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Well-Differentiated Human Airway Epithelial Cell Cultures

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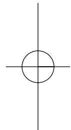
1. Introduction

The airway epithelium occupies a critical environmental interface, protecting the host from a wide variety of inhaled insults, including chemical and particulate pollutants and pathogens. The coordinated regulation of ion and water transport, mucous secretion, and cilia beating underlies mucociliary clearance. Physical trapping and removal of harmful substances, in combination with baseline or inducible secretion of antimicrobial factors, antioxidants, and protease inhibitors and recruitment of nonspecific inflammatory cells (neutrophils, monocytes), constitutes airway innate host defense.



Cystic fibrosis (CF) is a genetic disease in which impaired innate host defense results in repeated, severe airway infections. Airway epithelial cell cultures (AECCs) have been integral to our understanding of CF pathogenesis. Because CF is a monogenic, recessive, loss-of-function disorder, it is theoretically curable by gene therapy. However, the promise of gene therapy has not been fulfilled, mainly owing to vector inefficiency and safety concerns. AECCs will be an important tool for advancing gene therapy.

In addition to its key functional role in innate immunity, the airway epithelium modulates inflammation and adaptive immunity (dendritic cell function, specific T and B cells). Alterations in both innate and acquired immune function induced by the epithelium may contribute to the pathophysiology of asthma and chronic bronchitis. Many aspects of these profoundly important diseases remain poorly understood and AECCs will facilitate studies of both basic pathophysiology and development of novel therapies.

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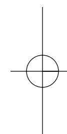
The epithelium itself responds to injury and becomes modified during the progression of disease. The changes range from mild and transient cytopathology to epithelial hyperplasia or metaplasia, and ultimately, in some cases, malignant transformation. The bronchial epithelium is the source of the world's most prevalent lethal cancer, caused principally, but not exclusively, by exposure to tobacco products. AECCs enable analysis of epithelial growth and differentiation and may prove useful for studies relating to prevention, detection, monitoring, and treatment of lung cancer.

Finally, a significant and growing number of drugs are administered as aerosols. Transepithelial transport properties, as well as positive and negative effects on host cells, are key parameters requiring assessment. Thus, human AECCs are integral to the study of basic and applied aspects of airway biology, disease, and therapy.

1.1. Historical Perspective and Milestones

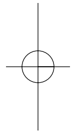
Epithelial cell cultures have been created from the human airway for more than 20 yr ***(1)***. In the original method, finely minced airway tissue fragments were explanted and epithelial cells were harvested as outgrowths. Alternatively, protease dissociation creates suspensions of free epithelial cells ***(2)***. The initial cell harvest usually contains some nonepithelial cells. Morphologic characteristics during passage in selective medium and immunostaining for cytokeratin have traditionally been used for cellular identification. Primary airway epithelial cells on plastic dishes can be repeatedly passaged. On plastic dishes, the cells assume a poorly differentiated, squamous phenotype. However, when freshly harvested or passaged primary airway epithelial cells are cultured under conditions enabling cellular polarization, a dramatic phenotypic conversion occurs, enabling the cells to more closely recapitulate their normal in vivo morphology. This was first recognized when animal or human airway epithelial cells were inoculated into devitalized tracheal or intestinal tubes and then implanted subcutaneously in compatible hosts ***(3,4)***. A similar effect occurs in vitro when masses of cells assume a three-dimensional spheroidal shape ***(5)*** or if cells are grown on or within thick collagen gels ***(6,7)***. However, the most widely utilized system enabling the cells to undergo mucociliary differentiation involves growing them on porous supports at an air–liquid interface, first shown by Whitcutt, Adler, and Wu ***(8)***. These cultures demonstrate vectorial mucus transport ***(9,10)***, high resistance to gene therapy vectors ***(11)***, and cell-type-specific infection by viruses ***(12)***, functions that cannot be studied using undifferentiated cells on plastic. The complex process of airway epithelial differentiation involves cell–matrix and cell–cell interactions, differentiation of mucous and goblet cells, and acquisition of characteristic epithelial ion transport properties. Numerous genes and proteins are induced during differentia-



tion, including those characteristically present in secretory and ciliated cells ***(13,14)***. Retinoic acid is essential to suppress or reverse squamous metaplasia in culture ***(15)***. Much remains to be learned about the complex program regulating mucociliary differentiation and phenotypic modulation of the airway epithelium.

