Chapter 7

Applications of Mouse Airway Epithelial Cell Culture for Asthma Research

Amjad Horani, John D. Dickinson, and Steven L. Brody

Abstract

Primary airway epithelial cell culture provides a valuable tool for studying cell differentiation, cell–cell interactions, and the role of immune system factors in asthma pathogenesis. In this chapter, we discuss the application of mouse tracheal epithelial cell cultures for the study of asthma biology. A major advantage of this system is the ability to use airway epithelial cells from mice with defined genetic backgrounds. The in vitro proliferation and differentiation of mouse airway epithelial cells uses the air–liquid interface condition to generate well-differentiated epithelia with characteristics of native airways. Protocols are provided for manipulation of differentiation, induction of mucous cell metaplasia, genetic modification, and cell and pathogen coculture. Assays for the assessment of gene expression, responses of cells, and analysis of specific cell subpopulations within the airway epithelium are included.

Key words Asthma, Trachea, Mouse, Air–liquid interface, Mucous cell, Ciliated cell

1 Introduction

1.1 Mouse Airway Epithelial Cell Models for Asthma Research

Asthma is characterized by remodeling and inflammation of the airway epithelium. Experimental models of asthma in mice allow control of genetic and environmental factors. In this chapter, the power of mouse genetics is extended to culture of mouse tracheal epithelial cells (mTEC). The use of airway epithelial cells from defined genetic strains of mice facilitates testing phenotypes relevant to asthma in a highly controlled environment and offers analysis of epithelial–immune interactions using syngeneic cells. Since its introduction, our mTEC protocol has been widely adapted for experimental purposes relevant to the study of asthma [1–12].

Protocols and assays provided in this chapter are diagrammed in Fig. 1. The basic mTEC culture protocol can be used to generate cell preparations with epithelial cell type constituents as found in vivo, including basal, ciliated, and secretory cells. The mTEC culture protocol results in a surface that is similar to that of the native mouse trachea [13]. While the method for the basic culture

Irving C. Allen (ed.), *Mouse Models of Allergic Disease: Methods and Protocols*, Methods in Molecular Biology, vol. 1032,
DOI 10.1007/978-1-62703-496-8_7, © Springer Science+Business Media, LLC 2013

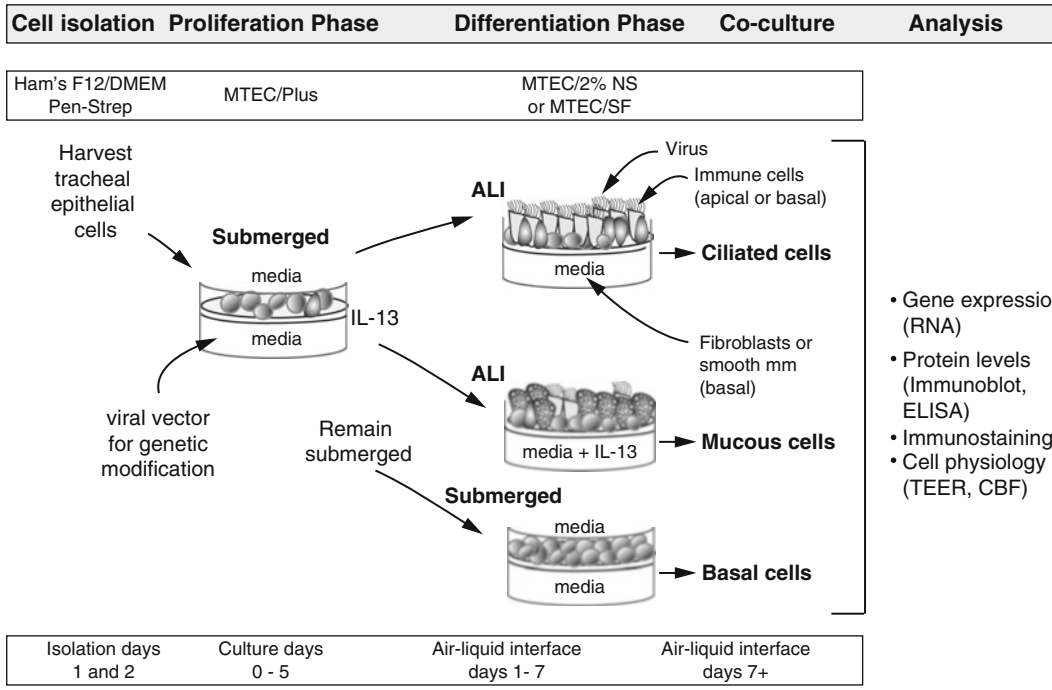

Fig. 1 Overview of culture and manipulation of mTEC. Each phase of the procedure is indicated, the culture media required is listed, and the timeline is noted. Tracheal epithelial cells are seeded onto supported membranes. During proliferation, cells are submerged in mTEC/Plus medium with retinoic acid (RA) for approximately 5 days until confluent. Differentiation uses the air–liquid interface (ALI) condition and either mTEC/NS or mTEC/SF medium (each with RA). Examples of manipulation of differentiation are shown. Mucous cells can be generated by treatment with IL-13 or cells held undifferentiated by submersion. Coculture with respiratory viruses or immune, fibroblast, or smooth muscle (mm) cells and assays are noted

system is included, the focus of this chapter is the manipulation and analysis of the culture system relevant to asthma. The reader is strongly encouraged to review detailed versions of the basic protocol [14].

1.2 Manipulation of mTEC and Coculture Conditions

The essential factors used for proliferation and differentiation of mTEC are similar to those used to culture airway epithelial cells from human and other species [15–17]. The culture of mTEC is critically dependent on the isolation of an adequate number of cells and subsequent proliferation of an amplifying progenitor cell population [13]. The mouse trachea harbors pluripotent epithelial basal cells within the epithelium, and the paratracheal glands located at the most proximal region of the trachea [8, 18]. Proliferation of these basal cells ultimately results in a confluent layer of cells that can readily differentiate using air–liquid interface (ALI) conditions and growth factor-enriched media. These conditions favor the differentiation of ciliated epithelial cells and a

smaller population of cells expressing the Clara cell marker, Scgb1a1 [10, 13]. If cells remain submerged, the undifferentiated basal cell population persists [19].

