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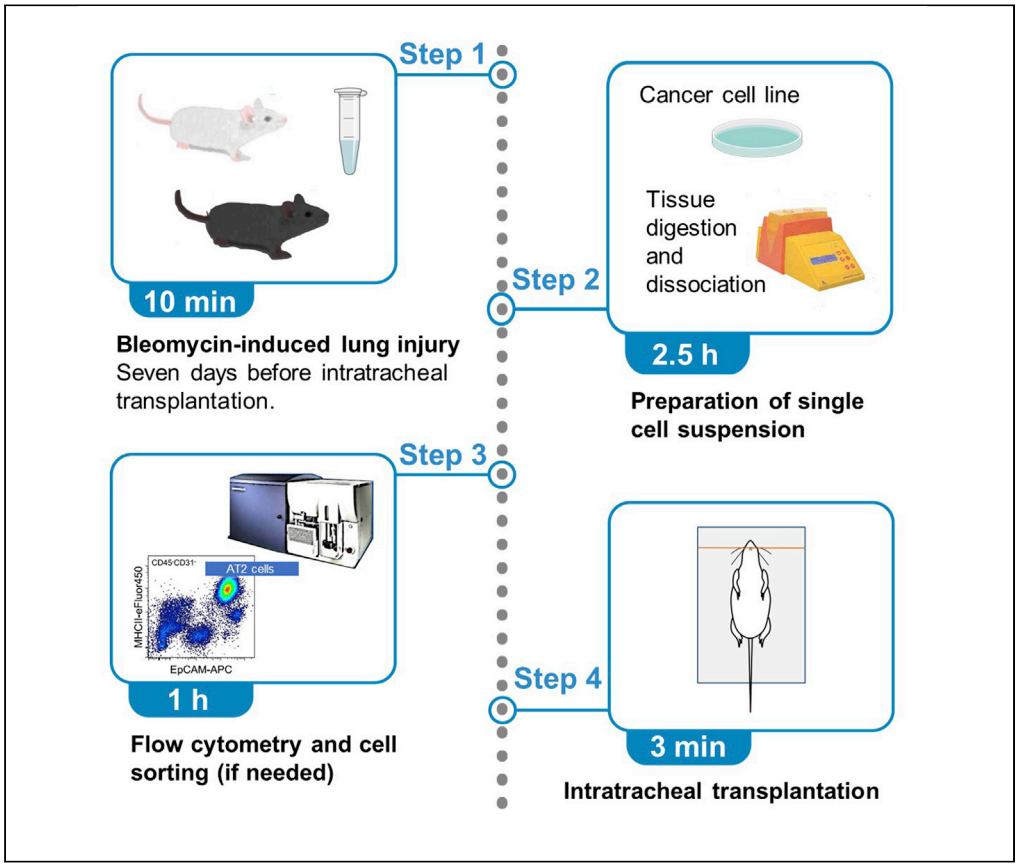
Protocol for cell-type-specific tissue reconstruction in the murine lung fibrogenic microenvironment



Pulmonary fibrosis is a process characterized by epithelial injury and fibroblast activation. It is also well recognized as a predisposition to lung cancer. Here, we present a protocol to establish an *in vivo* model to evaluate the dynamics of alveolar epithelial type 2 cells and lung cancer cells in the context of the lung fibrogenic microenvironment. Utilizing the cell transfer technique, we detail a basis for therapeutic approaches in pulmonary fibrosis and tools for precision medicine against lung cancer.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Intratracheal cell transplantation and engraftment to fibrogenic lung

Alveolar epithelial cell regeneration by exogenous cells

Patient-derived xenograft model of lung cancer for pharmacological study

Hasegawa et al., STAR Protocols 4, 102018
March 17, 2023
https://doi.org/10.1016/j.xpro.2022.102018

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Protocol for cell-type-specific tissue reconstruction in the murine lung fibrogenic microenvironment

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SUMMARY

Pulmonary fibrosis is a process characterized by epithelial injury and fibroblast activation. It is also well recognized as a predisposition to lung cancer. Here, we present a protocol to establish an *in vivo* model to evaluate the dynamics of alveolar epithelial type 2 cells and lung cancer cells in the context of the lung fibrogenic microenvironment. Utilizing the cell transfer technique, we detail a basis for therapeutic approaches in pulmonary fibrosis and tools for precision medicine against lung cancer.

