Chapter 15

Establishment of Respiratory Air–Liquid Interface Cultures and Their Use in Studying Mucin Production, Secretion, and Function

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Abstract

Primary cultures of human airway bronchial airways represent a valuable tool in understanding the roles of the epithelium, cilia, and the mucus layer in coordinating the clearance of mucus from the airways. The ability to obtain cells from both normal and diseased populations (such as cystic fibrosis and Chronic obstructive pulmonary disease (COPD)) allows researchers to investigate the disease phenotype on these processes. Furthermore, such cultures have provided investigators with a vast source of native airway mucus, devoid of external biological processes that occur in vivo, for biochemical and rheological studies. The primary goal of this chapter is to describe the culturing and use of human airway cultures grown under an in vivo-like air–liquid interface for use in a variety of mucus and mucociliary studies.

Key words: Airway epithelia, Mucus, Cilia, Mucociliary clearance, Mucus rheology

1. Introduction

The thin mucus layer lining the surfaces of the airways is vital for ensuring the sterility of the lungs from the constant bombardment of potentially infectious and toxic substances that are inhaled during normal tidal breathing. Mucus samples obtained from humans, as well as animals, have been invaluable for understanding many aspects of the normal biophysical properties of mucus and how these properties are altered in patients with chronic airway diseases, such as Chronic obstructive pulmonary disease (COPD) and cystic fibrosis. However, our growing understanding of mucus and the role of defects in mucociliary clearance on the pathogenesis of such airways diseases has most recently benefited immensely from the development of a highly differentiated primary human bronchial epithelial (HBE) cell culture system.

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Based on many years work with these primary cultures, it is clear that the presence of an air–liquid interface is critical to successfully recapitulate the normal airway epithelial biology in vivo. Specifically, when cultured under an air–liquid condition, these cultures differentiate into a state similar to that reached in vivo; i.e. a pseudostratified epithelia with basal cells, ciliated cells, and mucin-secreting cells. As a result of similarities between the well-differentiated primary human airway epithelia cultures grown at the air–liquid interface and the airways in vivo, many studies have focused on their use for investigating various biochemical and biophysical properties of mucus clearance. This chapter describes the techniques used to produce well-differentiated culture human airway epithelia cells for in situ and in vivo studies of mucus and mucociliary clearance.

2. Materials

2.1. Phosphate-Buffered Saline

80 g NaCl, 2.0 g KCl, 20.2 g $Na_2HPO_4 \cdot 7H_2O$, 2.0 g KH_2PO_4 in 10 L of ddH_2O.

2.2. Laboratory of Human Carcinogenesis Basal Medium

Laboratory of human carcinogenesis (LHC) basal medium can be purchased for small-scale production (Invitrogen, cat. no. 12680-013). For large-scale production, LHC basal medium powder can be specially ordered from Sigma-Aldrich (see Note 1). In a 5 L volumetric flask, dissolve the 5 L prepackaged mixture in 4 L of dH_2O. Add 5 g $NaHCO_3$, 150 mL of 200 mM L-glutamine (Sigma-Aldrich, cat. no. G7513), stir, and adjust pH to 7.2–7.4. Bring total volume up to 5 L. Filter into sterile 500 mL bottles using 0.2-μm Vacucap (VWR, West Chester, PA, cat. no. 28143-315). Store at 4°C.

2.3. Bronchial Epithelial Growth Medium

Bronchial epithelial growth medium (BEGM) is prepared using 100% LHC basal medium. For small-scale production, thawed additives are dispensed into media in the top of a bottle top filter unit. Note that some additives are not 1,000× stock solutions. For media made with homemade bovine pituitary extract (BPE) that is difficult to filter, use a 0.4-μm filter unit. For commercial BPE, a 0.2-μm filter is acceptable. To add homemade BPE to media, thawed BPE aliquots are first centrifuged at 1,500 × *g* for 10 min to remove debris and cryoprecipitate, prefiltered through a 0.8-μm syringe filter, and added to the media just as the last few milliliters of media are being filter-sterilized.

2.4. Airway Liquid Interface Medium (see Note 2)

Airway liquid interface (ALI) medium uses a 50–50 mixture of Dulbecco's modified Eagle medium (DMEM) (Gibco, Carlsbad, CA, cat. no. 11995-065) and LHC basal medium as its base. Additives are thawed and dispensed into base media at the proper

concentrations. ALI medium is then filtered according to small- or large-scale production. Note that some additives are not 1,000× stock solutions and that base ALI medium omits gentamicin and amphotericin.