Increased numbers of mucous secretory cells (goblet cells) is a cardinal feature of airway remodeling in asthma. Goblet cells are uncommon in the normal laboratory mouse airway, and are rarely found in standard mTEC preparations [13]. Goblet cell metaplasia can be induced in the cultured mTEC system by the addition of specific cytokines. Prolonged treatment with IL-13 induces the differentiation and proliferation of mucin (MUC5AC)-filled goblet cells and increases mucus secretion [1, 12, 20]. Likewise, IL-6 and IL-17 increase MUC5AC expression in cultured mouse airway cells [21]. In each case, the cytokine dose and treatment timing relative to the stage of cell differentiation impacts the extent of mucous cell metaplasia.

Cultured mTEC are devoid of immune cells; however, the preparations can be supplemented to study the interaction of airway epithelial cells and immune cells such as lymphocytes, dendritic cells, or neutrophils [11, 22, 23]. mTEC are grown on supported membranes with apical and basal chambers allowing apical basolateral surface interaction to study cell–cell interaction using mouse syngeneic immune cells, smooth muscle cells, or fibroblasts with specific genetic deficiencies. The coculture system may also be used in the study of host-pathogen responses to infection by respiratory viruses or bacteria [2, 3].

mTEC may be genetically modified using recombinant viruses for gene transfer [19, 24, 25] or treated with drugs or bioactive agents on the apical or basal surfaces. Finally, mTEC on supported membranes are amenable to analysis using multiple approaches to easily characterize the status of differentiation, gene expression, and ultrastructural features.

1.3 Approach to Protocols for mTEC Cultures

A timeline of the mTEC preparation protocol is shown Fig. 1. Materials (*see* Subheading 2) and Methods (*see* Subheading 3) are organized to match this sequence. Media and reagents required should be prepared prior to harvest. Isolation of tracheal epithelial cells is accomplished over 2 days. Trachea are harvested and incubated in pronase overnight. The following day epithelial cells are released from the trachea then isolated by differential adhesion of fibroblasts on culture plates, leaving mTEC in suspension. mTEC are seeded on supported, semi-permeable membranes in a media favoring proliferation, called mTEC plus. At this time, transduction with viral vectors can be used. Once a confluent layer of cells is established, an ALI condition is created. Media are changed to one with lower concentrations of growth factors using mTEC/NS, containing a serum with proprietary additives (NuSerum™), or mTEC/SF, a serum-free, defined medium. At this time, cytokine treatment or other interventions can be used to manipulate subsequent differentiation that occurs within 3–14 days.

2 Materials

2.1 Stock Components for mTEC Media

1. Prepare stock components prior to cell isolation (*see* Table 1, **Note 1**).

2.2 Media (See Table 2)

1. Ham's F-12/Pen-Strep is used for the harvest of cells. Fetal bovine serum, 10 % is added in some cell isolation steps.
2. mTEC/Basic is the core medium used to prepare proliferation and differentiation media.
3. mTEC/Plus is used to proliferate cells.
4. mTEC/NS (serum-containing; 2 % Nuserum) or mTEC/SF (serum-free) are used to differentiate cells at ALI.
5. RA Stock B (10,000×) must be freshly added to aliquots of media prior to each use (*see* Table 1 and **Note 1**).

2.3 Trachea Harvest (Day 1)

1. Mice from wild type or genetic defined strains (C57Bl/6, SV129/J, C57Bl/6-SV129/J hybrid, Balb/c, FVB, and Swiss Webster backgrounds) (**Note 2**).
2. Ethanol, 70 % for cleansing and wetting euthanized mice prior to dissection.
3. Freshly prepared 0.15 % Pronase (Sigma-Aldrich), in Ham's F-12/Pen-Strep at 0.15 % (w/v). Make 2–5 mL in a 15 mL tube, rock to mix, then filter sterilize.

2.4 Tracheal Epithelial Cell Isolation (Day 2)

1. Plastic sterile Petri dishes, 100 mm, for resected tracheas.
2. Ham's F12/Pen-Strep, on ice.
3. Fetal bovine serum (FBS, 3–5 mL), warmed to 37 °C.
4. Primaria™ (BD Bioscience) tissue culture plates to enhance fibroblast adherence.
5. DNase solution: Crude pancreatic DNase I (Sigma-Aldrich) 0.5 mg/mL, with bovine serum albumin, 1 mg/mL, in Ham's F-12/Pen-Strep. Filter, aliquot, and store in 5 mL aliquots at −20 °C.

2.5 Tracheal Epithelial Cell Seeding and Proliferation

1. Supported semipermeable membranes ("inserts") and culture plates. To get multiple samples, use 6.5 mm, 0.33 cm^2 polycarbonate (Transwell®, Corning) or polyester (polyethylene terephthalate, Transwell®-Clear) membranes with 0.4 μm pores. These fit into a 24-well plate. Include at least one clear membrane in each plate for inspection of cells by microscopy.
2. Rat tail collagen (type I, BD Biosciences). Dilute at 50 μg/mL in 0.02 N acetic acid (in tissue culture grade water) and filter. Store at 4 °C up to 8 weeks.

