germany) starting at one day before naphthalene treatment for a period of 14 days. Administer naphthalene intraperitoneally at a concentration of 0.275 mg per gram of body weight.

Note: For naphthalene treatment, female mice are preferably used since they are more susceptible to injury.

Note: Naphthalene stock solution (20 mg/mL) is prepared by dissolving naphthalene powder in corn oil at 20°C–25°C while vortexing in the dark.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC Rat monoclonal anti-PDGFRα	BioLegend	Cat#135907; RRID: AB_2043969
APC Rat IgG2a	BioLegend	Cat#400511; RRID: AB_1952838

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Naphthalene	Sigma-Aldrich	Cat#84679
Tamoxifen	Sigma-Aldrich	Cat#T5648
Insulin-Transferrin-Selenium	Biozym	Cat#41400-045
l-Glutamine	Gibco	Cat#11539876
PBS	Gibco	Cat#10010023
EDTA	Roth	Cat#6764.1
Matrigel matrix	Corning	Cat#356231
DMEM	Gibco	Cat#12491015
αMEM	Gibco	Cat#41061029
Dispase	Corning	Cat#354235
Collagenase IV	Gibco	Cat # 17104019
DNase I	Serva	Cat#18535.01
Heparin	Stemcell Technologies	Cat#07980
Y-27632 ROCK inhibitor	Stemcell Technologies	Cat#72304
rmFGF10	R&D	Cat#6224-FG-025/CF
rmHGF	R&D	Cat#2207-HG-025/CF
Ethanol	Merck	Cat#32305
Hepes	Gibco	Cat#12509079
Matrigel matrix	Corning	Cat #356231
HBSS	Gibco	Cat#14175095
FCS	Cell Biologics	Cat#6912
Pen/Strep/L-Glut	Merck	Cat#G6784
Corn oil	Sigma-Aldrich	Cat#C8267-500ML
Experimental models: organisms/strains		
Mouse: Tg(Acta2-cre/ERT2)12Pcn	Pierre Chambon (Wendling et al., 2009)	MGI: 3831907
Mouse: B6;129S6-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J	Jackson Laboratory	RRID: IMSR_JAX:007905
Mouse: B6N.129S6(Cg)-Scgb1a1$^{tm1(cre/ERT)Blh}$/J	Jackson Laboratory	RRID: IMSR_JAX:016225
Mouse: B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J	Jackson Laboratory	RRID: IMSR_JAX:007914
Software		
FlowJo	FlowJo, LLC	https://www.flowjo.com/solutions/flowjo RRID: SCR_008520
Other		
5 mL polypropylene round-bottom tube	Corning	Cat#352063
5 mL polystyrene round-bottom tube with cell-strainer cap	Corning	Cat#352235
60-mm glass petri dishes	Sigma-Aldrich	Cat#CLS7016560
Scalpel blades	Fisher Scientific	Cat# S95937A
Cell culture inserts 0.4 μm, 12 mm diameter	Millicell	Cat#PICM01250
24-Well cell culture plare	Greiner Bio-One	Cat#662160
Cell strainer 100 μm	Sarstedt	Cat#833945100
Cell strainer 70 μm	Sarstedt	Cat#833945070
0.2 μm filter	Sarstedt	Cat#831826001
BD FACSAriaTMIII Cell Sorter	BD Biosciences	Cat#648282
GentleMACS® Dissociator	MACS Miltenyi Biotec	Cat#130-093-235
Laminar hood Class II	Heraeus	Cat#50033851
Mettler-Toledo™ pH meter	Sigma-Aldrich	Cat#MT30130864

MATERIALS AND EQUIPMENT

All the chemicals and reagents should be kept sterile. Media and buffers should be prepared under a Class II biological hood and filter-sterilized using a 0.2 μm filter (Sarstedt). For pH adjustment, use 1 M HCl or NaOH (Merck).