2.5. Stock Additives for ALI and BEGM

Additives for media are filtered using 0.2 μM filters (unless product is sterile) and aliquots are stored at –20°C for up to 6 months.

1. Bovine serum albumin (BSA) (300 × 150 mg/mL): Add PBS directly to the BSA (Sigma-Aldrich, St. Louis, MO, cat. no. A7638) container to yield a concentration >150 mg/mL. Gently rock bottle at 4°C for 2–3 h until BSA is dissolved. Transfer to graduated cylinder and set volume to yield a final concentration of 150 mg/mL.
2. BPE (100× stock): Commercially prepared BPE is available from Sigma-Aldrich (cat. no. P1427) and is handled per manufacturer's instructions. It is used at a final concentration of 10 μg/mL. BPE can also be prepared from mature bovine whole pituitaries (Pel Freeze, Rogers, AR, cat. no. 57133-2). Thaw bovine pituitaries, drain, and rinse with chilled 4°C PBS. Add 2 mL of chilled PBS per gram of tissue. In a cold room, mince tissue in a Waring 2-speed commercial blender (Fisher Scientific, Pittsburgh, PA, cat. no. 14-509-17) at low speed for 1 min and then at high speed for 10 min. Aliquot suspension and centrifuge at 2,500 × *g* for 10 min at 4°C. Collect supernatant and centrifuge again at 10,000 × *g* for 10 min. Harvest the final BPE supernatant. Homemade BPE is difficult to filter and needs to be filtered during media preparation as described.
3. Insulin (5 mg/mL; 1,000× stock): Dissolve insulin (Sigma-Aldrich, cat. no. I6634) in 0.9N HCl.
4. Transferrin (10 mg/mL; 1,000× stock): Reconstitute transferrin, human-holo, natural (Sigma-Aldrich, cat. no. T0665) in PBS.
5. Hydrocortisone (0.072 mg/mL; 1,000× stock): Reconstitute hydrocortisone (Sigma-Aldrich, cat. no. H0396) in distilled water (dH_2O).
6. Triiodothyronine (0.0067 mg/mL; 1,000× stock): Dissolve triiodothyronine (Sigma-Aldrich, cat. no. T6397) in 0.001 M NaOH.
7. Epinephrine (0.6 mg/mL; 1,000× stock): Dissolve epinephrine (Sigma-Aldrich, cat. no. E4642) in 0.01N HCl.
8. Epidermal growth factor for BEGM, 50,000× for ALI (25 μg/mL; 1,000× stock): Dissolve human recombinant, culture-grade EGF (Atlanta Biological, Norcross, GA, cat. no. C100) in PBS.
9. Retinoic acid (concentrated stock = 1×10^{-3} M in absolute ethanol, 1,000× stock = 5×10^{-5} M in PBS with 1% BSA): Retinoic

acid (RA) is soluble in ethanol and is light sensitive. First, make a concentrated ethanol stock by dissolving 12.0 mg of RA (Sigma-Aldrich, cat. no. R2625) in 40 mL of 100% ethanol. Store in foil wrapped tubes at –20°C. To prepare the 1,000× stock, first confirm the RA concentration of the ethanol stock by diluting it 1/100 in absolute ethanol. Read the absorbance at 350 nm using a spectrophotometer and a 1 cm light path quartz cuvette, zeroed with a 100% ethanol solution. The molar extinction coefficient of RA in ethanol equals 45,000 at 350 nm. Thus, the absorbance of the diluted stock should equal 0.45. RA with absorbance readings below 0.18 should be discarded. If the absorbance equals 0.45, add 3 mL of 1×10^{-3} M ethanol stock solution to 53 mL PBS and add 4.0 mL of BSA 150 mg/mL stock (s). For absorbance values less than 0.45, calculate the needed volume of ethanol stock as 1.35/absorbance and adjust the PBS volume appropriately.