Table 1
Stock components for mTEC media

Stock	Components	Concentration	Aliquot size for 250 mL	Comments
Retinoic acid (RA) Stock A	Retinoic acid (50 mg) in 100 % ethanol, 33.3 mL	5×10^{-3} M	500 µL	Sigma-Aldrich Protect from light, use glass pipettes Filter sterilize, Store −80 °C up to 12 months
Retinoic acid Stock B (10,000×)	RA stock A, 0.5 mL in 100 % ethanol, 4.5 mL	5×10^{-4} M	500 µL	Filter sterilize Protect from light, avoid freeze-thaw Store −80 °C up to 6 months
I	Insulin, 50 mg in HCl (4 mM), 25 mL	2 mg/mL	1,250 µL for mTEC/Plus; 625 µL for mTEC/SF	Sigma-Aldrich Filter sterilize Store −20 °C
T	Transferrin (human), 100 mg plus 200 µL BSA (100 mg/mL) in HBSS, 19.8 mL	5 mg/mL	250 µL for mTEC/Plus; 250 µL for mTEC/SF	Sigma-Aldrich Filter sterilize Store −20 °C
EGF	Epidermal growth factor (mouse), 100 µg plus 200 µL BSA (100 mg/mL) in HBSS, 19.8 mL	5 µg/mL	1,250 µL for mTEC/Plus; 250 µL for mTEC/SF	BD Biosciences Filter sterilize Store −20 °C
CT	Cholera toxin, 1 mg plus 200 µL BSA (100 mg/mL) in HBSS, 19.8 mL	100 µg/mL	250 µL for mTEC/plus; 62.5 µL for mTEC/SF	Sigma-Aldrich Filter sterilize Store −20 °C
BPE	Bovine pituitary extract, 7.5 mg total protein in HEPES buffered saline	Varies with preparation	Volume of 7.5 mg protein, for 250 mL of mTEC/Plus or mTEC/SF	Frozen bovine pituitaries (Pel-Freeze) [15], or use Pel-Freeze BPE 57136 Store −80 °C
BSA	BSA (Fraction V), 5 g in HBSS, 50 mL	100 mg/mL	2.5 mL for mTEC/SF	Fisher, Filter sterilize Store −20 °C
Nu-Serum	NuSerum (contains 25 % serum), 5 mL	NA	5 mL for mTEC/NS	BD Biosciences Store −20 °C

3. Cell proliferation medium mTEC/Plus (*see* Subheading 2.2, **item 3** and **Note 1**).
4. Hemocytometer and trypan blue (0.4 % w/v), to assess cell viability.

2.6 Tracheal Epithelial Ciliated Cell Differentiation Using the ALI Condition

1. Cell differentiation media, either serum-containing mTEC/NS or serum-free mTEC/SF with defined components (*see* Subheadings 2.1 and 2.2, **item 4**).

2.7 Induction of Mucous Cell Metaplasia

1. Recombinant mouse IL-13 (Peprotech), IL-6 (R&D Systems), and IL-17 (R&D Systems).

2.8 Genetic Modification of mTEC with Viral Vectors

1. Use recombinant adenovirus or lentivirus, each with a titer of at least 10^7 infectious units/mL. Generate virus using standard protocols. Handle according to biosafety guidelines at the user's institution (*see* **Note 3**).

2.9 Cell Coculture Systems

1. Isolate lymphocytes, neutrophils or other immune cells, fibroblasts or smooth muscle cells, or others using specialized protocols.

2.10 Pathogen Infection Models

1. Infectious agents (e.g., influenza virus, respiratory syncytial virus, and bacteria) used in a coculture system should be handled using standard protocols and biosafety guidelines (*see* **Note 3**).

2.11 Analysis of mTEC Differentiation by Immunofluorescence

1. Fixative 4 % paraformaldehyde (*see* **Note 3**) in PBS. Prepared fresh or freshly defrosted. Use PBS to wash cells on the inserts.
2. Scalpel (#22) to cut the membrane from the plastic supports and forceps to hold membranes.
3. A blocking solution of 5 % donkey serum (Sigma-Aldrich), 3 % BSA and Add detergent Tween 0.2 %. (Sigma-Aldrich) in PBS.
4. Antibodies: Mucous cell marker, mouse anti-Muc5AC (Abcam), cilia marker, mouse anti-acetylated α-tubulin (clone 6-11B-1, Sigma-Aldrich) and anti-mouse, fluorescent-labeled secondary antibodies.
5. Mounting medium containing nuclear DNA stain Hoechst or 4′, 6 diamidino-2-phenylindole (DAPI) such as Vectashield® (Vector, Burlingame, CA). DNA binding chemicals are potentially carcinogenic (*see* **Note 3**).
6. Glass microscope slides and large cover slips (24 × 50 mm) to cover several pieces of mTEC membranes on a single slide.

2.12 Additional Methods to Assess Gene Expression and Differentiation of mTECs

1. mTEC flow cytometry; use 0.1 % EDTA or 0.25 % trypsin with 0.1 % EDTA in Cell Dissociation Solution (Sigma) for releasing cells from membrane (*see* Subheading 3.11, **step 1**). Use 2 % FCS in PBS (2 % FCS/PBS) for re-suspending the cells and staining with antibodies. Use standard protocols for flow cytometry.
2. Mini Cell Scrapers (Biotium) or 200 μL pipette tips for scraping cells off membranes.

3. RNA isolation from mTEC: Qiagan RNA Easy® Microkit and Kontes Pellet Pestle®.
4. DMEM to collect mucus and ATPγS (Sigma) 100 μM to induce mucus secretion.

2.13 Assessment of Cell Physiology

1. Transepithelial electrical resistance (Rt) using a Voltohmmeter with electrode "chopstick" pair (EVOM, World Precision Instruments).
2. Cilia beat frequency (CBF) measurement using specialized automated software (e.g., Sisson-Ammons Video Analysis, Ammons Engineering) [26], and an inverted microscope with phase contrast filters and objectives (20×), and high-speed video camera.

3 Methods

3.1 Media preparation

1. Prepare all stock components for media as described in subheading 2.1.
2. Prepare media (see Table 2).

3.2 Preparation of Materials for mTEC Isolation and Initiation of Culture

1. Coat the apical surface of the Transwell® insert membrane with rat tail type I collagen solution in the hood. Incubate plates at room temperature for 18–24 h or for a minimum of 4 h at 37 °C. Rinse apical and basal surfaces with sterile PBS three times then dry for 5 min. Prepare three inserts per trachea harvested.
2. Cells may be cultured on standard tissue culture plastic when coated with rat tail collagen, but will not differentiate to ciliated types.