1.2. Summary and Purpose

Compared to undifferentiated cells on plastic, human airway epithelial cell cultures maintained at an air–liquid interface (ALI) represent a quantum leap toward the in vivo biology, and are an excellent model to probe airway epithelial function. Although they have been used for studies too numerous to cite here, the technical requirements, financial commitment, and experimental limitations inhibit their use in many laboratories. The approximate cost of passage 1 airway epithelial cells from a commercial supplier is $569 (U.S. dollars) per 0.5×10^6 cells. As a point of reference, expansion and subculture of this number of cells would typically generate 25 passage 2 ALI cultures 12 mm in diameter. An alternative is direct procurement of cells from human tissues, but this requires establishment of working relationships with surgeons and pathologists and compliance with appropriate regulations. Furthermore, the media is complex, with expensive individual components. The University of North Carolina established a Tissue Procurement and Cell Culture Core in 1984, under the auspices of the Cystic Fibrosis Foundation, to provide standardized cell cultures. From 1984 to 2003, the Core prepared cells from more than 6030 human tissue specimens, adopting new technologies to extend research capabilities. The purpose of this chapter is to share our detailed protocols and "tricks of the trade," thus, enabling others to overcome barriers toward using this relevant cell culture model.



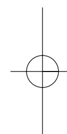
For many years, the dogma was that only fresh primary cells seeded at high density could reliably form well-differentiated cultures, and that differentiation was dependent on a proprietary cell-culture supplement, Ultroser G (Biosepra SA, Cergy-Sainte-Christophe, France). U.S. importation of Ultroser G requires a permit from the Department of Agriculture. A breakthrough paper published by Gray et al. ***(16)*** showed successful differentiation of subcultured human airway epithelial cells with no proprietary reagents. These procedures significantly enhance the ability to study differentiation-dependent functions and have also increased the number and area of well-differentiated cultures produced from each tissue sample. The ability to store primary cells as frozen stocks stabilizes cell availability, enables the simultaneous production of cultures at different stages of maturity from the same patient sample, allows repeat experiments with the same specimen, and permits simultaneous performance of experiments with replicate cultures derived from multiple patients. The proce-

dures detailed below represent an extension of the original methods given by Lechner and Laveck ***(17)***, strongly influenced by the methodology of Gray et al. ***(16)***, which evolved during years of practical experience in our laboratory.

2. Materials

2.1. Tissue Procurement

Airway epithelial cells can be extracted from nasal turbinate or polyp specimens, trachea, or bronchi procured locally through cooperation of surgeons and pathologists in accordance with relevant institutional, local, and national regulations. Surgical nasal specimens not requiring histopathologic examination or excess nonaffected portions of lung tissue after gross examination by a pathologist, such as bronchi after lobectomy or pneumonectomy for lung cancer, are common sources. These are transported to the laboratory in a specimen cup on wet ice containing a physiologic solution [sterile saline, phosphate-buffered saline (PBS), lactated Ringer's solution, or tissue-culture medium]. Lungs from potential organ donors are frequently unsuitable for transplantation as a result of age, smoking history, or acute injury such as aspiration, pulmonary edema, or pneumonia, but are useful for research. These can be obtained by development of appropriate protocols with federally designated organ procurement agencies that normally oversee collection and distribution of donated organs. In the U.S., nonprofit organizations such as the National Disease Research Interchange (www.ndriresource.org) facilitate provision of human biomaterials for research. When establishing protocols with organ suppliers, the laboratory must set criteria for organ acceptability (*see* **Note 1**). Lung tissues may be retrieved at time of autopsy but, in our experience, removal within several hours of time of death is necessary. Finally, one can circumvent the need for tissue procurement by purchasing human airway epithelial cells (www.cambrex.com).