For complete details on the use and execution of this protocol, please refer to Miyata et al. (2022).[1]

BEFORE YOU BEGIN

Institutional permissions

This protocol requires a murine bleomycin (BLM)-induced lung injury model, human lung parenchymal cells, and cancer cells. Institutional approvals for animal and clinical protocols with informed consent from patients are required to start this procedure.

The study protocols were approved by the Animal Research Committee of Kyoto University (ID: MedKyo 13533). Written informed consent for the use of lung parenchyma or cancer tissue and for subsequent use in the *in vivo* study was obtained according to the protocols approved by the Kyoto University Hospital Institutional Review Board (approved numbers: R1280 and R1486).

Bleomycin-induced lung injury

Timing: 10 min /mouse

The first step of this protocol is to generate a BLM-induced lung injury model 7–10 days before intratracheal cell transplantation.

1. Anesthetize mice.
   a. Measure the body weight (BW) of each mouse.
   b. Anesthetize mice with 3%–5% of isoflurane. Troubleshooting 1.

2. Administer BLM solution into the lung.
   a. Prepare BLM solution in 100 μL of PBS.

   Note: An appropriate dose of BLM depends on mice strains and usually ranges from 1 mg/kg to 2 mg/kg BW.

   b. Hung the mouse with its front teeth on a rubber band at a 45-degree angle.[2,3] Trouble-shooting 2.
   c. Administer BLM solution into the laryngeal space by pipette while pulling the tongue out of the oral cavity with a small cotton swab (Methods video S1).

   Note: Keep the tongue out until the BLM solution is entirely aspirated.

   d. Wait for the mice to recover entirely from the anesthesia while in the prone position in the cage.

⚠ CRITICAL: The severity of the BLM model affects the engraftment efficiency of transplanted cells. The dosage of BLM should be titrated depending on mouse strains.

⚠ CRITICAL: Stabilize the depth of anesthesia to create a BLM model with consistent severity. Practice the procedure until the researcher is fully trained so as to administer BLM solution by the time mice start awakening from anesthesia.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat anti-mouse CD31 (PECAM-1) Monoclonal Antibody (390), PE-Cyanine7 (dilution 1:100)	eBioscience	Cat# 25-0311-82
Rat anti-mouse CD45 Monoclonal Antibody (30-F11), PE-Cyanine7 (dilution 1:200)	eBioscience	Cat# 25-0451-82
Rat anti-mouse CD326 (EpCAM) Monoclonal Antibody (G8.8), APC (dilution 1:100)	eBioscience	Cat# 17-5791-82
Rat anti-mouse MHC Class II (I-A/I-E) Monoclonal Antibody (M5/114.15.2), eFluor™ 450 (dilution 1:150)	eBioscience	Cat# 48-5321-82
Chemicals, peptides, and recombinant proteins		
Bleomycin	Nihon Kayaku	CAS# 9041-93-4
Hank's Balanced Salt Solution (HBSS)	Wako Pure Chemical Industries	Cat# 084-08965
Collagenase type I	Gibco	Cat# 17100017
Dispase	Corning	Cat# 354235
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich	Cat# D5030
Antibiotic-antimycotic	Life Technologies	Cat# 15240096
4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)	Life Technologies	Cat# 15630080
Fetal bovine serum (FBS)	Biosera	Cat# FB-1365
Low melting point (LMP) agarose	Wako Pure Chemical Industries	Cat# 11408

