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Isolation of Murine Alveolar Type II Epithelial Cells

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[Abstract] We have optimized a protocol for isolation of alveolar type II epithelial cells from mouse lung. Lung cell suspensions are prepared by intratracheal instillation of dispase and agarose followed by mechanical disaggregation of the lungs. Alveolar type II epithelial cells are purified from these lung cell suspensions through magnetic-based negative selection using a Biotin-antibody, Streptavidin-MicroBeads system. The purified alveolar type II epithelial cells can be cultured and maintained on fibronectin-coated plates in DMEM with 10% FBS. This protocol enables specific investigation of alveolar type II epithelial cells at molecular and cellular levels and provides an important tool to investigate *in vitro* the mechanisms underlying lung pathogenesis.
Keywords: Alveolar type II epithelial cells, Lung, Biotin, Streptavidin, Dispase, Agarose

[Background] Alveolar type II epithelial cells play critical roles in alveolar integrity maintenance, surfactant protein synthesis and secretion, and defense against pulmonary infection of bacteria and viruses. Recent studies using mouse lung cancer models have proven that alveolar type II epithelial cells are a key cell of origin of adenoma/adenocarcinoma induced by chemical carcinogens and oncogenic mutations (Qu *et al*., 2015; Zhou *et al.*, 2015 and 2017). To further expand our understanding of the role of alveolar type II epithelial cells in lung pathogenesis *in vivo*, isolation of alveolar type II epithelial cells is needed to allow for a precise mechanism analysis *in vitro*. Based on previous studies (Corti *et al.*, 1996; Rice *et al.*, 2002), a modified method was used in our laboratory to isolate highly purified, viable and culturable alveolar type II epithelial cells from mice (Zhou *et al.*, 2015; Sun *et al.*, 2016).

Materials and Reagents

1. Needles (BD, catalog number: 305167) or tapes
2. 10 ml syringe (BD, catalog number: 309604)
3. 27 gauge needle (BD, catalog number: 305109)
4. Nylon string (Dynarex, catalog number: 3243)
5. 22 G x 1” Exel Safelet Catheter (Exel International, catalog number: 26746)
6. 1 ml syringe (BD, catalog number: 309659)
7. 15 ml tubes (VWR, catalog number: 89039-666)
8. 60 mm non-coated cell culture dish (Greiner Bio One International, catalog number: 628160)

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9. Cell strainer (70 µm) (Fisher Scientific, catalog number: 22-363-548)
10. Cell strainer (40 µm) (Fisher Scientific, catalog number: 22-363-547)
11. Nylon mesh (25 µm) (ELKO filtering, catalog number: 03-25/19)
12. MS column (Miltenyi Biotec, catalog number: 130-042-201)
13. Fibronectin-coated plate (Corning, catalog number: 354402)
14. Mice (THE JACKSON LABORATORY)
15. 70% ethanol (Decon Labs, catalog number: 2701)
16. Dispase (1 mg/ml dissolved in PBS) (Roche Diagnostics, catalog number: 4942078001)
17. 1% low melting point agarose (Dissolved in PBS, autoclaved, aliquoted and stored at 4 °C) (Lonza, catalog number: 50100)
18. DMEM (Lonza, catalog number: 12-604F)
19. DNase I (Roche Diagnostics, catalog number: 10104159001)
20. Biotinylated anti-CD45 (Miltenyi Biotec, catalog number: 130-101-952)
21. Biotinylated anti-CD16/CD32 (Miltenyi Biotec, catalog number: 130-101-895)
22. Streptavidin MicroBeads (Miltenyi Biotec, catalog number: 130-048-101)
23. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10437028)
24. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9625)
25. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541)
26. Disodium hydrogen phosphate heptahydrate ($Na_2HPO_4·7H_2O$) (Fisher Scientific, catalog number: BP331-500)
27. Potassium phosphate monobasic (KH_2PO_4) (Acros Organics, catalog number: 205925000)
28. Bovine serum albumin (BSA) (MP Biomedicals, catalog number: 199898)
29. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E26282-500G)
    Note: This product has been discontinued.
30. Penicillin-streptomycin (Lonza, catalog number: 17-602E)
31. Phosphate buffered saline (PBS) (see Recipes)
32. Labeling buffer (see Recipes)