10. Phosphorylethanolamine (70 mg/mL; 1,000× stock): Dissolve phosphorylethanolamine (Sigma-Aldrich, cat. no. P0503) in PBS.
11. Ethanolamine (30 μL/mL; 1,000× stock): Dilute ethanolamine (Sigma-Aldrich, cat. no. E0135) in PBS.
12. Zinc solution (0.863 mg/mL; 1,000× stock): Dissolve zinc sulfate (Sigma-Aldrich, cat. no. Z0251) in dH_2O. Store at room temperature.
13. Penicillin–streptomycin (100,000 U/mL Pen and 100 mg/mL Strep; 1,000× stock): Dissolve penicillin-G sodium (Sigma-Aldrich, cat. no. P3032) and streptomycin sulfate (Sigma-Aldrich, cat. no. S9137) in dH_2O for a final concentration of (100,000 U/mL and 100 mg/mL, respectively).
14. Gentamicin (50 mg/mL; 1,000× stock): Sigma-Aldrich, cat. no. G1397. Store at 4°C. For BEGM only.
15. Amphotericin B (250 μg/mL; 1,000× stock): Sigma-Aldrich, cat. no. A2942. Used for BEGM only.
16. Salt Stock solution (1,000× stock): Combine 0.42 g ferrous sulfate (Sigma-Aldrich, cat. no. F8048), 122.0 g magnesium chloride (J.T. Baker, Phillipsburg, NJ, cat. no. 2444), 16.17 g calcium chloride-dihydrate (Sigma-Aldrich, cat. no. C3881), and 5.0 mL hydrochloric acid (HCl) to 800 mL of dH_2O in a volumetric flask. Stir and bring total volume up to 1 L. Store at room temperature.
17. Trace elements solution (1,000× stock): Prepare seven separate 100 mL stock solutions. Using a volumetric 1-L flask, fill to the 1-L mark with dH_2O. Remove 8 mL of dH_2O. Add 1.0 mL of each stock solution and 1.0 mL of HCl (conc.). Store at room temperature.

3. Methods

3.1. Establishment of Respiratory Air–Liquid Interface Cultures

HBE cell culture systems serve as a good in-vitro model system of the airway epithelium. As cultures can be developed from both normal and diseased (CF/COPD) lungs, they allow for a direct comparison of differences engendered by lung disease. The HBE cell culture systems have been shown to produce mucus, maintain a 7 μm periciliary layer (PCL) (1), and facilitate coordinated transport of the mucus layer (2, 3). Biophysically, these cell culture systems have been used to assay the effect of shear stress on volume regulation and ATP release (4, 5) as well measuring the force of both individual (6) and patches of cilia (7).The methodology for isolating airway cells from donor lungs and culturing HBE cultures, well described by Fulcher et al. (8), is briefly summarized below. In addition to procuring HBE cells from human donor lungs, there are commercially available sources of human airway epithelial cells.

1. HBE cells are extracted from lungs from potential organ donors that are frequently unsuitable for transplantation but useful for research. HBE cells are also obtained from excess surgical pathology specimens procured through cooperating surgeons and pathologists using protocols in accordance with relevant regulations. In both cases, they are transported to the laboratory in a container of physiologic solution on wet ice, and used within 24 h after removal.
2. Airways from these lungs are manually dissected and all connective tissue removed. Cleaned segments are washed in phosphate-buffered saline (PBS) and placed in a DMEM solution containing antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin), proteases (0.1% (w/v), type XIV), and DNases (1 mg/mL) and gently rocked at 4°C overnight. The epithelial layer is gently scraped and the cell supernatant is harvested. To end the dissociation process, a final concentration of 10% (v/v) fetal bovine serum is added. Cells are centrifuged at $500 \times g$ for 5 min at 4°C. The pellet is resuspended in F-12 medium and counted using a hemocytometer.
3. Cells are plated on Type I/III collagen-coated plastic dishes ($2–6 \times 10^6$ cells per 10 mm dish) in an antibiotic supplemented (100 U/mL penicillin and 100 mg/mL streptomycin) BEGM (8) for 3–5 days.
4. Primary cells are passaged at 70–90% confluence. Rinse cells twice with PBS and add 3 mL of Trypsin (0.1% (w/v))/EDTA (1 mM). Incubate for 5–10 min at 37°C to free cells from the culture dish. Gently tap dish after the incubation and harvest the cells and transfer to a tube containing 3 mL of STI media

(1 mg/mL soybean trypsin inhibitor in F12 culture medium) on ice. Repeat if necessary.

