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Expansion of Human Airway Basal Stem Cells and Their Differentiation as 3D Tracheospheres

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Abstract

Although basal cells function as human airway epithelial stem cells, analysis of these cells is limited by in vitro culture techniques that permit only minimal cell growth and differentiation. Here, we report a protocol that dramatically increases the long-term expansion of primary human airway basal cells while maintaining their genomic stability using 3T3-J2 fibroblast coculture and ROCK inhibition. We also describe techniques for the differentiation and imaging of these expanded airway stem cells as three-dimensional tracheospheres containing basal, ciliated, and mucosecretory cells. These procedures allow investigation of the airway epithelium under more physiologically relevant conditions than those found in undifferentiated monolayer cultures. Together these methods represent a novel platform for improved airway stem cell growth and differentiation that is compatible with high-throughput, high-content translational lung research as well as human airway tissue engineering and clinical cellular therapy.

Keywords: Lung, Stem cells, Epithelial cells, Goblet cells, Cilia, Adult stem cells, Cell culture techniques, Primary cell culture

1 Introduction

Human airways represent a key environmental barrier whose dysregulation in diseases such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and cancer is a major cause of worldwide morbidity and mortality [1]. Human airways are composed of a pseudostratified ciliated and mucosecretory epithelium responsible for protecting against inhaled particulate matter, pathogens, and other airborne toxicants. Supporting this epithelium are multipotent basal stem cells characterized primarily by cytokeratin 5 (CK5) and P63 expression [2, 3]. Given the clear physiological importance of maintaining human airway homeostasis, establishing techniques for investigating basal stem cell growth and differentiation is of significant fundamental and translational biomedical relevance [4].

Historically, the majority of airway stem cell research has involved either in vitro studies of undifferentiated, immortalized cell cultures or in vivo animal models that exhibit only limited

human translational applicability [1]. To overcome this, researchers have traditionally used human air–liquid interface (ALI) cultures in which a confluent monolayer of human airway basal cells is grown at an air interface in medium containing retinoic acid to encourage ciliated and mucosecretory cell differentiation [5, 6]. Despite the use of primary human cells in ALI models [7], this technique remains extremely time-consuming and is poorly suited to high throughput, high content translational medicine approaches. In addition, evidence suggests that basal cells rapidly lose their capacity for multipotent differentiation and undergo premature senescence after only a small number of passages. This failure to maintain airway stem cells in vitro presents a considerable barrier for their use in translational medicine.

In this chapter, we describe a protocol for improved human airway stem cell expansion that maintains their multipotent differentiation capacity. Originally developed for epidermal keratinocyte stem cells, this technique relies upon the coculture of primary human epithelial cells with mitotically inactivated 3T3-J2 fibroblast feeder cells [8]. Cocultures of epithelial and J2 feeder cells are grown in medium containing the Rho-associated kinase (ROCK) inhibitor Y-27632 [9–11]. Successful application of this procedure to airway epithelial cells provides large numbers of basal cells that exhibit physiologically relevant differentiation, karyotype stability, and maintenance of telomere length [12]. In addition, we also provide two distinct methods for the differentiation and visualization of lung basal cells as 3D airway tracheospheres. These techniques build on previous 3D culture systems in which either ciliated or mucosecretory differentiation was achieved [2, 13, 14]. The methods described here, based on several recently published studies, permit normal ciliated and mucosecretory cell differentiation [12, 15, 16] and are compatible with high-throughput, high-content analyses. Altogether, these methods provide a platform to expand and differentiate large numbers of airway basal epithelial cells from patient endobronchial biopsy samples. These techniques have uses in translational medicine, tissue engineering, and human cellular therapy.

2 Materials

2.1 Human Airway Epithelial Cell Isolation

1. RPMI medium with L-glutamine (Gibco, Thermo Fisher). Storage at 4 °C.
2. Dispase (Corning). Storage at −20 °C.
3. 0.25 % trypsin–EDTA (Gibco, Thermo Fisher). Storage at −20 °C.
4. Neutralization medium: Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose, L-glutamine, and pyruvate

(Gibco, Thermo Fisher) plus 10 % fetal bovine serum (FBS) and 1× penicillin–streptomycin (Gibco, Thermo Fisher). Storage at 4 °C.