2.2. Media

Two closely related media are used for culturing airway epithelial cells. Bronchial epithelial growth medium (BEGM) is used when the initial cell harvests are plated on collagen-coated plastic dishes or to expand passaged cells on plastic. ALI medium is used to support growth and differentiation on porous supports. BEGM composition is given in **Table 1** and the differences between BEGM and ALI are illustrated in **Table 2**. All base media and additives can be purchased commercially (*see* www.biosource.com for LHC basal media and additives and as specified below for others). Bovine pituitary extract (BPE) can be purchased commercially or made from mature bovine pituitaries. The decision whether to make BPE depends on the volume of media needed and, thus, savings realized.

Table 1
BEGM and ALI Composition

Additive		Final Concentration In Media	Company	Cat. no.
Bovine serum albumin		0.5 mg/mL	Sigma-Aldrich	A7638
Bovine pituitary extract, homemade[a]		0.8% (v/v)	Pel Freeze	57133-2
Bovine pituitary extract, commerical[a]		10 µg/mL	Sigma-Aldrich	P1476
Insulin		0.87 µ*M*	Sigma-Aldrich	I6634
Transferrin		0.125 µ*M*	Sigma-Aldrich	T0665
Hydrocortisone		0.1 µ*M*	Sigma-Aldrich	H0396
Triiodothyronine		0.01 µ*M*	Sigma-Aldrich	T6397
Epinephrine		2.7 µ*M*	Sigma-Aldrich	E4642
Epidermal growth factor		25 ng/mL—BEGM 0.50 ng/mL—ALI	Atlanta Biological	C100
Retinoic acid		5×10^{-8} *M*	Sigma-Aldrich	R2625
Phosphorylethanolamine		0.5 µ*M*	Sigma-Aldrich	P0503
Ethanolamine		0.5 µ*M*	Sigma-Aldrich	E0135
Zinc sulfate		3.0 µ*M*	Sigma-Aldrich	Z0251
Penicillin G sulfate		100 U/mL	Sigma-Aldrich	P3032
Streptomycin sulfate		100 µg/mL	Sigma-Aldrich	S9137
Gentamicin[b]		50 µg/mL	Sigma-Aldrich	G1397
Amphotericin[b]		0.25 µg/mL	Sigma-Aldrich	A2942
Stock 4	Ferrous sulfate	1.5×10^{-6} *M*	Sigma-Aldrich	F8048
	Magnesium chloride	6×10^{-4} *M*	J.T. Baker	2444
	Calcium chloride	1.1×10^{-4} *M*	Sigma-Aldrich	C3881
Trace Elements	Selenium	3.0 µ*M*	Sigma-Aldrich	S5261
	Manganese	0.1 µ*M*	Sigma-Aldrich	M5005
	Silicone	50 µ*M*	Sigma-Aldrich	S5904
	Molybdenum	0.1 µ*M*	Sigma-Aldrich	M1019
	Vanadium	0.5 µ*M*	Sigma-Aldrich	A1183
	Nickel sulfate	0.1 m*M*	Sigma-Aldrich	N4882
	Tin	0.05 µ*M*	Sigma-Aldrich	S9262

[a]*See* **Subheading 2.3.2.**
[b]Not in ALI.

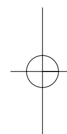
Table 2
Differences Between ALI and BEGM Medium

	ALI	BEGM
Base media	LHC Basal : DMEM-H 50 : 50	LHC Basal 100%
Base Antibiotics	Pen/Strep (100 U/mL/100 μg/mL)	Pen/Strep (100 U/mL/100 μg/mL) Gentamicin 50 μg/mL Amphotericin 0.25 μg/mL
EGF	0.50 ng/mL	25 ng/mL
$CaCl_2$	1.0 m*M*	0.11 m*M*

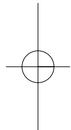
2.3. Stock Additives for ALI and BEGM

Additives for media are filtered using 0.2-μ*M* filters (unless product is sterile) and aliquots are stored at –20°C for up to 6 mo.