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dulbecco's phosphate-buffered saline (DPBS)	Wako Pure Chemical Industries	Cat# 045-29795
Isoflurane	Pfizer	Cat# v002139
Pentobarbital	Kyoritsu Seiyaku	CAS# 76-74-4
Heparin sodium	Mochida Pharmaceutical	CAS# 9005-49-6
DNase I (1 mg/mL)	Sigma-Aldrich	Cat# D4527-40KU
RBC lysis buffer	eBioscience	Cat# 00-4300-54
7-Amino-actinomycin D (7-AAD) (dilution 1:50)	BD Biosciences	Cat# 55816
Experimental models: Cell lines		
A549	ATCC	CCL-185
NCI-H441	ATCC	HTB-174
NCI-H1975	ATCC	CRL-5908
NCI-H226	ATCC	CRL-5826
NCI-H460	ATCC	HTB-177
NCI-H1299	ATCC	CRL-5803
PC-9	IBL cell bank	RRID: CVCL_B260
Lewis lung carcinoma	Taiho Pharmaceutical	RRID: CVCL_5653
Experimental models: Organisms/strains		
Mouse: C57BL/6J (8–10 weeks, male)	The Jackson Laboratory	Strain code: 632
Mouse: CB17.Cg-Prkdc^{scid}Lyst$^{bg\text{-}J}$/CrlCrlj (8–10 weeks, male)	The Jackson Laboratory	Strain code: 250
Mouse: C57BL/6-Tg (CAG-EGFP) (8–10 weeks, male)	Dr. Masataka Asagiri	Strain code: 329
Other		
20-gauge catheter	Terumo	Cat# SR-SFA2032
gentleMACS™ Dissociator	Miltenyi Biotec	Cat# 130-093-235
gentleMACS C tube	Miltenyi Biotec	Cat# 130-093-237
40 μm cell strainer	Falcon	Cat# 352340
70 μm cell strainer	Falcon	Cat# 352350
FACS: Aria III	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

Protease mix (for 1 sample)

Reagent	Final concentration	Amount
HBSS	N/A	1.8 mL
Collagenase type I	300 U/mL	3.5 mg
Dispase	5 U/mL	200 μL
$CaCl_2$ (1 M)	1.25 mM	2.5 μL
Total	**N/A**	**2 mL**

Note: Protease mix should be prepared just before use.

Processing medium (for 1 sample)

Reagent	Final concentration	Amount
DMEM with Antibiotic-Antimycotic	N/A	4.5 mL
HEPES (1 M)	25 mM	125 μL
FBS	10%	0.5 mL
Total	**N/A**	**5 mL**

Note: Processing medium can be stored at 4°C for 1 month.

1% low melting point (LMP) agarose solution.

To make a 1% LMP agarose solution, dissolve 100 mg of LMP agarose in 10 mL of PBS. Store at 45°C until use.

Note: 1% low melting point agarose solution should be prepared just before use.

Sorting buffer		
Reagent	Final concentration	Amount
PBS(-)	N/A	97 mL
FBS	3%	3 mL
Total	**N/A**	**100 mL**

Note: Sorting buffer should be prepared just before use.

Antibody mix for the isolation of AT2 cells by FACS (for 1 sample)		
Reagent	Final concentration	Amount
CD326 (EpCAM) Monoclonal Antibody (G8.8), APC	1:100	1 μL
MHC Class II (I-A/I-E) Monoclonal Antibody (M5/114.15.2), eFluor™ 450	1:150	0.75 μL
CD45 Monoclonal Antibody (30-F11), PE-Cyanine7	1:200	0.5 μL
CD31 (PECAM-1) Monoclonal Antibody (390), PE-Cyanine7	1:100	1 μL
Sorting buffer		96.75 μL
Total	**N/A**	**100 μL**

Note: Antibody mix for the isolation of AT2 cells by FACS should be prepared just before use.

STEP-BY-STEP METHOD DETAILS

Sample collection, enzymatic tissue digestion, and mechanical dissociation

Timing: 60 min

This part details how to collect samples, perform enzymatic digestion, and mechanical dissociation.

For the isolation of alveolar epithelial type 2 (AT2) cells,

1. Anesthetize mice by intraperitoneal injection of pentobarbital (40–50 mg/kg) with 10 U of heparin sodium, and dissect the mice.
   a. Expose the trachea and intubate a 20-gauge catheter into the trachea (Methods video S2).
   b. Open the abdominal cavity, and euthanize mice via transection of the abdominal aorta.