Digestion medium		
Reagent	Final concentration	Amount
DMEM	n/a	48.75 mL
HEPES	25 mM	1.25 mL
DNase I	10 U/mL	0.005 mL
Total		**50 mL**

Note: This medium should be freshly made and kept at 4°C for a maximum of 2 h.

MACS buffer		
Reagent	Final concentration	Amount
PBS	n/a	46.05 mL
EDTA	2 mM	3.7 mL
FCS	0.5%	0.25 mL
Total		**50 mL**

Note: Adjust PH to 7.2 and store at 4°C for no longer than 2 months.

Organoid medium		
Reagent	Final concentration	Amount
αMEM	n/a	44.45 mL
FCS	10%	5 mL
Pen/Strep/L-Glut	100 U/mL /0.1 mg/mL /2 mM	0.5 mL
Insulin-transferrin-selenium	10 μg/mL-5.5 μg/mL–6.7 ng/mL	0.5 mL
Heparin	4 μg/mL	0.05 mL
Total		**50 mL**

Note: Store at 4°C for no longer than 2 weeks.

Note: For the initial culture, prepare 5 mL of organoid medium containing 10 μM of PBS-reconstituted ROCK inhibitor.

Alternatives: DMEM may also be used.

RBC lysis buffer		
Reagent	Final concentration	Amount
NH4Cl	155 mM	90 g
KHCO3	10 mM	10 g
EDTA	0.1 mM	370 mg

Note: Dissolve in 1 L of ddH2O and adjust PH to 7.4.

Note: Store at 20°C–25°C for no longer than 2 months.

STEP-BY-STEP METHOD DETAILS

Lung homogenate preparation

Timing: 2.5 h

Two weeks after naphthalene treatment, prepare the lung homogenates needed for the co-culture from *Acta2-Cre-ERT2; tdTomato*^{flox} mice and *Scgb1a1*$^{Cre\text{-}ERT}$*; tdTomato*^{flox} mice.

Note: Female 12- to 14-week-old *Scgb1a1*$^{Cre\text{-}ERT}$*; tdTomato*^{flox} mice can be used without tamoxifen treatment.

1. Euthanize animals by exposure to CO_2 followed by cervical dislocation.
2. Sterilize the mouse thorax and abdomen with 80% ethanol.
3. Open the thoracic cavity to expose the lungs and heart using surgical scissors.
4. Use a 25 G needle to perfuse the lungs with 20 mL of sterile Hank's Balanced Salt Solution (HBSS, Life Technologies) through the right ventricle until the lungs are free of blood.
5. Lung harvest:
   a. To obtain lung cell suspensions from *Acta2-Cre-ERT2; tdTomato*^{flox} mice:
      i. Harvest the lungs and cut them into small pieces (~2 mm) in a small petri dish using a blade.
      ii. Place the lung pieces in 15 mL Falcon tubes and incubate in 5 mL of 0.5% collagenase type IV in HBSS for 45 min at 37°C while rotating.
   b. To obtain lung cell suspensions from *Scgb1a1*$^{Cre\text{-}ERT}$*; tdTomato*^{flox} mice:
      i. Make a small incision in the exposed trachea to insert a shortened 21 G cannula (firmly fixed).
      ii. Instill 1.5 mL of dispase through the trachea.
      iii. Remove the cannula and immediately tie the trachea using a surgical suture.
      iv. Harvest the lungs and place them in separate Eppendorf tubes containing 2 mL of dispase
      v. Incubate for 40 min at 20°C–25°C.
      vi. Remove the trachea and heart using surgical scissors.
      vii. Homogenize the lungs in 10 mL of digestion medium using GentleMACS® Dissociator (MACS Miltenyi Biotech).

⚠ CRITICAL: Be careful not to digest lungs longer than 45 min. This could lead to significant loss of cells.

6. Filter the homogenates through 70 μm and 40 μm nylon cell strainers (BD Biosciences).
7. Centrifuge at 500 ×*g* for 10 min at 4°C.

Note: Wash the filters using 5 mL of DMEM if necessary.