3.3 Trachea Harvest (Day 1)

1. In the tissue culture hood, prepare two 100 mm dishes (non-tissue culture) with 10 mL cold sterile Ham's F-12/Pen-Strep on ice, to hold resected tracheas.
2. Immerse the euthanized mouse in 70 % ethanol.
3. Expose the trachea. Incise the abdominal skin circumferentially, and then invert the entire layer of skin toward the head to reveal the neck. Separate the neck muscles and open the thoracic cavity to expose the trachea and mainstem bronchi.
4. Resect the trachea. Bluntly dissect the trachea from the posterior surface of the esophagus. Cut the trachea just distal to the larynx, leaving the larynx intact. Place the trachea in the dish of Ham's F-12/Pen-Strep on ice.
5. In the hood, strip off adherent tissues from trachea with a small forceps. Place each cleaned trachea in a dish of cold sterile Ham's F-12/Pen-Strep on ice.

Table 2
mTEC media

Media name	Components and amount (final concentration)		Comments
Ham's F-12/ Pen-Strep	Pen/Strep (1,000×)	500 µL (100 U Penicillin 100 µg Streptomycin)	Store at 4 °C
	Ham's F-12	Add to 500 mL final volume	
mTEC/basic	1 M HEPES	7.5 mL (15 mM)	Filter sterilize
	Glutamine 200 mM	10 mL (4 mM)	Store at 4 °C
	$NaHCO_3$ 7.5 %	2.0 mL (3.6 mM)	Stable up to 6 weeks
	Ampho B (250 µg/mL)[a]	500 µL (0.25 µg/mL)	
	Pen/Strep (1,000×)	500 µL (100 U Pen/100 µg Strep)	
	DMEM/F-12	Add to 500 mL final volume	
mTEC/Plus[b] (High concentration growth factors)	I	1,250 µL (10 µg/mL)	Filter sterilize
	T	250 µL (5 µg/mL)	Store at 4 °C
	CT	250 µL (0.1 µg/mL)	Stable up to 6 weeks
	EGF	1,250 µL (25 ng/mL)	
	BPE[c]	TBD µL (7.5 mg protein/250 mL)	
	FBS	12.5 mL (5 % v/v)	
	mTEC Basic medium	Add to 250 mL final volume	
mTEC/NS[b] (*Nu*Serum)	NuSerum	5.0 mL	Store at 4 °C
	mTEC Basic medium	Add to 250 mL final volume	Stable up to 6 weeks
mTEC/SF[b] (*S*erum-*F*ree media)	I	625 µL (5 µg/mL)	Filter sterilize
	T	250 µL (5 µg/mL)	Store at 4 °C
	CT	62.5 µL (0.025 µg/mL)	Stable up to 6 weeks
	EGF	250 µL (5 ng/mL)	
	BPE[c]	TBD µL (7.5 mg protein/250 mL)	
	BSA stock	2.5 mL (1 mg/mL)	
	mTEC Basic medium	Add to 250 mL final volume	

[a]Amphotericin B: do not filter
[b]RA, stock B 10,000×: add 1 µL to each 10 mL of mTEC/Plus, mTEC/NS, mTEC/SF immediately prior to adding media to cells
[c]BPE: TBD, to be determined, concentration varies with preparation. Use an amount to provide 7.5 mg protein/250 mL of medium

6. Cut each trachea lengthwise to open and submerge in freshly made pronase.
7. Incubate at 4 °C overnight (18–24 h).

3.4 Tracheal Epithelial Cell Isolation (Day 2)

1. Thaw the DNase solution on ice. Thaw and warm FBS to 37 °C in a water bath.
2. Gently invert the tube containing the tracheas in pronase about five times. Warm the tube to room temperature for 10 min and mix gently.

3. Add warmed FBS to a final concentration of 10 % and invert again gently 15–20 times (down and up is a single cycle) to dislodge epithelial cells.
4. Remove each trachea from the tube with a Pasteur pipette and place it in a new 15 mL tube with 3 mL of Ham's F-12/10 % FBS. Invert the tube 15 times.
5. Remove tracheas from the tube and place in a third 15 mL tube containing 3 mL of Ham's F-12/10 % FBS. Invert the tube 15 times.
6. Using a Pasteur pipette, remove and discard the tracheas.
7. Combine the contents of all three tubes containing the enzyme-released cells. Centrifuge at $500 \times g$, 4 °C, for 10 min.
8. Carefully aspirate the supernatant, and re-suspend the cells in DNase solution (~200 μL per trachea).
9. Put the tube on ice for 5 min, then collect the cells by centrifugation at $500 \times g$, 4 °C, for 5 min.
10. Resuspend the cells in mTEC/Basic medium containing 10 % FBS, using 2–3 mL per 10 tracheas. Plate the cells in a Primaria™ tissue culture dish. Incubate at 37 °C, 5 % CO_2 for 3–4 h. During this incubation, allow the fibroblasts to attach, while the epithelial cells remain nonadherent and suspended in the medium.
11. Gently swirl the medium in the culture dish. Carefully collect the supernatant containing the nonattached epithelial cells and place in a sterile tube.
12. Gently rinse the dish one or two times with warm mTEC Basic medium/10 % FBS to recover additional airway epithelial cells, collect the wash and add to a sterile cell collection tube (from **step 11**). Avoid excessive force that detaches fibroblasts.
13. Centrifuge at $500 \times g$, 4 °C, for 5 min.
14. Aspirate the supernatant and re-suspend the cell pellet in a small, measured volume (e.g., 100–200 μL/trachea) of mTEC/Plus medium with fresh RA. Do not try to pipette the cell clumps vigorously to form a single cell suspension.
15. Calculate the number of viable cells with a hemocytometer. Cells viability by trypan blue exclusion should be greater than 90 %. Single cells and clumps will be present. Do not overestimate cell numbers within clumps, or include red blood cells. Yields are $1–2 \times 10^5$ cells per trachea.