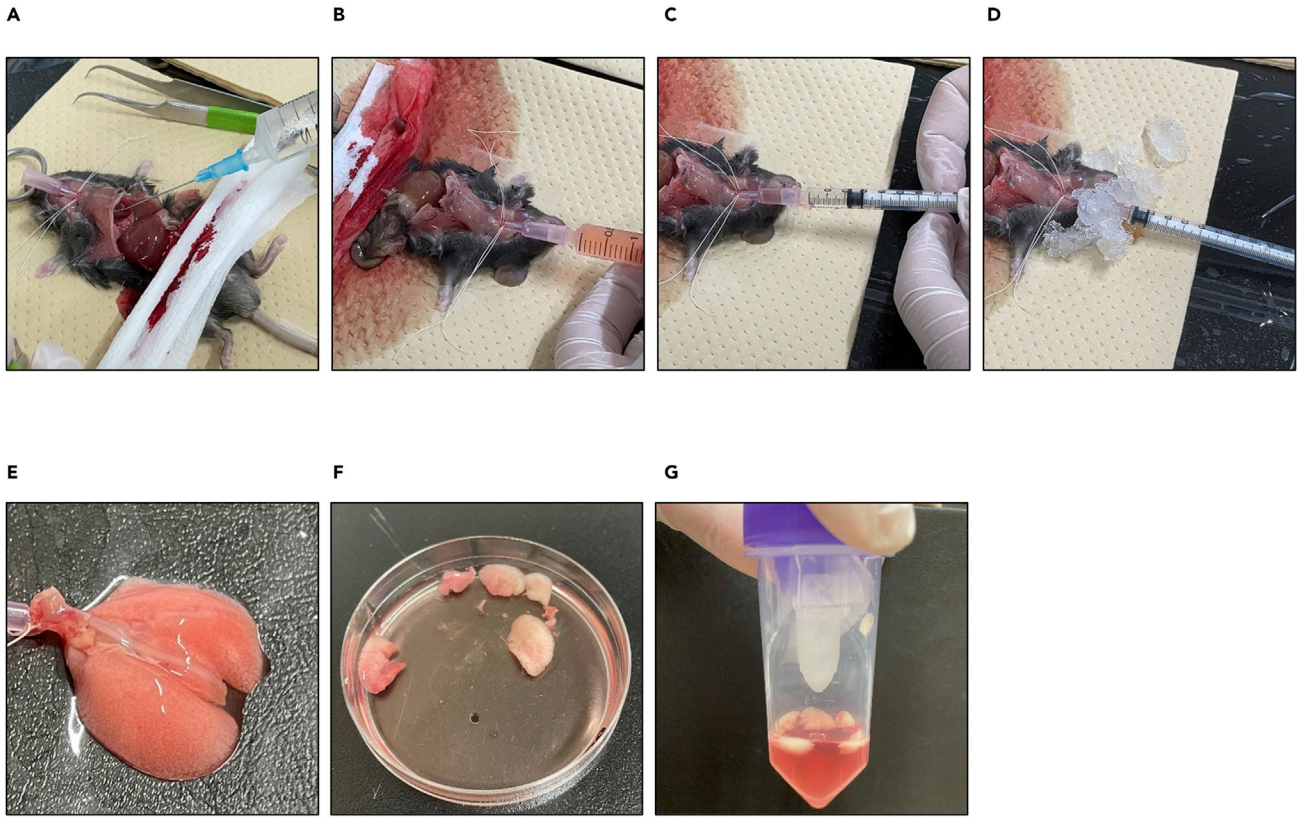

Figure 1. Tissue digestion of murine lungs

(A) Perfusion with saline from the right cardiac ventricle.
(B) Protease mix instillation from the catheter inserted into the trachea.
(C) Low-melting point (LMP) agarose instillation from the catheter.
(D) The ice pack for solidification of LMP agarose.
(E) Murine lungs removed from the thoracic cavity after protease mix instillation.
(F) Lobes are separated from lung minimizing bronchus tissue contamination.
(G) Separation of each lobe after incubation with protease mix.

c. Open the thoracic cavity and perfuse the lungs with 10 mL of cold 0.9% saline from the right ventricle (Figure 1A).