Note: The optimal yield expected at this stage for both cell types is between 15 ×10^6 to 30×10^6 cells per lung.

Primary cell isolation

Timing: 3 h

After the preparation of *Acta2-Cre-ERT2; tdTomato*^{flox} and *Scgb1a1*$^{Cre\text{-}ERT}$*; tdTomato*^{flox} lung cell suspensions, use a cell sorter (For e.g., BD FACSAria™ III) to isolate mesenchymal and club cells, respectively.

8. Stain Acta2-Cre-ERT2; tdTomato^{flox} cells

a. Resuspend cells in 50 μL of anti-PDGFRα antibodies (1:100) or isotype control in MACS buffer for 20 min on ice in the dark.
b. Wash cells with 100 μL of MACS buffer.
c. Centrifuge cells at 500 × *g* for 10 min at 4°C.
d. Resuspend the cell pellet in 300 μL of MACS buffer.

9. Resuspend the cell suspension obtained from *Scgb1a1*$^{Cre\text{-}ERT}$*; tdTomato*^{flox} mice in 300 μL of MACS buffer.

Filter cells with a final volume of 500 μL of MACS buffer using 40 μm mesh into FACS tubes for cell sorting.

10. Set the appropriate gating strategy (Figure 1).
    a. Isolate PDGFRα+ tdTom+ cells from the lung homogenates of *Acta2-Cre-ERT2; tdTomato*^{flox} mice (Figure 1A) (troubleshooting 1).
    b. Isolate tdTom+ cells from the lung homogenates of *Scgb1a1*$^{Cre\text{-}ERT}$*; tdTomato*^{flox} mice (Figure 1B) (troubleshooting 2).
11. Perform cell sorting into FACS tubes containing 350 μL organoid medium using an 85 μm nozzle (troubleshooting 3).

Note: The expected viability post-sort is around 96% for mesenchymal cells and 86% for club cells.

Organoid culture

Timing: 14 days

Following cell isolation, co-culture mesenchymal and epithelial cells for 14 days to generate bronchiolospheres.

12. Centrifuge flow-sorted cells for 5 min, 500 × *g* at 4°C.
13. Resuspend the cells in organoid medium.
    a. Adjust cell concentrations as follows:
       i. 2×10^4 PDGFRα+ tdTom+ cells per 25 μL of medium per cell culture insert.
       ii. 1×10^3 SCGB1A1+ cells per 25 μL of medium per cell culture insert.

Note: The expected number of sorted cells per mouse is ~15,000 PDGFRα+ tdTom+ mesenchymal cells and ~100,000 SCGB1A1+ club cells.

Note: For each culture, between 15,000 and 20,000 mesenchymal cells and a minimum of 5,000 epithelial cells are required.

⚠ CRITICAL: It is advisable to perform a cell viability and quantification assay before the culture if the total number of isolated cells is lower than expected.

14. Mix both cell populations to end up with a volume of 50 μL of organoid medium per insert.
15. Dilute PDGFRα+ tdTom+ cells and SCGB1A1+ cells in 50 μL (per insert) with cold growth factor-reduced Matrigel at a 1:1 ratio as described before (Quantius et al., 2016) (troubleshooting 4).

⚠ CRITICAL: Mix well but carefully to avoid bubble formation.

⚠ CRITICAL: Work as fast as possible to avoid Matrigel polymerization in the tube.