3.5 Tracheal Epithelial Cell Seeding and Proliferation

1. Seed cells at 1.0×10^5 cells/cm^2. Density is critical for proliferation and differentiation. Higher seeding density may be required if cultures are manipulated by cytokine treatment or gene transfer.

2. Add the cell suspension to the apical chamber and gently move the plate to distribute the cells on the membrane. Add mTEC/Plus with RA to the basal compartment. This is culture day 0.
3. On Day 3, change the medium in apical and basal compartments. Adherent cells should appear elongated and in islands.
4. On Day 5, the cells are typically confluent, but may require 7 days. Transepithelial cell resistance is typically greater than 1,000 Ω cm^2 (*see* **Note 4**).

3.6 Induction or Inhibition of Ciliogenesis

1. Confluent cells can be differentiated to induce ciliated cells using ALI conditions and either mTEC/NS or mTEC/SF. This time point is ALI day 0. Aspirate media from the apical chamber and supply fresh medium only to the basal chamber. The apical surface should remain dry (*see* **Note 4**).
2. Change the medium every other day, including freshly added RA.
3. Follow cells by inspection and microscopy. The apical surface should remain dry or have a small ring of mucus that can be washed with warm media or PBS. Cells develop a cobblestone appearance. Beating cilia may be seen by microscope as early as ALI day 5. Ciliated cells gradually increase in number, to over 30 % of the surface by ALI day 14.
4. The cells can be maintained at ALI for over 2 months without loss of differentiation.
5. Inhibition of differentiation. To maintain basal cells in a polarized state, block ciliogenesis and minimize proliferation, culture the cells with mTEC/Plus until confluent, then change the media to mTEC/NS or mTEC/SF with RA and keep cells submerged by applying media to the apical compartment. Reversion to the air–liquid interface condition can induce differentiation.
6. Absence of retinoic acid in the media prevents normal airway differentiation in both submerged and ALI conditions.

3.7 Induction of Mucous Cell Metaplasia

1. Seed the cells at $1 \times 10^5/cm^2$ or higher. The extent of mucous cell metaplasia will increase when seeding cells at $1.5 \times 10^5/cm^2$.
2. Add 10 ng/mL of mouse IL-13 to mTEC/Plus media at seeding day 3, which is 2 days prior to establishing ALI. Titrate the dose of IL-13 for the desired degree of mucus cell metaplasia, using a range of 1–100 ng/mL.
3. Upon creation of ALI, add fresh IL-13 to the mTEC/NS or mTEC/SF media in the basal compartment and provide fresh IL-13 with each media change. Wash the cell surface to remove mucus.

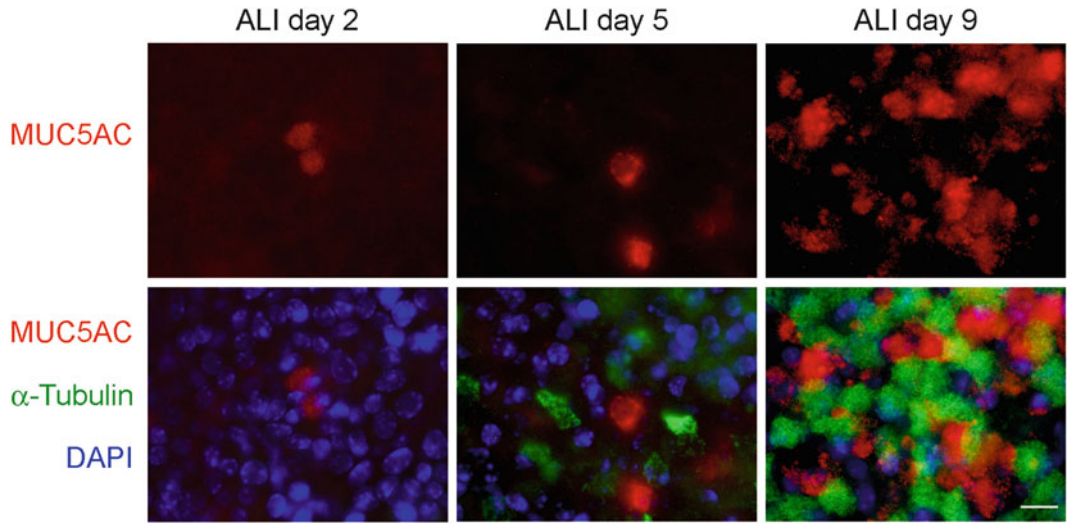

Fig. 2 Induction of mucous cells in mTEC models the asthmatic airway. mTEC were treat with IL-13 (10 ng/mL, in mTEC/NS medium) at ALI day 0 and immunostained at indicated times for the mucous marker MUC5AC (*red*) and cilia marker acetylated α-tubulin (α-tubulin, *green*). DNA is stained with DAPI (*blue*). Photomicrographs, en face of fixed membranes mounted on glass slides. Bar = 10 μm

4. Harvest cells after 3–21 days to assay for mucous cell markers, such as MUC5AC, using quantitative real time PCR, immunostaining, or ELISA (*see* Subheadings 3.11 and 3.12 and Fig. 2).
5. Treatment of well-differentiated mTEC at ALI with 10 ng/mL of IL-13 for 3–14 days will result in mucous cell metaplasia. This metaplasia will be less abundant than levels observed following earlier treatments with the cytokine.
6. 10 ng/mL of IL-6 or IL-17 (the dose can range from 1 to 200 ng/mL) may also be used to induce mucous cell metaplasia using a protocol that is similar to the IL-13 treatment.