Note: Cutting the right ventricle just after perfusion prevents the blood from perfusing the lungs again.

2. Fill the lungs with protease mix.
   a. Instill 1.5 mL of protease mix into the lungs through the intubated catheter (Figure 1B).
   b. Instill 0.2 mL of 1% LMP agarose solution through the catheter (Figure 1C).
   c. Cover the trachea with ice and wait for 2 min (Figure 1D).

Note: Instillation of protease mix from the catheter inserted into the trachea facilitates tissue dissociation with a small amount of protease mix.

3. Remove the lungs from the thoracic cavity (Figure 1E). Troubleshooting 3.
4. Perform enzymatic digestion and mechanical dissociation of the lungs.
   a. Incubate the resected lungs in 2 mL of HBSS at 37°C for 30 min.
   b. Separate each lobe (Figure 1F) and transfer them to a gentleMACS C tube containing 5 mL of processing medium (Figure 1G).

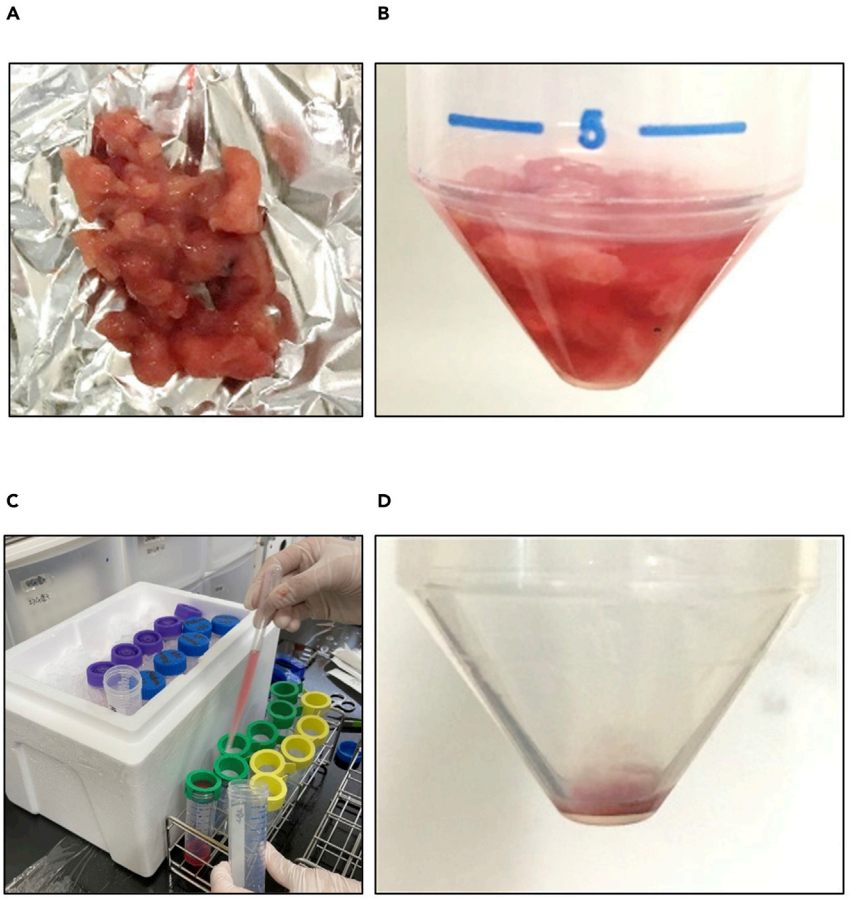

Figure 2. Tissue digestion of human lungs and preparation of single cell suspension

(A) Human lung parenchymal tissues minced into 10–50 mm^3 fragments.

(B) Human lung parenchymal tissues before incubation in protease mix.

(C) Transfer of cell suspension through cell strainers.