5. Transport medium: αMEM (Gibco, Thermo Fisher) plus 1× penicillin–streptomycin (Gibco, Thermo Fisher), 1× gentamicin (Gibco, Thermo Fisher) and 1× amphotericin B (Fisher Scientific). Storage at 4 °C.

2.2 3T3-J2 Feeder Cell Culture

1. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose, L-glutamine, and pyruvate (Gibco, Thermo Fisher). Storage at 4 °C.
2. Bovine serum (Gibco, Thermo Fisher). Storage at −80 °C.
3. Penicillin–streptomycin (Gibco, Thermo Fisher). Storage at −20 °C.
4. Mitomycin C (Sigma-Aldrich): 0.4 mg/ml in sterile PBS. Storage at −20 °C.
5. 0.05 % trypsin–EDTA (Gibco, Thermo Fisher). Storage at −20 °C, 4 °C short-term.
6. Complete fibroblast culture medium: 500 ml DMEM plus 45 ml bovine serum and 1× penicillin–streptomycin. Storage at 4 °C.

2.3 Human Airway Epithelial Cell Culture

1. Phosphate-buffered saline (PBS; Sigma).
2. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose, L-glutamine, and pyruvate (Gibco, Thermo Fisher). Storage at 4 °C.
3. Fetal bovine serum (FBS; Gibco, Thermo Fisher; *see* **Note 1**). Storage at −80 °C long-term, −20 °C until use.
4. Penicillin–streptomycin (Gibco, Thermo Fisher). Storage at −20 °C.
5. Ham's F-12 nutrient mixture with L-glutamine (Gibco, Thermo Fisher). Storage at 4 °C.
6. Gentamicin (Gibco, Thermo Fisher): 100×. Storage at 4 °C.
7. Amphotericin B (Fisher Scientific): 250 μg/ml, 1000×. Storage at −20 °C.
8. Hydrocortisone (Sigma-Aldrich). *See* **Note 2** for storage conditions.
9. Recombinant human EGF (Thermo Fisher). *See* **Note 2** for storage conditions.
10. Insulin, 1000× stock (Sigma-Aldrich): 5 mg/ml in distilled water (add glacial acetic acid dropwise to dissolve and sterile filter). Storage at −20 °C.

11. Cholera toxin (Sigma-Aldrich): 1 mg/ml (11.7 μM) in distilled water. Storage at 4 °C.
12. Y-27632 (Cambridge Bioscience): 5 mM in distilled water. Storage at −20 °C.
13. Complete epithelial culture medium: 373 ml serum-containing DMEM (500 ml DMEM plus 50 ml FBS and 1× penicillin–streptomycin), 125 ml Ham's F-12, 0.5 ml gentamicin, 0.5 ml amphotericin B, 0.5 ml hydrocortisone/EGF (*see* **Note 2**), 0.5 ml insulin, 0.5 ml Y-27632, 4.3 μl cholera toxin. Storage at 4 °C for up to 2 weeks.
14. Profreeze Chemically Defined Freezing Medium (2×; Lonza).

2.4 3D Human Tracheospheres (Lumen-in)

1. Matrigel, basement membrane matrix, growth factor reduced (BD Biosciences; *see* **Note 3**). Storage at −20 °C.
2. Ultra-low attachment 96-well plate (Corning).
3. Bronchial epithelial growth medium with supplements (BEGM; Lonza). Medium stored at 4 °C, supplements at −20 °C.
4. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose, L-glutamine, and pyruvate (Gibco, Thermo Fisher). Storage at 4 °C.
5. Y-27632 (Cambridge Bioscience): 5 mM in distilled water. Storage at −20 °C.
6. All-*trans* retinoic acid (Sigma): 10 mM in 100 % EtOH (50 mg in 16.642 ml). Storage at −80 °C (*see* **Note 4**).
7. Tracheosphere medium: 50 % DMEM (no serum, no antibiotics), 50 % bronchial epithelial basal medium (plus all of the BEGM supplements except amphotericin B, triiodothyronine, and retinoic acid). Storage at 4 °C. Supplemented with 5 μM Y-27632 for cell seeding (but not subsequent feeds). Always supplemented with 100 nM all-*trans* retinoic acid at the time of use.