3.8 Genetic Modification of mTEC with Viral Vectors

1. Generate viral particles using the desired recombinant adenovirus or lentivirus vectors for over expression or gene silencing using established methods.
2. Lentivirus generated by the producer cell line in culture should be collected in mTEC/Plus medium. Dilute adenovirus into mTEC/Plus medium.
3. Resuspend freshly isolated mTEC cells in virus media using a multiplicity of infection of 25–200. Adenovirus should be titered in the mTEC medium.
4. To achieve a high percentage of adenovirus transfected cells at the time of establishing ALI, seed cells at $1.5–2.0 \times 10^5/cm^2$. This speeds the time of reaching ALI. Alternatively, mTEC can be transfected with adenovirus 2 days prior to establishing ALI.

5. Add the cells in the virus-containing media to the apical chambers. The basalateral chamber should be filled with the same infection media.
6. Change the basolateral media 16–18 h after transduction.
7. If the lentivirus also codes for an antibiotic resistance gene (i.e., puromycin), the antibiotic can be added to the culture media approximately 48 h after infection. When using a selection strategy, it is important to use a g high viral titer (approximately 1×10^7 infectious particles/mL) and a high seeding density. An infection of at least 50 % of the cells will allow recovery of a confluent layer so that an ALI condition can be created.

3.9 Cell Coculture Systems

1. Proliferate or differentiate mTEC to desired status, *see* Protocols 3.6–3.8. Transwell membrane pore density varies depending on composition and manufacturer and may affect immune cell migration. mTEC culture is less successful on membranes with pores greater than 0.4 μm. However, this pore size will accommodate neutrophil migration [22].
2. Tissue culture plates containing wells with cultured smooth muscle cells, fibroblasts, or other types may be prepared to receive inserts with mTEC.
3. If mTEC are well differentiated (older than ALI day 7) or undifferentiated and submerged, then the immune cell culture media can be used without concern for significant loss of mTEC differentiation over a 5–7 day period.
4. Apply immune cells, such as lymphocytes or neutrophils, or other cell types directly on the apical surface of mTEC or add to the lower chamber.
5. If immune cell contact with the basolateral aspect of the mTEC is desired, then the Transwell insert with confluent mTEC is inverted and set into the well of a tissue culture plate. Immune cells are then applied to the basal surface of the membrane, held in place by surface tension or if necessary a collar of ethanol rinsed Parafilm®. The plate must be covered to avoid evaporation of media on the basal surface.
6. Immune cells can be recovered from either compartment by gentle aspiration and the immune cells can be collected with the mTEC by scraping the surface to release cells. The immune cells can then be assayed as desired by experimental method such as flow cytometry.

3.10 Pathogen Infection Models

1. Proliferate or differentiate mTEC to desired status, *see* Protocols 3.6–3.8. Antibiotics may be removed from mTEC media as needed.
2. Apply viruses or bacteria directly on the apical surface of mTEC. Initial multiplicity of infection is 0.01–10 infectious

particles/cell for respiratory viruses. Titrate exposure time and multiplicity of infection to the desired effect. mTEC can support some viruses over many weeks.

3. If contact of pathogen interaction with the basolateral aspect of mTECs are desired, assemble inserts as described in Protocol 3.9, **step 5**.
4. Collect cells on whole membranes for immunostaining, as cell suspensions, lysates or as required for assay, *see* Protocols 3.11 and 3.12.

3.11 Analysis of mTEC Differentiation and Mucus Cell Metaplasia by Immunofluorescent Staining

1. Fix the cells. Wash the cells gently with PBS, aspirate the PBS, and then fill the chambers with 4 % paraformaldehyde to cover the membranes (this chemical is toxic, *see* **Note 3**). Incubate at room temperature for 10 min without rocking. Remove and properly discard the paraformaldehyde. Wash the cells on the membrane three times for 5 min each, by adding PBS to both chambers and slowly rocking the plate.
2. Cut the membrane from the plastic support. Do not allow the membrane to dry. Invert the insert and with a scalpel, cut the membrane from the plastic ring support. Prepare a wet surface for delivery of the membrane by adding 1 mL of PBS to a tissue culture dish. Position the insert, with the basal surface on the plate, and use fine forceps to release the membrane. To produce multiple samples from one membrane, cut into quarters by rocking the scalpel blade across the membrane. Notch the outer bottom corner of each quarter to orient to the cell surface. Handle the membrane at the edge with fine forceps. Fixed cells on membranes can be stored in sterile PBS at 4 °C in a Parafilm® sealed plate for several months.
3. Block nonspecific antibody binding on the cells (*see* Subheading 2.12, **item 3**). Transfer the membrane pieces to a 24- or 96-well plate. Cover the membrane with blocking solution and slowly rock at room temperature for 30–60 min.
4. Immunostain cells with an anti-cilia (acetylated α-tubulin) or anti-mucous (MUC5AC) antibody (*see* Fig. 2). Simultaneous use of two mouse primary antibodies can be achieved using Fab labeling of one antibody (e.g., Zenon, Life Technologies). Dilute primary antibody in the blocking solution (*see* Subheading 2.12, **item 3**). Use an isotype-matched antibody as a control. Incubate with cells on a piece of membrane for 1 h at room temperature or overnight at 4 °C. Wash membrane with PBS three times for 5 min each. Add a fluorescent-labeled secondary antibody for 30 min, and wash three times.
5. Mount immunostained membranes on slides. Transfer the membrane to a glass microscope slide and apply 10–20 μL of

mounting medium containing DAPI. Inspect the membrane under the fluorescence microscope if the membrane orientation is not certain. Apply the coverslip and seal the edges with nail polish. Examine by fluorescent microscopy.