(D) The cell pellet derived from human lung parenchyma after red blood cell lysis.

c. Dissociate tissues in a gentleMACS C tube with the preset program B_01.

d. Add 100 μL of DNase I and incubate at 37°C for 3 min.

⚠ CRITICAL: Complete perfusion with saline can remove blood from the lungs and improve the sorting efficiency of AT2 cells by FACS.

For the isolation of human lung parenchymal cells or lung cancer cells,

5. Obtain human lung parenchyma or cancer tissues from patients undergoing lung resection due to lung cancer.

   Note: Maintain human lung parenchyma or cancer tissues on ice from sample collection to mince.

6. Mince lung parenchyma or cancer tissues into small fragments (10–50 mm^3) using a pair of sterilized scissors (Figure 2A).
7. Perform enzymatic digestion and mechanical dissociation.
   a. Transfer tissue fragments into 50 mL conical tubes containing 5 mL of protease mix and incubate at 37°C for 1 h (Figure 2B).

b. Transfer digested tissues to a gentleMACS C tube.
c. Dissociate tissues in a gentleMACS C tube with the preset program B_01.
d. Add 100 μL of DNase I and incubate at 37°C for 3 min.

Preparing single-cell suspension (common for murine AT2 cells and human lung parenchymal or cancer cells)

Timing: 25 min

This part details how to make the single suspension, which is common for the isolation of murine AT2 cells and human lung parenchymal or cancer cells.

8. Filter cell suspension through cell strainers (Figure 2C).
   a. Place 70 μm and 40 μm cell strainers on 50 mL conical tubes and moisten them with a small amount of processing medium.
   b. Transfer the digested solutions to the 70 μm cell strainer.
   c. Rince the cell strainer with 2 mL of processing medium.
   d. Transfer the flow-through to the 40 μm cell strainer.
   e. Rince the cell strainer with the remaining processing medium.
9. Centrifuge cell suspension at 250 × *g* for 8 min at 8°C.
10. Discard the supernatant, resuspend the cell pellet with 4 mL of RBC lysis buffer, incubate for 3 min at 20°C, and add 20 mL of PBS.
11. Centrifuge at 250 × *g* for 8 min at 8°C and discard the supernatant (Figure 2D).
12. Resuspend the cell pellet in sorting buffer for cell isolation or in PBS for intratracheal transplantation.

Note: Isolate murine AT2 cells by FACS for transplantation. Cancer cells can be transplanted without further cell selection.

Isolation of murine AT2 cells by FACS

Timing: 1 h

This part details the isolation of murine AT2 cells using FACS.[4] The following steps are designed for acquisition on a BD FACS Aria III.

13. Prepare cell suspension for AT2 cell isolation by FACS.
   a. Resuspend cells in sorting buffer (4–6 × 10^6 cells/mL of live cells).
   b. Transfer 2.5 mL of cell suspension into a 5 mL round-bottom tube.
   c. Centrifuge at 250 × *g* for 5 min at 8°C.
   d. Aspirate the supernatant and resuspend cells in 100 μL of the antibody mix.
   e. Incubate for 20 min at 4°C in the dark.
   f. Add 1 mL of sorting buffer and centrifuge at 250 × *g* for 5 min at 8°C.
   g. Resuspend cells in 350 μL of sorting buffer.

Note: Keep the tube on ice in the dark until sorting.

14. Isolate AT2 cells by FACS.
   a. Add 7 μL of 7-AAD and incubate for 5 min on ice in the dark.
   b. Briefly vortex the tube and pass samples through a cell strainer before sorting. Troubleshooting 4.