16. Add 100 μL of mixed cells in Matrigel on top of a 12-mm cell culture insert.

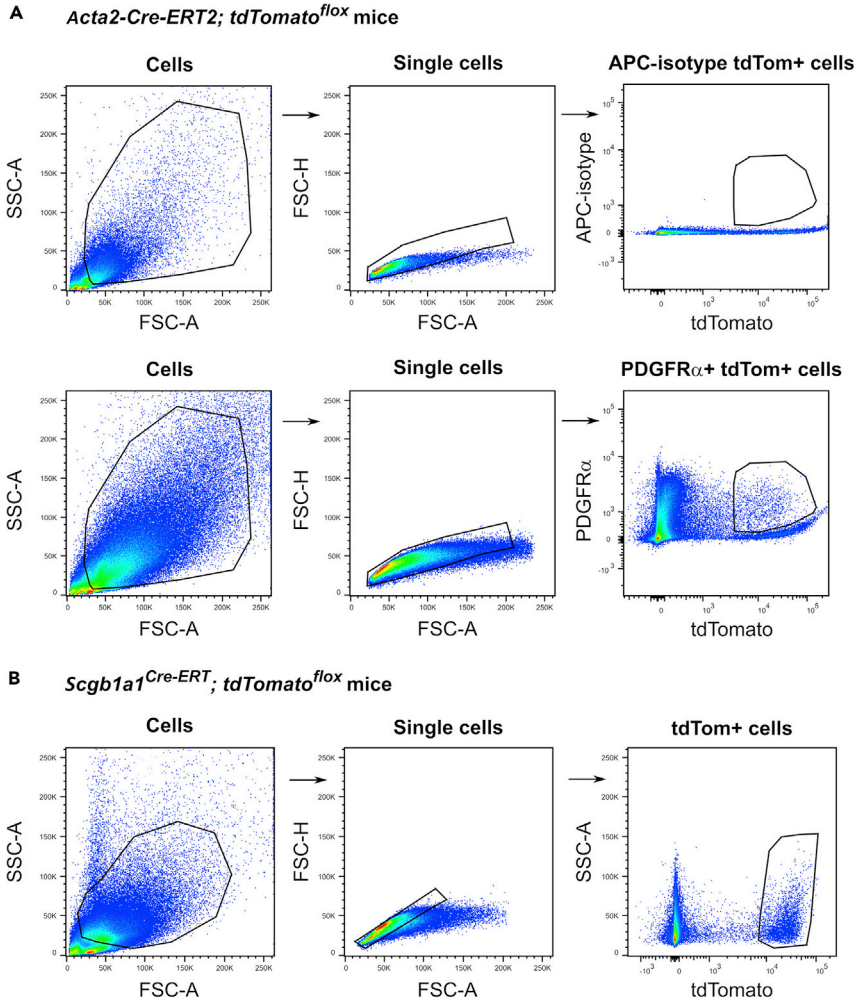

Figure 1. Gating strategy for sorting mesenchymal and epithelial cell populations

(A) Representative flow cytometric dot plots of isotype control and PDGFRα and tdTomato expression in mesenchymal cells isolated from lung homogenates of *Acta2-Cre-ERT2; tdTomato*^{flox} mice 14 days after naphthalene injury.

(B) Representative flow cytometric dot plots of tdTomato expression in club cells isolated from lung homogenates of *Scgb1a1*$^{Cre\text{-}ERT}$*; tdTomato*^{flox} mice.

Note: Add the cell mixture drop by drop placing the pipette in the middle of the insert to ensure cell dispersion into the entire insert.

17. Place inserts in a 24-well plate.
18. Incubate for 15 min at 37°C to allow Matrigel polymerization.
19. Remove the insert and add 350 μL of organoid medium into each well.

⚠ CRITICAL: Add ROCK inhibitor at a concentration of 10 μM to the organoid medium for 14–18 h, then change to regular organoid medium.

20. Place back the insert into the well containing the organoid medium and incubate at 37°C with 5% CO_2 for at least 2 weeks.
21. Change medium three times per week for at least 14 days to generate bronchiolospheres (troubleshooting 5).