2.5 Immunofluorescence Staining of Tracheospheres

1. 4 % (w/v) paraformaldehyde in PBS. Heat to 65 °C to dissolve and adjust pH to 7.2.
2. HistoGel Specimen Processing Gel (Thermo Scientific; *see* **Note 5**).
3. Blocking solution (10 % FBS in PBS).
4. DAPI (4′,6-diamidino-2-phenylindole; Molecular Probes). Stock solution: 10 μg/ml.
5. Mounting medium (Immu-mount, Thermo Scientific).

3 Methods

3.1 Preparation of 3T3-J2 Feeder Layers

1. Maintain 3T3-J2 cells at low passage numbers (*see* **Note 6**) under subconfluent conditions (*see* **Note 7**) in 37 °C, 5 % CO_2 incubators with weekly splits of 1:8–1:10.
2. For feeder layer preparation, add mitomycin C (4 μg/ml; final concentration) to flasks of 3T3-J2 cells in fibroblast culture medium for 2 h (*see* **Note 8**).
3. Wash cells with PBS, harvest cells with 0.05 % trypsin–EDTA and replate at a density of at least 20,000 feeder cells/cm^2 in fibroblast culture medium (*see* **Note 9**).
4. Allow 3T3-J2 cells to adhere and spread overnight before adding epithelial cells the following day (*see* **Note 10**).

3.2 Isolation of Human Airway Epithelial Cells

1. Derivation of human cells should be approved by the relevant local ethics committee.
2. Human airway epithelial cells are derived from brushings or biopsies taken during bronchoscopy procedures. Samples are transported to the laboratory on ice as soon as possible.

3.2.1 Cell Seeding from Brushings

1. Brushes are collected in 15 ml falcon tubes in transport medium.
2. Thoroughly vortex tubes to remove cells from the brush.
3. Centrifuge tubes, with brushes inside, at 300 × *g* for 5 min.
4. Carefully remove transport medium and resuspend the cell pellet in epithelial culture medium (*see* **Note 11**).
5. Seed cell suspension on preprepared T25 feeder layers, incubate at 37 °C (5 % CO_2) and change the medium after 2 days.
6. Epithelial colonies become evident after 2–3 days (*see* **Note 12**).

3.2.2 Cell Seeding from Biopsies

1. For explant cultures, biopsies are seeded directly onto preprepared feeder layers with a minimal covering of epithelial culture medium (Fig. 1a; *see* **Note 13**) or they can be digested to form a cell suspension prior to culture.
2. To digest a biopsy, carefully transfer the biopsy to an eppendorf tube containing 16 U/ml dispase in RPMI. Digest for 20 min at room temperature.
3. Neutralize the digestion by adding an equal volume of neutralization medium.
4. Transfer the tissue to a sterile petri dish and dissect away epithelium (*see* **Note 14**).