5. Pool harvested cells and centrifuged at $500 \times g$ for 5 min at 4°C. The supernatant is then aspirated and the pellet is resuspended in ALI media. The number of viable cells obtained are then determined using trypan blue exclusion or similar technique. At this point, cells can be plated on permeable supports or cryopreserved using an F-12-based freezing media (containing 30 mM HEPES, 10% (v/v) FBS, and 10% (v/v) DMSO).
6. The isolated HBE cells are plated onto collagen Type IV (human placental, Sigma) coated porous supports (Transwell Clear, Corning) at a density of $1.0–2.5 \times 10^5$ cells/cm^2.
7. To facilitate the formation of air–liquid interface cultures, remove the apical medium and rinse the surface of the porous support with gentle washings with PBS (0.5 mL/cm^2) after 24 h following initial plating and daily over the subsequent 7–10 days until confluent.
8. The media in the basolateral compartment is replaced with the appropriate volume of medium as specified for the support. Change the medium every other day until the cells become confluent and generate an air–liquid interface.
9. During the normal maintenance, HBE cultures need to be feed with fresh ALI every 2–3 days. Cultures should receive an apical PBS wash once a week to remove accumulating mucus.
10. After 4–6 weeks, the cultures will differentiate into ciliated cells and mucus-producing goblet cells. While the time to full differentiation is often variable between preparations, this stage can be monitored by observing the presence of beating cilia and the production of a mucus layer (Fig. 1).
11. With proper care, air–liquid interface HBE cultures can be maintained for a period of several months.

3.2. Collection of HBE Mucus for In Vitro Studies

By collection and reconcentrating these washings, mucus from HBE cell culture can be "made to order," thereby serving as a physiologically relevant model system for in-vitro mucus experiments. These systems have been shown to produce mucus, maintain a 7 μm PCL (1), and facilitate coordinate transport of the mucus layer (2, 3). Further, mucus collected from these model systems has been shown to be chemically similar to normal sputum (10), with roughly 75% overlap in the proteins detected in both specimens. Finally, the physical properties of HBE mucus versus % solids (a marker of mucus concentration) fall well within the range of those demonstrated by sputum, while being much more predictable (Table 1, Fig. 2). Further, mucus harvested from the HBE system and concentrated to normal (2.5% solids) and CF-like (8% solids) has been shown to be physiologically relevant model system