Equipment

1. CO_2 chamber
2. Biosafety cabinet
3. Styrofoam board
4. Forceps (Roboz Surgical Instrument, catalog number: RS-5135)
5. Scissors (Roboz Surgical Instrument, catalog number: RS-6802)
6. Water bath incubator (Thermo Fisher Scientific, Thermo Scientific™, model: Barnstead™ 18000A-1CE)
7. Shaker (Thermo Fisher Scientific, Thermo Scientific™, model: Barnstead™ 2314)

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8. Centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: IEC CL40R, catalog number: 11210927)
9. MACS MultiStand (Miltenyi Biotec, catalog number: 130-042-303)
10. Hemocytometer (Hausser Scientific, catalog number: 3110)

Procedure

A. Preparation of crude single lung cell suspension
1. Sacrifice a mouse by CO_2 inhalation in a CO_2 chamber.
2. Bring the mouse to a biosafety cabinet.
3. Dampen the mouse with 70% ethanol.
4. Place the mouse front side up on dissecting Styrofoam board and fix the arms and legs with needles or tape.
5. Use scissors to make incision in the skin from abdomen to neck, and tear skin with forceps to expose thoracic cage and neck.
6. Gently remove the muscle around the neck to expose the trachea.
7. Carefully cut the ribs to expose the heart and lungs.
8. Perfuse the lung with 0.9% NaCl, using a 10 ml syringe fitted with a 27 gauge needle, through the right ventricle of heart until it is visually free of blood.
9. Use forceps to put a ~10 cm-long nylon string under the trachea.
10. Insert the 22 G x 1” Exel Safelet Catheter into the trachea, remove stylet hub, and tie a knot with a nylon string to secure catheter and trachea together firmly.
11. Slowly inject 2 ml of dispase into the lung, and allow the lung to collapse for 5 min.
12. Quickly load a 1 ml syringe with 0.5 ml of 1% low melting point agarose (brought from 4 °C storage, thawed in a 70 °C water bath, and then kept in a 45 °C water bath in a melted status), replace the dispase syringe with the agarose-containing syringe, and gently infuse the lung with the loaded agarose. Leave the syringe in place, and immediately cover the lung with ice and incubate for 2 min to promote the solidification of agarose.
13. Remove the lung from animal to a 15 ml tube containing 2 ml of dispase, incubate for 45 min at room temperature on a shaker.
    Note: Shaking helps dispase digestion and cell release, but the exact speed of shaking is not critical.
14. Transfer the lung to 7 ml of room-temperature DMEM with 0.01% DNase I in a 60 mm non-coated cell culture dish. The digested tissue is carefully teased from bronchi with scissors and forceps.
15. The resulting cell suspension is successively filtered through 70 µm and 40 µm cell strainers, and then 25 µm nylon mesh.

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B. Magnetic labeling

1. Count the filtered cells by a hemocytometer, centrifuge cell suspension at 300 *x g* for 10 min at 4 °C, and then resuspend in 400 µl of labeling buffer per 10^7 cells.
2. Add 5 µl of each biotinylated anti-CD45 and biotinylated anti-CD16/CD32, mix well and incubate for 10 min at 4 °C.
3. Wash cells with 1 ml of labeling buffer and centrifuge at 300 *x g* for 10 min at 4 °C.
4. Resuspend cell pellet in 90 µl of labeling buffer per 10^7 cells.
   Note: In steps B4-B8, if working with fewer than 10^7 cells, use the same volume as indicated.
5. Add 10 µl of Streptavidin MicroBeads per 10^7 cells.
6. Mix well and incubate for 15 min at 4 °C.
7. Wash cells with 1 ml of labeling buffer and centrifuge at 300 *x g* for 10 min at 4 °C.
8. Resuspend the cell pellet in 500 µl of labeling buffer.

C. Magnetic separation

1. Place an MS column in the magnetic field of a suitable MACS MultiStand.
2. Prepare column by rinsing with 0.5 ml of labeling buffer.
3. Apply cell suspension onto the column. Collect flow-through containing unlabelled cells, representing the alveolar type II cells.
4. Wash column 3 times with 0.5 ml of labeling buffer each. Collect unlabelled cells that pass-through, and combine with the effluent from step C3.