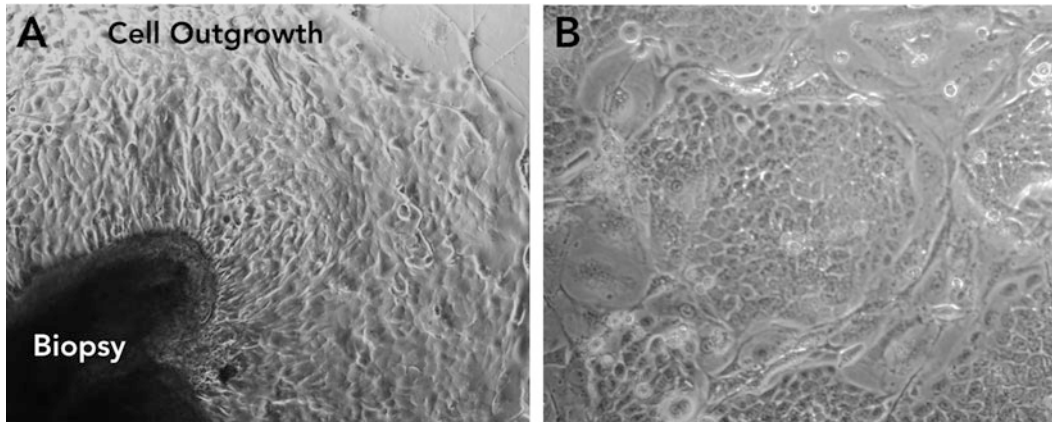

Fig. 1 (**a**) Phase-contrast image of epithelial cell outgrowth from an endobronchial biopsy grown on a 3T3-J2 feeder layer in medium containing the ROCK inhibitor Y-27632 (3T3+Y). (**b**) Phase-contrast image of colonies of subcultured human airway epithelial cells grown in 3T3+Y

5. Transfer to an eppendorf containing 0.1 % trypsin–EDTA (diluted in RPMI) and incubate at 37 °C for 30 min. Remove cells from the incubator and agitate by pipetting every 10 min.
6. Neutralize the trypsin by addition of at least the same volume of neutralization medium and pipette thoroughly.
7. Combine the neutralized digest with the neutralized dispase solution from **step 3**.
8. Centrifuge cells at 300 × *g* for 5 min.
9. Remove supernatant and resuspend the pellet in epithelial culture medium.
10. Seed cell suspension on preprepared T25 feeder layers, incubate at 37 °C (5 % CO_2) and change the medium after 2 days.
11. Epithelial colonies become evident after 2–3 days.

3.3 Maintenance of Human Airway Basal Epithelial Cells

1. During normal maintenance epithelial cells are fed with fresh complete epithelial medium three times per week (10 ml per T75 or equivalent for other vessel surface areas) and stored in 37 °C, 5 % CO_2 incubators.
2. Passage epithelial cell cultures at 80–90 % confluence (Fig. 1b).
3. Remove epithelial culture medium and wash cells once with PBS.
4. Add 0.05 % trypsin–EDTA (*see* **Note 15**) for 2 min at room temperature, gently tap flasks and confirm the removal of feeder cells, which are substantially more trypsin-sensitive than epithelial cells, under a microscope.

5. Remove the trypsin and wash with PBS to remove remaining feeder cells.
6. Add 0.05 % trypsin–EDTA and incubate at 37 °C until epithelial cells detach from the flask (typically this takes 5 min; *see* **Note 16**).
7. Neutralize the trypsin solution with the same volume of epithelial culture medium, centrifuge at 300 × *g* for 5 min and resuspend the pellet in epithelial culture medium.
8. For continued passage, perform a 1:5 split and seed cells onto a preprepared feeder layer in complete epithelial culture medium. Incubate in a 37 °C, 5 % CO_2 incubator.
9. To cryopreserve, freeze 0.5 × 10^6 cells/vial in 200 µl total volume using Profreeze freezing medium according to the manufacturer's instructions.

3.4 3D Tracheosphere Culture

1. Coat the base of a non-adherent 96-well plate with 25 % prechilled Matrigel in tracheosphere medium (30 µl per well) and transfer to an incubator (37 °C, 5 % CO_2) for at least 20 min (*see* **Note 17**).
2. Differentially trypsinize cultured airway basal cells from feeder cells as described above (**step 3** in Section 3.3). For one 96-well plate, resuspend 270,000 basal cells in 7 ml tracheosphere medium containing 5 % prechilled Matrigel and 5 µM Y-27632. Add 65 µl cell suspension per well (2500 cells per well, *see* **Note 18**). Return the plate to an incubator (37 °C, 5 % CO_2; *see* **Note 19**).
3. Feed cells by adding 70 µl tracheosphere medium (without Y-27632) on days 3, 8, and 14 of culture (Fig. 2a).
4. On day 18 of culture tracheospheres are well differentiated with basal cells, goblet cells and ciliated cells present (Fig. 1; *see* **Note 20**).