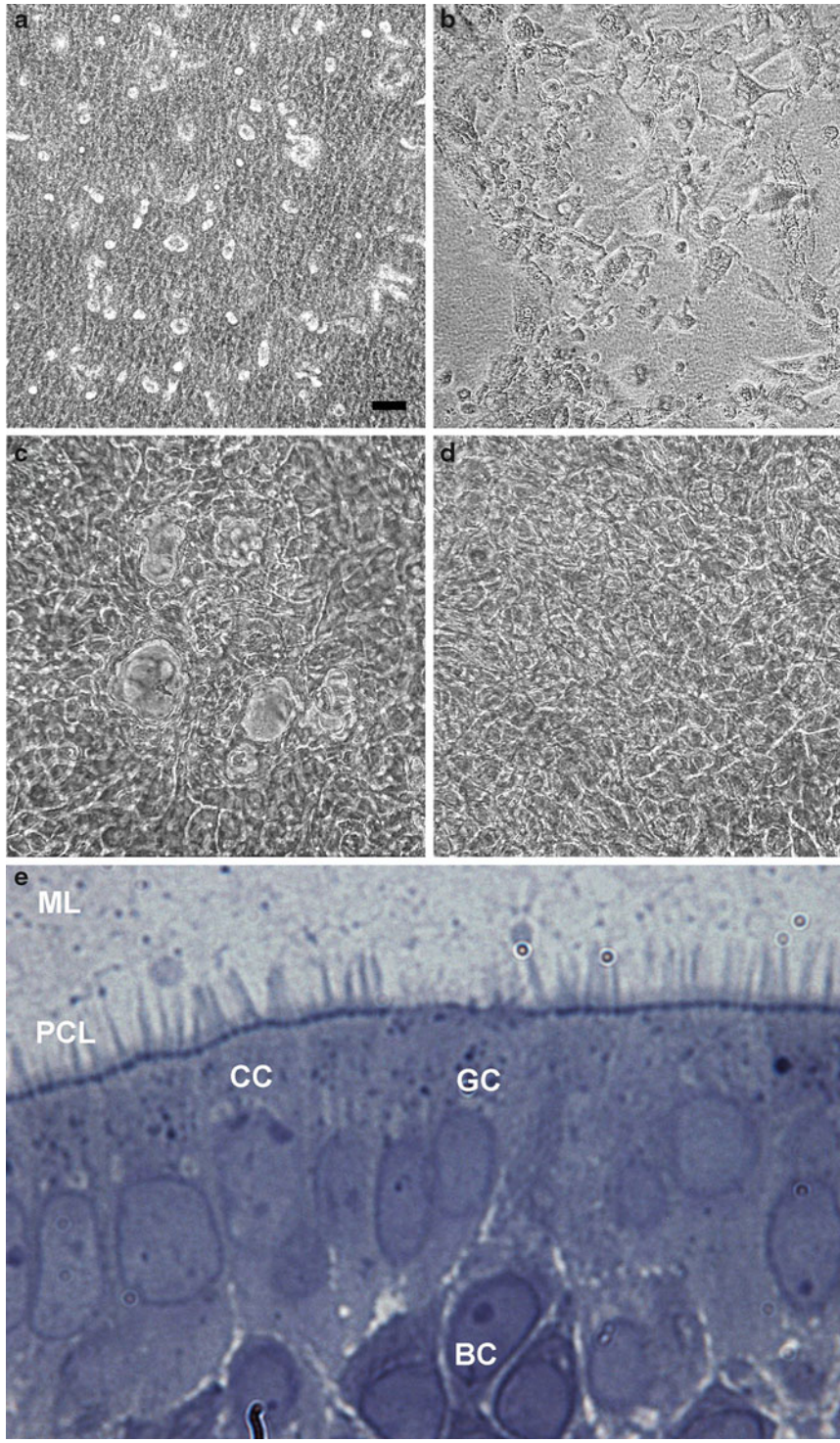

Fig. 1. Growth of Primary HBE cell cultures. Representative XY images of (**a**) freshly plated cells, (**b**) cells at 3–5 days, spreading across the culture support, (**c**) culture at day 10–12, nearly confluent with only small holes left to be filled in, and (**d**) culture at day 14, fully confluent. Bar = 20 μm. (**e**) XZ cross-section of a fully differentiated HBE culture (at 28 days) fixed with Os-PFC (to preserve the overlying mucus layer) and stained with Richardson's (1, 9). *ML* mucus layer; *PCL* periciliary layer; *CC* ciliated cell; *GC* goblet (mucus secreting) cell; and *BC* basal cell.

Table 1
Published rheological properties of human mucus and sputum

References	η_0 (Pa·s)	G_{inf} (Pa)
Puchelle (12), human (recurrent bronchitis)	24.8	6.2
Puchelle (12), human (mild chronic bronchitis)	11.4	0.76
Puchelle (12), human (severe chronic bronchitis)	12.5	0.87
Baconnais (13), human	0.71	N/A
Baconnais (13), human (CF)	0.16	N/A
Puchelle (14), human	24.7	1.7
Dawson (15), human (CF)	60	15.5
Hill (3), 2.5% (w/w) human cell culture mucus	0.12	0.87
Hill (3), 8% (w/w) human cell culture mucus	3.3	4.7

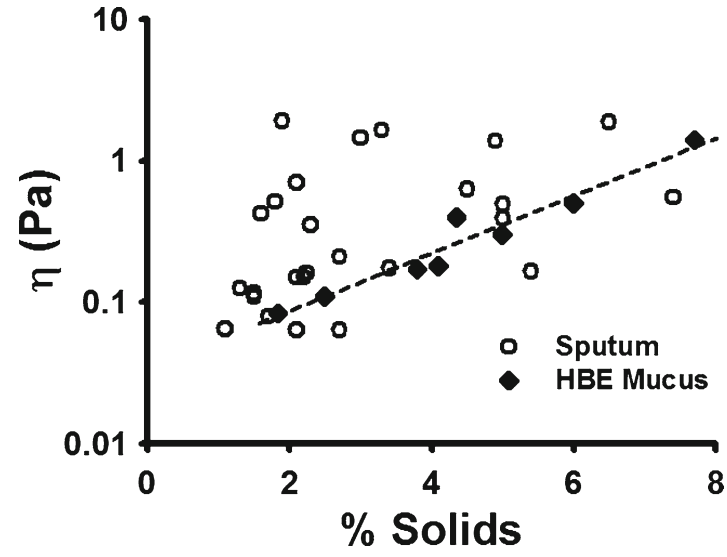

Fig. 2. Comparison of the complex viscosity (η) of HBE mucus (*filled diamonds*) versus sputum samples obtained from various individuals (*open circles*).

for predicting the affect of the biophysical properties of the mucus layer on bacterial biofilm formation (3). Below we describe the methodology used in harvesting and reconstituting mucus from HBE cultures.