3.12 Additional Methods to Assess Differentiation and Gene Expression in mTEC

1. Flow cytometry of mTEC. To obtain a cell suspension use 0.1 % EDTA in Cell Dissociation Solution. To detect an intracellular protein, use 0.25 % trypsin with 0.1 % EDTA in Cell Dissociation Solution. Put the appropriate solution in both chambers, place in the tissue culture incubator for 5–20 min. Aspirate the basal compartment. Release the cells from the apical compartment by mixing with a mini cell scraper or a pipette tip. Transfer the cells to a tube. Add 2 % FCS/PBS, 200 μL (0.33 cm^2 membrane) to the apical chamber, pipette to recover additional cells. Repeat this twice more and pool all washes. Pass the cell suspension through a 70 μM cell strainer to obtain a single cell suspension. Centrifuge the cells at $500 \times g$, 4 °C, for 5 min. Resuspend the cell pellet in 2 % FCS/PBS. The typical yield is 2×10^5 cells per 0.33 cm^2 insert. Proceed using relevant flow cytometry protocols.
2. Protein blot analysis of mTEC. Put the plate with the inserts on ice, wash the cells twice with ice cold PBS. Add 25–30 μL of the appropriate lysis buffer on the apical surface of a 0.33 cm^2 of membrane. Incubate at 4 °C, rocking slowly. After 20 min, pipette and gently scrape the surface to release cells. Transfer the lysate solution to a microcentrifuge tube on ice and process using standard protocols. Typical yield of protein is 25–40 μg per 0.33 cm^2 membrane.
3. RNA isolation. Collect cells in the Qiagan RNA Easy® Microkit. Freeze at −80 °C then break cells with the Kontes Pellet Pestle® for 90 s. Continue according to the manufactures' instructions. The typical RNA yield is 6–10 μg per cm^2 membrane. Analyze expression by real time PCR.
4. Collection of mucus secretions [27]. Gently wash the apical insert surface with 100 μL of warm DMEM, incubate 10 min and repeat three more times. Determine the period of baseline secretion to be sampled (e.g., 1–24 h). Then add 100 μL of warm DMEM, incubate at 37 °C for 10 min, repeat three times and pool washings as baseline readings. Then stimulate with 100 μM ATPγS, and repeat collection after 30–60 min. A conventional ELISA assay with a monoclonal antibody for MUC5AC can then be used to measure the amount of a secreted mucin.
5. Electron microscopy for SEM or TEM. Wash cells with cold PBS. Fix the cells in 2.5 % glutaraldehyde in sodium cacodylate buffer at 4 °C overnight on the membrane. Consultation with the EM facility should guide sample preparation.

3.13 Physiologic Measurements of Cell Function

1. Transepithelial Electrical Resistance (Rt). Use a voltohmmeter to assess junction integrity and maturation. A typical Rt for a confluent membrane is greater than 1,000 Ω cm^2. It is highest early in ALI and decreases with differentiation.
2. Immerse the probe in 70 % ethanol, air dry, and rinse with sterile water or PBS.
3. Add medium to both chambers for the measurement of Rt. A coated membrane without cells is used to a obtain baseline Rt. The baseline value is subtracted from the observed Rt from the membrane with cells to obtain the Rt.
4. Cilia beat frequency. Wash the apical surface with warm PBS at least 1 h prior to measuring cilia beat frequency measurement. Image at least five fields (*see* Subheading 2.13, **item 2**).

4 Notes

1. Media preparation
   Reagents and components for cell isolation should be filter sterilized using a 0.22 μM syringe filter with low protein binding (e.g., Pall PN 4602). Aliquot stock components in volumes appropriate for preparing 250 mL of media. Retinoic acid (RA, stock B 10,000×) should be freshly added to media at a final concentration of 5×10^{-8} M. RA supplemented media should be used within 48 h. If prior fungal contamination has occurred, 0.25 μg/mL of Amphotericin B should be added to media without filtering and should be used until the cells are changed to ALI conditions. Sustained use of Amphotericin B over several weeks should be avoided due to toxicity.
2. Mice
   The growth and differentiation of mTEC from mice ages 4 weeks to over 18 months is similar. Isolation of cells from younger and smaller mice is more technically challenging and the total cell number recovered is diminished. Some strain-dependent differences (e.g., SV129, FVB) in proliferative populations can be overcome by increasing the seeding density.
3. Cautions regarding potentially toxic materials
   The fixatives paraformaldehyde and glutaraldehyde are toxic. The DNA binding compounds Hoechst, DAPI, and sodium cacodylate are potentially carcinogenic. These reagents and viruses used for gene transfer or pathogen studies should be handled according to biosafety guidelines at the user's institution.
4. Troubleshooting mTEC preparations
   Failure of cells to proliferate to confluence or differentiate may have several causes including the following: (1) not resecting the entire trachea, especially that containing the paratracheal

glands (so that basal cell numbers are insufficient); (2) inadequate pronase activity; (3) fibroblast contamination; or (4) infection. To correct these issues, assure proper dissection of the complete length of the trachea (but avoid the larynx), increase the number and force of "shakes" of tracheas in pronase, assure the pronase is fresh, and increase the pronase concentration (e.g., to 0.20 %). Increasing the time of pronase digestion does not significantly improve cell yield; however, pronase activity varies with supplier and lot. To minimize the fibroblast contamination, allow adequate time for adherence of fibroblast in the culture dish and avoid over-washing the culture dish after fibroblast adherence. Low levels of fungal contamination may inhibit cell growth. The addition of Amphotericin B 0.25 μg/mL to stock media or antifungal/antibiotic, Primocin™ 50 mg/mL (InvivoGen) may be helpful.

Acknowledgments

This work was supported by awards to S.L.B. from the National Institute of Health and the Children's Discovery Institute of Saint Louis Children's Hospital and Washington University.