3.5 Immunofluorescence Staining of Tracheospheres

1. To collect tracheospheres, pipette the well contents into chilled PBS on ice using 200 µl pipette tips with the ends cut off. Centrifuge at 200 × *g* for 3 min. Fix tracheospheres by resuspension in 4 % PFA for 30 min.
2. Using the same centrifuge settings, wash once with PBS and resuspend the tracheosphere pellet in pre-warmed HistoGel specimen processing gel. Pipette the HistoGel onto Parafilm as small droplets appropriate for embedding and allow it to gel at room temperature for 10 min.
3. Process, embed and stain the tracheospheres according to standard histology protocols (Fig. 2b–d).

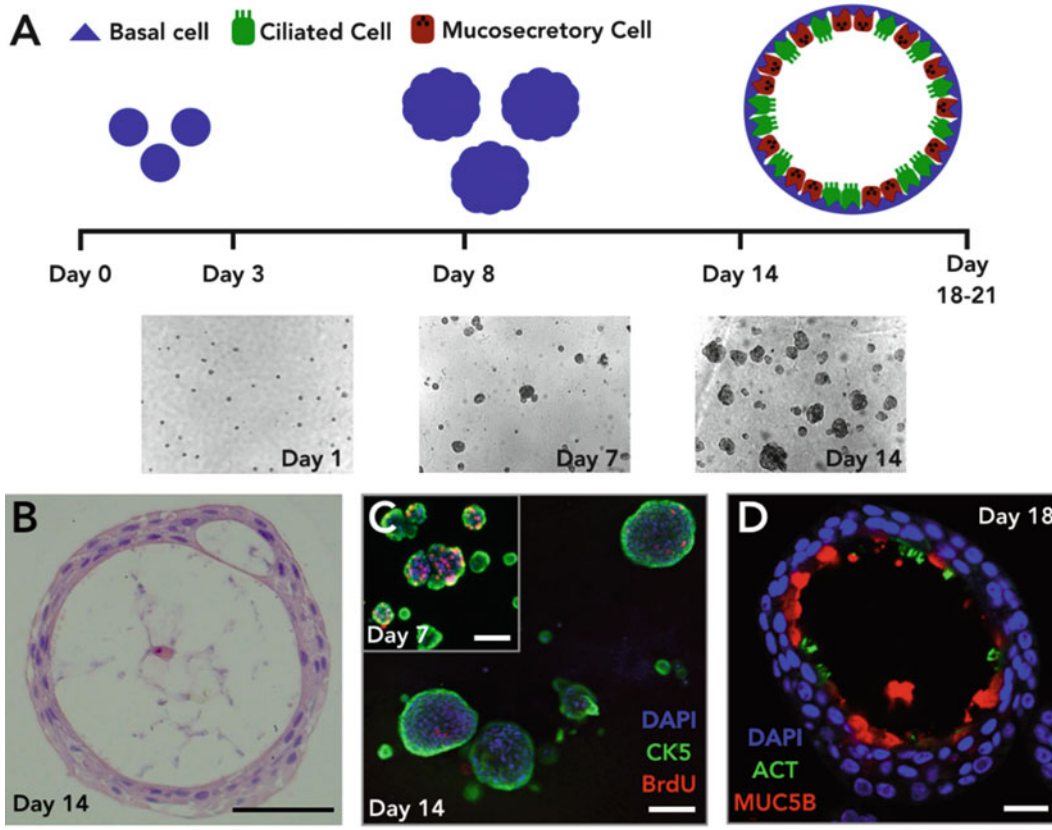

Fig. 2 (**a**) Schematic representation of airway tracheosphere formation in 3D Matrigel culture with brightfield images showing the time course of tracheosphere growth. (**b**) Hematoxylin and eosin (H&E) staining of a tracheosphere. Scale bar = 50 μm. (**c**) Whole-mount immunofluorescence staining of tracheospheres at day 7 and day 14 shows that BrdU uptake is high in the early stages of culture, as tracheospheres form, but reduces over time. Scale bar = 100 μm. (**d**) Immunofluorescence staining of tracheospheres shows the differentiation of cultured primary human airway basal cells into both ciliated (ACT; *green*) and mucosecretory (MUC5B; *red*) cells in tracheospheres. Scale bar = 20 μm

4 Notes

1. Batch testing of FBS for the ability to support rapid expansion and serial passage of primary human epithelial cells is essential.
2. Hydrocortisone/EGF stock is prepared by dissolving hydrocortisone at 0.5 mg/ml in 100 % ethanol. 1 ml hydrocortisone is added to 19 ml DMEM containing 2.5 μg recombinant human EGF. Hydrocortisone/EGF stock aliquots are stored at −20 °C.
3. A bottle of Matrigel must be thawed on ice for >6 h. Once thawed, Matrigel should be aliquoted into prechilled, sterile eppendorf tubes and stored at −20 °C. Freeze-thaw Matrigel a

maximum of three times. Matrigel should be kept on ice throughout the tracheosphere seeding process.