D. Alveolar type II cell culture

1. The combined effluents are centrifuged at 300 *x g* for 10 min at 4 °C. The cell pellet is resuspended to 10^6 cells per ml in DMEM with 10% FBS, and cultured for 12 h in non-coated cell culture plate in a humidified, 5% CO_2 incubator at 37 °C to remove residual mesenchymal cells attached to plate.
   Note: Generally, 4-5 million cells will be retrieved per mouse lung, and ~4 ml medium will be used to resuspend the isolated cells at 10^6 cells per ml.
2. Cell suspensions with unattached cells (mainly alveolar type II epithelial cells) are gently collected and centrifuged at 300 *x g* for 10 min at 4 °C. The pellet is resuspended in DMEM with 10% FBS and cultured on a fibronectin-coated plate. The typical morphology of alveolar type II epithelial cells is shown in Figure 1. Meanwhile, genomic sequencing of cultured alveolar type II epithelial cells is performed to detect the mutations of *K-Ras* gene (Figure 2).

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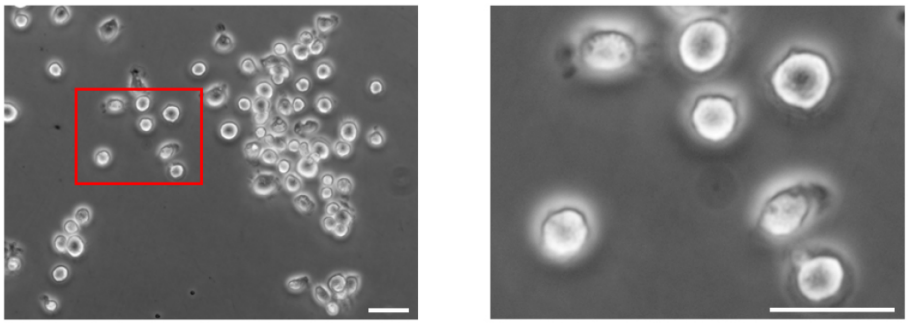

Figure 1. Isolated murine alveolar type II epithelial cells cultured in complete DMEM on fibronectin-coated plate for 5 days. Image on the right is the magnification of the red-box area on the left. Scale bars = 20 μm.

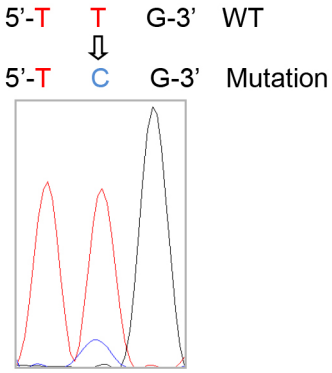

Figure 2. The codon 61 mutation of *K-Ras* gene in alveolar type II epithelial cell. Q61 codon: CAA (reverse complementary: TTG).

Data analysis

1. Morphology of isolated alveolar type II epithelial cells.
2. Genome DNA is extracted from isolated alveolar type II epithelial cells from urethane-treated mice. Then the exon 3 of *K-Ras* gene is PCR-amplified and subsequently sequenced to detect codon 61 mutations.

Notes

1. Injection of agarose helps push dispase solution deep into alveoli. The solidified agarose can prevent the reflux of dispase solution, and also reduce the contamination of cells from bronchi, such as Clara cells.
2. If the isolated alveolar type II epithelial cells are to be used for culture, carry out all steps in sterile conditions with autoclaved dissection tools.
3. Using this protocol, about 4-5 x 10^6 alveolar type II epithelial cells per mouse can be retrieved. The isolated cells can be cultured and maintained for at least 8 days. It is better to use freshly

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isolated/cultured alveolar type II epithelial cells. Freezing of the cells for storage is not recommended.

Recipes

1. Phosphate-buffered saline (PBS) (1 L)
   8.0 g NaCl
   0.2 g KCl
   1.15 g $Na_2HPO_4 \cdot 7H_2O$
   0.2 g KH_2PO_4
   Adjust to pH 7.4
2. Labeling buffer
   PBS, pH 7.2
   0.5% BSA
   2 mM EDTA

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