1. As a part of routine maintenance, cultures are washed with ~1.5 μL PBS/mm² of culture area (i.e., ~1,000 μL per 30 mm diameter dish, ~150–200 μL per 12 mm diameter dish).

2. Once added, PBS is left on the culture for 30 min at 37°C before removal. For cultures that demonstrate heavy mucus production, a second 30 min wash can be beneficial for mucus harvesting. Care must be taken to ensure that cells are not disturbed during collection of the lavage solution.
3. Because these cell cultures can produce mucus for up to 6 months, this procedure can be performed repeatedly on a weekly or biweekly basis. To maintain sterility, it is recommended to perform the lavage procedure in a laminar flow hood using sterile solutions.
4. Once collected, cells and larger debris are collected by a low-speed centrifugation ($300 \times g$ for 5 min). The pellet is discarded and the washings are pooled and stored at 4°C until sufficient volume is obtained (see Note 3).
5. When one has harvest ~1 L of pooled washing the concentration step can begin. Here, washings are loaded into 3,800 Da molecular weight cut off dialysis bags and loaded into a container filled with polymer absorbent (Spectra/Gel) for 1–5 days at 4°C to concentrate the mucus. Absorbent is replaced daily.
6. Midway through concentration, mucus is next dialyzed against PBS containing 500 μM $MgCl_2$, and 800 μM $CaCl_2$ at 4°C to establish the proper salt balance (3).
7. Once mucus has reached its final concentration (typically when 200- to 1,000-fold reduction in volume is achieved), it is once again dialyzed against PBS.
8. The concentration of the final product is determined by placing a sample aliquot (~50–100 μL) on a preweighed piece of foil which is then placed in an 80°C oven overnight to dry the sample. The final concentration of solid material (% solids) is then determined.

Once the dry weight of the sample is determined, the concentration of mucins in mucus is assessed by differential refractometry (11). A 500 μL sample is chromatographed on a Sephacryl S-1000 column (Amersham Pharmacia) and eluted with 200 mM sodium chloride/10 mM EDTA at a flow rate of 0.5 mL/min. The column effluent is passed through an in-line Dawn EOS laser photometer coupled to a Wyatt/Optilab DSP inferometric refractometer to measure light scattering and sample concentration, respectively. The concentration of the mucin is calculated by integrating the refractive index peak associated with the material eluted in the void volume of the column and employing a value for the refractive-index increment (dn/dc) of 0.165 mL/g, which has been measured previously at 650 nm. The reproducibility of this procedure is typically within 5%. The total protein content of a given mucus sample is determined by similar methods, but with a Sephadex

G-25 column and a $dn/dc = 0.170$. The non-mucin content of the mucus sample is then determined by subtraction of the mucin content from the total protein (mucin and non-mucin) content.

3.3. In Vitro Measures of Mucociliary Transport

The lung is continually under assault by pathological and noxious materials inhaled during every breath that we take. Lung health is maintained because these inhaled particles and pathogens are trapped within a blanket of mucus and swept from the lung by a continuous flow of mucus generated by the beating of cilia. The failure of mucus clearance in the environmentally damaged lungs, in COPD or asthma, leads to severe health problems as the lung tissue is destroyed by the inflammation response to microbial infections that cannot be cleared.

While insight into airway surface liquid and cilia beat parameters (height regulation, mucus secretion, beat frequency, etc.) have been obtained, the understanding of the full phenomena of mucus clearance has remained elusive. Key to understanding potential links between genetic disorders and failure of mucus transport may lie in the two essential components of the mucociliary clearance system, cilia and mucus. However, animal models have been the only source of true clearance models. This is due to the lack of an established methodology for promoting coordination of beating cilia across the length of a cell culture system in a suitable geometry.

One of the key advantages of this well-differentiated human airway culture system is the ability to generate cultures with coordinated cilia beating that produces vectoral mucus transport. We have observed that anywhere from 10 to 25% of well-differentiated HBE cell cultures develop millimeter sized patches in which the cilia spontaneously coordinate their beating in a circular pattern, yielding rotational (the so-called "mucus hurricanes"), reflecting the circular boundary of the culture support for flowing mucus (see Figure 3, below). The protocol below is designed to facilitate the production of cell cultures with transporting mucus and the approach used by our laboratory to measure the rate of transport.