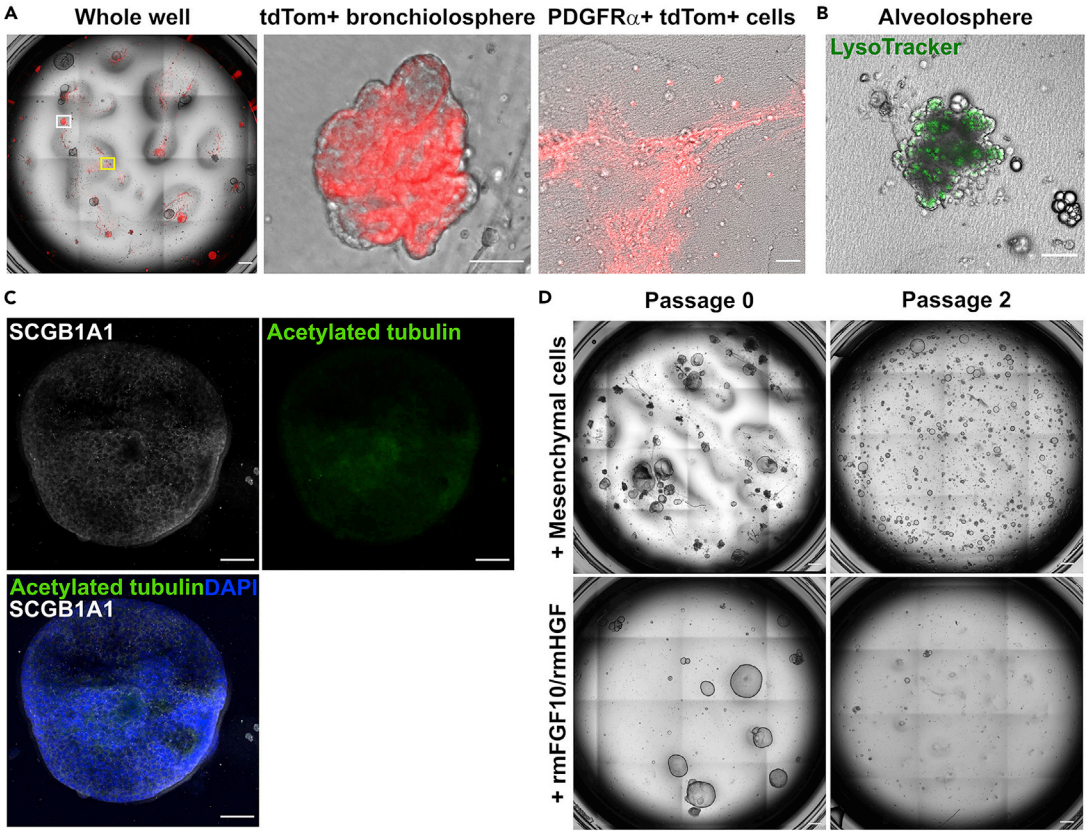

Figure 2. Bronchiolosphere formation after co-culture of mesenchymal and club cells

(A) Whole-well overview (left), representative image of a bronchiolosphere (white square; middle) and a mesenchymal cell cluster (yellow square; right) at day 14 of co-culture of PDGFRα+ tdTom+ cells and SCGB1A1+ club cells. Scale bars represent 100 μm.

(B) Representative confocal image of an alveolosphere stained for LysoTracker (stains lamellar bodies in AECII). Scale bar represents 100 μm.

(C) Representative confocal image of a bronchiolosphere stained for SCGB1A1 (club cells) and acetylated tubulin (ciliated cells). Scale bars represent 50 μm.

(D) Whole-well overview of bronchiolospheres grown in the presence or absence of mesenchymal cells before and after passaging. Note that bronchiolospheres grown in the absence of mesenchymal cells were supplemented with rmFGF10 and rmHGF. Scale bars represent 100 μm.

Note: Bronchiolospheres can be kept in culture for at least 4 weeks.