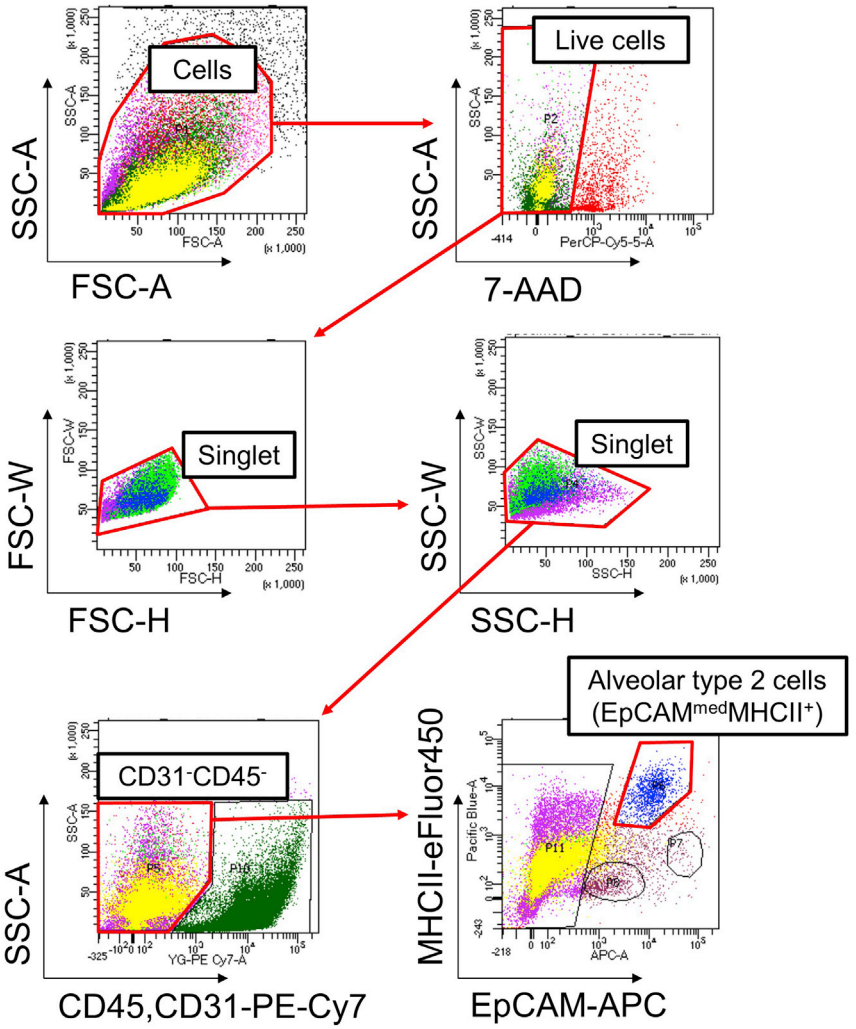

Figure 3. Gating strategy for alveolar type 2 cell isolation

After excluding debris, dead cells, and doublets, AT2 cells are identified as the EpCAM^{med}MHCII^{+} population among CD45^{-}CD31^{-}cells.

c. Start running the samples. After gating cells in the plot of forward scatter (FSC)-area versus side scatter (SSC)-area plot, identify dead cells as 7-AAD positive cells followed by doublet exclusion in the plots of FSC-height vs. FSC-width and SSC-height vs. SSC-width.

d. Identify AT2 cells as EpCAM^{med}MHCII^{+} cells among CD45^{-}CD31^{-} cells (Figure 3).

e. Sort AT2 cells into a 15 mL conical tube containing 11 mL of processing medium.

15. Centrifuge at 300 × *g* for 10 min at 8°C and resuspend cells in PBS.

16. Count the total cell number and adjust it to the appropriate cell number for AT2 cell transplantation (usually 400,000 cells/100 μL).

Note: An expected number of sorted AT2 cells from 8–10-week-old mice is approximately 800,000 cells/lung.

Intratracheal transplantation

Timing: 3 min/mouse

This part details how to administer cell suspension into the BLM model by oropharyngeal aspiration.

17. Anesthetize BLM model mice with 3% isoflurane for 2 min. Troubleshooting 5.
18. Hung the mouse with its front teeth on a rubber band at a 45-degree angle.
19. Apply 100 μL of single cell suspension (AT2 cells or lung cancer cells) for oropharyngeal aspiration (Methods video S1).

Note: The procedure is the same as oropharyngeal aspiration of BLM.