4. Retinoic acid is extremely light sensitive. Care should be taken to minimize light exposure during preparation and storage, for example by foil wrapping falcon tubes. Aliquoted 10 mM stocks can be stored for up to 1 month at −80 °C. We prepare a 1:1000 (10 μM) stock and dilute this 1:100 to prepare tracheosphere medium (final conc. 100 nM).
5. A 10 ml vial of HistoGel should be preheated to 65 °C in a water bath for 30 min. HistoGel can be aliquoted into 1.5 ml eppendorf tubes to reduce this time to 10 min and sets quickly at room temperature so should be kept at 65 °C until use.
6. We thaw fresh 3T3-J2 cells after passage 12 as the cells are susceptible to undergoing spontaneous transformation, proliferating more rapidly and adopting a cuboidal morphology compared with the usual spread appearance of fibroblasts.
7. 3T3-J2 cultures should never be allowed to become over confluent as their morphology and sensitivity to irradiation/mitomycin C can be affected, leading to poor quality feeder layers.
8. Alternatively, 3T3-J2s can be mitotically inactivated by 40 Gy (4000 rd) irradiation.
9. After mitotic inactivation, 3T3-J2 cells are fragile and care should be taken when pipetting the cells as excessive stress can cause feeder layers to degenerate more rapidly.
10. Co-seeding of 3T3-J2 and epithelial cells in complete epithelial culture medium produces poor quality feeder layers. Mitotically inactivated fibroblasts do not spread, as when seeded in fibroblast culture medium, and feeder layers degenerate more quickly. Thus, it is important to be well organized in the planning of experiments using cocultured cells.
11. Care should be taken to wash the brush with epithelial culture medium to free as many cells as possible. We use at least two rounds of washing with fresh medium.
12. Contamination of cultures with human fibroblasts has not been a problem in our lab. It is thought that the combination of the feeder layers and culture medium additives suppress the growth of fibroblasts.
13. We do not change the medium on explant biopsies for the first 7 days of culture in order to avoid the risk of detaching the biopsy by moving the flask. Epithelial outgrowths are normally visible in the second week and can be serially passaged as described in **step 3** in Section 3.3.
14. In practice, it is difficult to see the epithelium at this stage—after proper dispase digest the biopsy will appear "fluffy"—but

we do find that pulling apart the biopsy at this stage improves the efficiency of the trypsin step.

15. TrypLE Select (Gibco, Thermo Fisher), a recombinant trypsin that avoids the use of porcine trypsin in human cultures, can be used in place of 0.05 % trypsin–EDTA for passaging epithelial cells with no negative impact on the quality of cultures.
16. Epithelial cells should be treated with care during passaging. We remove cells following trypsinization by gently tapping flasks rather than by washing. Care should also be taken to avoid excessive pipetting of epithelial cells during resuspension.
17. To maximize the well volume available for addition of culture medium, the Matrigel layer at the well base is minimal. Non-adherent culture plates are used to prevent cells in close proximity to the culture plastic from losing 3D structure.
18. Video microscopy studies indicate that tracheospheres formed from cells at this density are not clonal but multiple basal cells in the initial culture can migrate and interact to form tracheospheres.
19. Tracheospheres are very sensitive to culture conditions. We use dedicated incubators, with extra humidification, for airway differentiation assays in order to reduce the number of times incubator doors are opened and to better maintain constant humidity, CO_2, etc.
20. The abundance of cilia in tracheospheres increases over time. Cilia are observed at day 14, but longer culture periods produce better results. This must be balanced with the ability to continue to add culture medium to wells. End-point assays can be performed between day 14 and day 21.

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