EXPECTED OUTCOMES

Using this detailed protocol for the isolation and 3D co-culture of lineage-labeled mesenchymal and epithelial cell populations, it is expected that tdTom+ bronchiolospheres will develop after 14 days of culture (Figure 2A). Notably, repair-supportive mesenchymal cells (RSMCs) classical spindle-shape morphology can also be observed around the bronchiolospheres (Figure 2A). The typical morphology of bronchiolospheres has been addressed in a previous publication (Vazquez-Armendariz et al., 2020). Besides bronchiolospheres, a low percentage of Lysotracker+ alveolospheres can also be detected (14%±3%) (Figure 2B). At day 14 of culture, these organoids mostly express the club cell marker SCGB1A1 with only a few acetylated tubulin+ ciliated cells (Figure 2C). Nevertheless, abundant beating cilia can be observed within bronchiolospheres at day 16 of culture, indicating the presence of mature ciliated cells (Methods video S1). At this stage, the supportive role of mesenchymal cells for club-cell growth can be assessed by quantification of bronchiolosphere colony-forming efficiency (CFE) and size, as well as identification of ciliated cell differentiation by immunofluorescence/live imaging. Bronchiolospheres can be passaged up to at least 5 times by using freshly isolated mesenchymal cells. Alternatively, the mesenchymal component can be substituted by a mixture of recombinant mouse (rm)FGF10 and rmHGF; however, the latter approach leads to

significant reduction in bronchiolosphere CFE, lifespan until passaging and ciliated cell differentiation (Figure 2D).

LIMITATIONS

Although most of the organoids are bronchiolospheres (~86%), other types of organoids such as alveolospheres can also be generated (~14%). This is due to the nature of isolated mesenchymal cells; in this case, RSMCs. If RSMCs are substituted by airway smooth muscle cell (ASMC)-enriched cells (PDGFRα- tdTom+), only bronchiolospheres will be generated albeit at significantly lower CFE (Moiseenko et al., 2020). Note that this bronchiolosphere model can also be used to test the ability of other mesenchymal cell populations to support club cell growth *in vitro* such as EpCAM- CD31- CD45- SCA-1+ resident mesenchymal cells.

TROUBLESHOOTING

Problem 1

There is no access to mesenchymal cells (step #10a).

Potential solution

Club cells can be cultured in organoid medium containing 50 ng/mL rmFGF10 and 30 ng/mL rmHGF. Bronchiolospheres will develop after 14 days albeit at significantly lower numbers (Figure 2D).

Problem 2

SCGB1A1+ club cell number is not enough for co-culturing with mesenchymal cells (step #10b).

Potential solution

SCGB1A1+ club cells can be expanded in vitro by culturing them in organoid medium containing 50 ng/mL rmFGF10 and 30 ng/mL rmHGF. The resulting bronchiolospheres can then be digested using 1 mg/mL dispase (BD Biosciences) for 10 min at 4°C, washed with MACS buffer twice by centrifugation at 300 ×*g* for 10 min and then passaged and expanded until co-culture with the mesenchymal cells can be performed (Figure 2D).

Problem 3

Slow cell sorting due to the presence of red blood cells (RBCs) in the sample (step #11).

Potential solution

Use an RBC lysis buffer as an additional step before sorting. Incubate cell suspensions in RBC lysis buffer for 90 s, wash with DMEM containing 10% FCS, centrifuge at 500 × *g* for 10 min at 4°C, and resuspend in 300 μL of MACS buffer.

Problem 4

Matrigel polymerization during co-culture preparation (step #15).

Potential solution

Keep Matrigel, organoid media and samples on ice during the entire culture preparation procedure. Keep a box of sterile 200 μL pipette tips at −20°C to use them exclusively for Matrigel mixing and transfer of the cells.

Problem 5

Bronchiolospheres do not develop even when the right number of cells per well was cultured (step #21).

Potential solution

Exclude dead cells by adding a live/dead cell marker before the sort of both cell types. For instance, add 1 μL of SYTOX™ Blue (Invitrogen) per mL of sample just before sorting.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Elie El Agha (Elie.El-Agha@innere.med.uni-giessen.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze any data sets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100594.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

A.I.V.-A. and E.E.A. designed and wrote this protocol. W.S., S.H., and E.E.A. provided guidance during the experiments and manuscript preparation. All authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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