Note: The application volume depends on the ethical approval of the animal protocol in each institution. Adjust the cell concentration so as to follow the approval.

⚠ CRITICAL: When anesthetizing mice, monitor the respiratory pattern of the mice carefully. The thoracic movement of the mice becomes shallow very acutely after starting the anesthesia.

EXPECTED OUTCOMES

Transplanted AT2 cells engraft, proliferate, and differentiate into AT1 cells in BLM-injured lungs. When evaluated 11 days after transplantation, about 3% of lung epithelial cells are derived from transplanted cells. Transplanted lung cancer cells form multiple cancer foci with endogenous murine mesenchymal cells.

The engraftment rate of murine lung cancer cell line LLC and human lung cancer cell lines (A549, H441, PC9, H1975, H226, H460, and H1299) is 100%.

AT2 cells and cancer cells including cell lines do not engraft in PBS-administered control lungs.

We can histologically evaluate the temporal and spatial dynamics of engrafted cells. In lung cancer models, cancer foci formed in mice are histologically relevant to that of surgically resected ones. We can also isolate engrafted cells again from mice lungs by FACS for further analyses.[1]

Of importance, the xenograft model has to be performed in immunodeficient mice.[5,6]

LIMITATIONS

The efficiency of AT2 cell engraftment is relatively low. When 400,000 cells are transplanted, we can isolate significantly lower engrafted cells (~30,000/lungs) 11 days after transplantation. The severity of BLM-injured lungs affects the efficiency of cell engraftment. The more lungs are damaged, the more cells tend to engraft. In contrast, lung cancer cells easily engraft and proliferate rapidly in the lungs. The cell number needs to be adjusted to optimize analyses.

TROUBLESHOOTING

Problem 1

The severity of BLM-induced lung injury varies between each mouse or experiment (related to before you begin).

Potential solution

Anesthetize mice deep enough to decrease their respiratory rate to 20 /min before administration of BLM. This allows for making a consistent model.

Problem 2

The mouse falls off the rubber band (related to before you begin).

Potential solution

Set the rubber band to the platform more tightly. Or, make sufficient tension to the rubber band when pulling the tongue of the mouse during the administration of BLM.

Problem 3

Inflated lungs shrink while removing them from the thoracic cavity (related to step 3).

Potential solution

Do not poke lung lobes too much. Some leakage of protease mix from the lungs does not affect dissociation efficiency.

Problem 4

The FACS sample line is clogged (related to step 14).

Potential solution

Be sure to pass samples through the cell strainer just before starting cell sorting. Aggregated cells clog the sample line, which makes the cell sorting process unstable.

Problem 5

Mice do not aspirate cell suspension (related to step 17).

Potential solution

BLM-injured mice are more susceptible to anesthesia. Inspiratory capacity is lower compared to the baseline. Be careful not to anesthetize mice at the same level as the BLM administration.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact, Atsuyasu Sato (atsuyasu@kuhp.kyoto-u.ac.jp).

Materials availability

All reagents are commercially available.

Data and code availability

No datasets or codes were generated in this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.102018.

ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI Grant Number (JP18K08176 and JP20H03770). The authors would like to thank A. Saito for technical assistance with the experiments. We also acknowledge the work and helpful discussion by our laboratory members (N. Chiba, H. Miyamoto, S. Nishikawa, K. Takahashi, and T. Toyazaki in the Department of Thoracic Surgery; T. Mizutani, Y. Hamakawa, K. Uemasu, K. Tanimura, S. Sato, and S. Muro in the Department of Respiratory Medicine). Cell isolation and analysis by FACS were performed at the Medical Research Support Center, Graduate School of Medicine, Kyoto University.

AUTHOR CONTRIBUTIONS

Conceptualization, K.H., R.M., A.S.; Methodology, K.H., R.M., A.S.; Investigation, K.H., R.M.; Formal analysis, K.H., R.M.; Writing- Original Draft, K.H., R.M.; Visualization, K.H., R.M.; Writing- Review & Editing, T.H., H.D., A.S.; Supervision, A.S.; Funding Acquisition, A.S., T.M.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

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