References

1. Lankford SM, Macchione M, Crews AL, McKane SA, Akley NJ, Martin LD (2005) Modeling the airway epithelium in allergic asthma: interleukin-13-induced effects in differentiated murine tracheal epithelial cells. In Vitro Cell Dev Biol Anim 41:217–224
2. Ibricevic A, Pekosz A, Walter MJ, Newby C, Battaile JT, Brown EG, Holtzman MJ, Brody SL (2006) Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. J Virol 80:7469–7480
3. Brockman-Schneider RA, Amineva SP, Bulat MV, Gern JE (2008) Serial culture of murine primary airway epithelial cells and ex vivo replication of human rhinoviruses. J Immunol Methods 339:264–269
4. Mebratu YA, Dickey BF, Evans C, Tesfaigzi Y (2008) The BH3-only protein Bik/Blk/Nbk inhibits nuclear translocation of activated ERK1/2 to mediate IFNgamma-induced cell death. J Cell Biol 183:429–439
5. Nakagami Y, Favoreto S Jr, Zhen G, Park SW, Nguyenvu LT, Kuperman DA, Dolganov GM, Huang X, Boushey HA, Avila PC, Erle DJ (2008) The epithelial anion transporter pendrin is induced by allergy and rhinovirus infection, regulates airway surface liquid, and increases airway reactivity and inflammation in an asthma model. J Immunol 181:2203–2210
6. Sel S, Rost BR, Yildirim AO, Sel B, Kalwa H, Fehrenbach H, Renz H, Gudermann T, Dietrich A (2008) Loss of classical transient receptor potential 6 channel reduces allergic airway response. Clin Exp Allergy 38: 1548–1558
7. Tachdjian R, Mathias C, Al Khatib S, Bryce PJ, Kim HS, Blaeser F, O'Connor BD, Rzymkiewicz D, Chen A, Holtzman MJ, Hershey GK, Garn H, Harb H, Renz H, Oettgen HC, Chatila TA (2009) Pathogenicity of a disease-associated human IL-4 receptor allele in experimental asthma. J Exp Med 206: 2191–2204
8. Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, Hogan BL (2009) Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc Natl Acad Sci USA 106:12771–12775
9. Wong AP, Keating A, Lu WY, Duchesneau P, Wang X, Sacher A, Hu J, Waddell TK (2009) Identification of a bone marrow-derived epithelial-like population capable of repopulating injured mouse airway epithelium. J Clin Invest 119:336–348
10. Ghosh M, Brechbuhl HM, Smith RW, Li B, Hicks DA, Titchner T, Runkle CM, Reynolds SD (2011) Context-dependent differentiation of multipotential keratin 14-expressing

tracheal basal cells. Am J Respir Cell Mol Biol 45:403–410
11. Deppong CM, Xu J, Brody SL, Green JM (2012) Airway epithelial cells suppress T cell proliferation by an IFNgamma/STAT1/TGFbeta-dependent mechanism. Am J Physiol Lung Cell Mol Physiol 302:L167–L173
12. Zeki AA, Thai P, Kenyon NJ, Wu R (2012) Differential effects of simvastatin on IL-13-induced cytokine gene expression in primary mouse tracheal epithelial cells. Respir Res 13:38
13. You Y, Richer EJ, Huang T, Brody SL (2002) Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. Am J Physiol Lung Cell Mol Physiol 283:L1315–L1321
14. You Y, Brody SL (2013) Culture and differentiation of mouse tracheal epithelial cells. In: Randell SH, Fulcher M L (eds) Epithelial cell culture protocols, 2nd edn. Humana Press, New York, pp 123–143
15. Lechner J, LaVeck M (1985) A serum-free method for culturing normal human bronchial epithelial cells at clonal density. J Tissue Cult Meth 9:43–48
16. Whitcutt MJ, Adler KB, Wu R (1988) A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. In Vitro Cell Dev Biol 24: 420–428
17. Wu R (1997) Growth and differentiation of tracheobronchial epithelial cells. In: MacDonald J (ed) Growth and development of the lung. Deckker, New York, pp 211–241
18. Borthwick DW, Shahbazian M, Krantz QT, Dorin JR, Randell SH (2001) Evidence for stem-cell niches in the tracheal epithelium. Am J Respir Cell Mol Biol 24:662–670
19. You Y, Huang T, Richer EJ, Schmidt JE, Zabner J, Borok Z, Brody SL (2004) Role of f-box factor foxj1 in differentiation of ciliated airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 286:L650–L657
20. Tyner JW, Kim EY, Ide K, Pelletier MR, Roswit WT, Morton JD, Battaile JT, Patel AC, Patterson GA, Castro M, Spoor MS, You Y, Brody SL, Holtzman MJ (2006) Blocking airway mucous cell metaplasia by inhibiting EGFR antiapoptosis and IL-13 transdifferentiation signals. J Clin Invest 116:309–321
21. Chen Y, Thai P, Zhao YH, Ho YS, DeSouza MM, Wu R (2003) Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. J Biol Chem 278:17036–17043
22. Farberman MM, Ibricevic A, Joseph TD, Akers KT, Garcia-Medina R, Crosby S, Clarke LL, Brody SL, Ferkol TW (2011) Effect of polarized release of CXC-chemokines from wild-type and cystic fibrosis murine airway epithelial cells. Am J Respir Cell Mol Biol 45:221–228
23. Kreisel D, Lai J, Richardson SB, Ibricevic A, Nava RG, Lin X, Li W, Kornfeld CG, Miller MJ, Brody SL, Gelman AE, Krupnick AS (2011) Polarized alloantigen presentation by airway epithelial cells contributes to direct CD8+ T cell activation in the airway. Am J Respir Cell Mol Biol 44:749–754
24. Schmid A, Bai G, Schmid N, Zaccolo M, Ostrowski LE, Conner GE, Fregien N, Salathe M (2006) Real-time analysis of cAMP-mediated regulation of ciliary motility in single primary human airway epithelial cells. J Cell Sci 119:4176–4186
25. Pan J, You Y, Huang T, Brody SL (2007) RhoA-mediated apical actin enrichment is required for ciliogenesis and promoted by Foxj1. J Cell Sci 120:1868–1876
26. Sisson JH, Stoner JA, Ammons BA, Wyatt TA (2003) All-digital image capture and whole-field analysis of ciliary beat frequency. J Microsc 211:103–111
27. Abdullah LH, Wolber C, Kesimer M, Sheehan JK, Davis CW (2012) Studying mucin secretion from human bronchial epithelial cell primary cultures. Methods Mol Biol 842: 259–277