1. These studies rely on the use of well-differentiated HBE cultures prepared using the approach listed in the section above. To facilitate differentiation into well-ciliated cultures necessary for mucus transport studies, initial cell seeding densities as high as $250–500 \times 10^3$ cells/cm^2 can be used.
2. A primary requirement for producing cultures which are capable of generating transporting mucus is the degree of ciliation. Typically, cultures with >70% of the surface area with beating cilia are required (see Note 4).
3. The mucus layer that is produced in HBE cultures is not "cleared" as it is in vivo, due to the constraints of the fixed Transwell culture support. The result is a continual accumulation of mucus. In order to maintain a "flowable" mucus layer during ciliogenesis, it is necessary to remove accumulated

mucus on a frequent basis. The next few steps are designed to maximize the chances of generating cultures with rotational transport.

(a) First, the accumulated mucus is regularly removed from the luminal surface of the HBE cell cultures. This is accomplished by three incubations with an isotonic salt solution (e.g., PBS) for 10 min at 37°C. At the completion of the washing procedure, any remaining fluid is suctioned from the apical surface and the cultures returned to the incubator. Typically, this procedure is performed three times per week following confluence.

(b) Second, because mucus layer on the surface of an HBE culture is very thin (typically between 10 and 50 μm, depending on washing interval), and therefore very small volumes (microliters), it is very susceptible to dehydration, even in incubators at 90% humidity (see Note 5).

4. Following culture confluence, it can take anywhere from 3 to 5 weeks for cultures to reach >70% surface area ciliation.
5. As noted above, rotational coordination of cilia typically occur in less than 25% of all well-ciliated cultures. Therefore, prior to transport studies it is necessary to prescreen for cultures exhibiting rotational transport. This can be done by observing the movement of cellular detritus following the addition of a small volume of fluid (~20 μL/cm^2) to the apical surface in the absence of any exogenous label. Only cultures which exhibit rotational mucus transport are used for the transport studies.
6. Depending on the type of study being performed, endogenous mucus is allowed to accumulate for 1–3 weeks prior to the transport assay. During this time, only the basolateral media is changed.
7. To better visualize transport of mucus during the experiments, 0.5–1.0 μm fluorescent microspheres (FluoSpheres, Invitrogen) are added to the luminal surface 24 h prior to the transport studies (see Note 6). To resolve single microspheres, the microspheres are diluted 1:10,000 in PBS and added to the cultures (at 20 mL/cm^2). The excess fluid is absorbed over the 24 h. The rate of mucus transport during the experiment is determined from time-lapse images of the microspheres using a low power (10×) fluorescent microscope. The microspheres appear as streaks when taken as 2–5 s exposure images (Fig. 3a).
8. During the experiment, images are taken at desired time points and/or after the addition of mucus altering agents, depending on the experiment being performed.
9. Because the mucus layer is quite thin (10s of micrometer), the mucus layer is susceptible to evaporation during data analysis which can significantly alter the transport measurements. Such evaporation can be prevented by the addition of perfluorocarbon

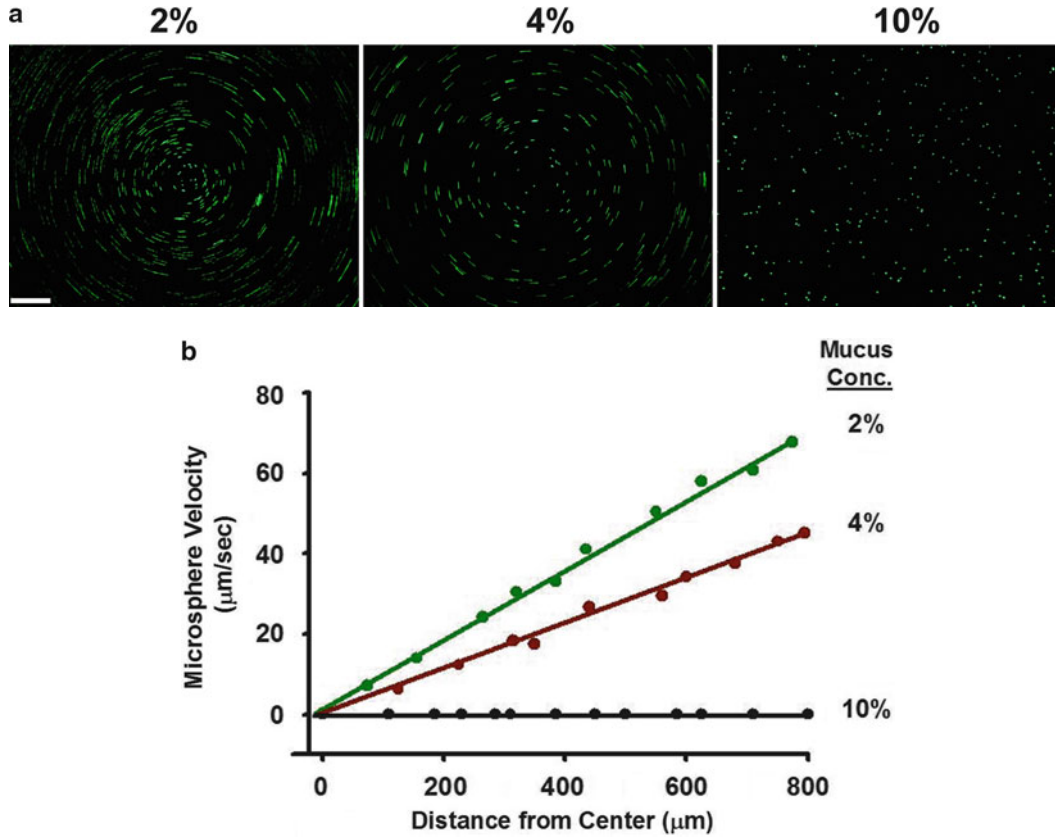

Fig. 3. Determination of mucus transport rates in HBE cultures. (**a**) Example 2-s time-lapse images of 1.0 μm fluorescent microspheres (1.0 μm, *green*) in cultures with 2, 4, and 10% mucus (% solids). (**b**) Plot of microsphere velocity (μm/s).

(such as Fluorinert FC-3283, 3M) to the luminal surface (100 μL volume/cm^2). This can be done with no noticeable alteration in transport rate and can be easily removed to add additional test agents.

10. Because the coordination of mucus transport is rotational, the microsphere-streaks in the center of the culture will have a different length than at the periphery. Image analysis software (such as Image J, NIH) can be used to determine the length of the streaks.
11. Linear regression analysis of the length of the streak plotted versus the distance from the center of the transporting mucus (Fig. 3b) results in an angular velocity of the beads. For general comparison, transport rates can be normalized at a given distance from the center of rotation (e.g., 0.5 mm).
12. In the example in Fig. 3a, b, the transport rate versus distance is shown for three different mucus concentration ranging from normal airway mucus (~2% solids) to cystic fibrosis-like (10% solids), where mucus transport ceases (17). Here, the time-lapse image of the microspheres appears as spots rather than streaks.

4. Notes

1. If anticipated usage of LHC basal medium exceeds 550 L/year, powdered stock can be custom ordered from Sigma-Aldrich.
2. For more details regarding LCH, ALI, and BGEM Media, see Fulcher et al. (8).
3. Degradation of mucins during storage is a potential shortcoming of this mucus preparation methodology. In general, we observe a roughly 25% decrease in the molecular weight and radius of gyration of mucins, as determined by dynamic light scattering, over a 1 month period. To prevent this effect from becoming larger, it is recommended that mucus be prepared from pooled washings that have been stored less than 1 month.
4. During differentiation into ciliated cells and associated ciliogenesis, coordination of cilia beating pattern is necessary in the formation into mucus transporting cultures. While we do not know how coordination of cilia is formed, recent work in fish and amphibian development indicates that ciliary orientation at the apical membrane of vertebrate ciliated cells is a two-step process beginning with tissue patterning, followed by a flow-based refinement phase in which the effective stroke of the beat aligns tightly with the direction of fluid flow (16).
5. To maintain proper mucus flow during ciliogenesis, it is necessary to use well-humidified incubator, which can be accomplished by having multiple water pans in the incubator, as the humidity is proportional to the surface are of the water exposed to the air. Using this approach, we observe near saturating conditions. It is useful to regularly measure the humidity in an incubator using a hygrometer.
6. Alternatively, small volumes (nanoliter) containing microspheres can be aerosolized onto the culture using a ultrasonic nebulizer (Aeroneb, Aerogen) to avoid the addition of a relatively large volume (microliter) which might alter mucus properties.

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