1. Bovine serum albumin (BSA) (300X 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. 100X BPE: 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 2500*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 in **Subheading 2.4.2.**
3. 1000X insulin (5 mg/mL): Dissolve insulin (Sigma-Aldrich, cat. no. I6634) in 0.9 *N* HCl.
4. 1000X transferrin (10 mg/mL): Reconstitute transferrin, human-holo, natural (Sigma-Aldrich, cat. no. T0665) in PBS.
5. 1000X hydrocortisone (0.072 mg/mL): Reconstitute hydrocortisone (Sigma-Aldrich, cat. no. H0396) in distilled water (dH_2O).
6. 1000X triiodothyronine (0.0067 mg/mL): Dissolve triiodothyronine (Sigma-Aldrich, cat. no. T6397) in 0.001 *M* NaOH.
7. 1000X epinephrine (0.6 mg/mL): Dissolve epinephrine (Sigma-Aldrich, cat. no. E4642) in 0.01 *N* HCl.



8. 1000X epidermal growth factor for BEGM, 50,000X for ALI (25 µg/mL): 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, 1000X 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 1000X 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 cuvet, blanked on 100% ethanol. 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 (*see* **Subheading 2.3., item 1**). 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. 1000X phosphorylethanolamine (70 mg/mL): Dissolve phosphorylethanolamine (Sigma-Aldrich, cat. no. P0503) in PBS.
11. 1000X ethanolamine (30 µL/mL): Dilute ethanolamine (Sigma-Aldrich, cat. no. E0135) in PBS.
12. 1000X Stock 11 (0.863 mg/mL): Dissolve zinc sulfate (Sigma-Aldrich, cat. no. Z0251) in dH_2O. Store at room temperature.



13. 1000X Penicillin–streptomycin (100,000 U/mL and 100 mg/mL): 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. 1000X gentamicin (50 mg/mL): Sigma-Aldrich, cat. no. G1397. Store at 4°C. Used for BEGM only.
15. 1000X amphotericin B (250 µg/mL): Sigma-Aldrich, cat. no. A2942. Used for BEGM only.
16. 1000X Stock 4: 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. 1000X trace elements: Prepare seven separate 100 mL stock solutions (*see* **Table 3**). 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.

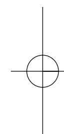
2.4. Making LHC Basal Medium, BEGM, and ALI Medium

The overall approach to making media depends on the culture scale of the individual laboratory. For example, purchase of pre-made base media and addi-

Table 3
Stock Solutions for Trace Elements

Component	Sigma-Aldrich Cat. no.	Amount/ 100 mL	Molarity
Selenium ($NaSeO_3$) highly toxic	S5261	520 mg	30.0 m*M*
Manganese ($MnCl_2 \bullet 4H_2O$) harmful	M5005	20.0 mg	1.0 m*M*
Silicone ($Na_2SiO_3 \bullet 9H_2O$) corrosive	S5904	14.2 g	500 m*M*
Molybdenum [$(NH_4)_6Mo_7O_{24}$ $4H_2O$]	M1019	124.0 mg	1.0 m*M*
Vanadium (NH_4VO_3) highly toxic	A1183	59.0 mg	5.0 m*M*
Nickle ($NiSO_4 \bullet 6H_2O$) toxic	N4882	26.0 mg	1.0 m*M*
Tin ($SnCl_2 \bullet 2H_2O$) corrosive	S9262	11.0 mg	500 µ*M*

tives may represent a logical choice for small-scale efforts. However, laboratories making large quantities of media may choose to make base media and additive stocks in house. Small batches, i.e., 500 mL or 1 L of BEGM or ALI medium, are easily assembled within the reservoir of a bottle top filter, whereas 6-L quantities are made in a volumetric flask and are sterilized by pumping through a cartridge filter. Both scales of media preparation are illustrated below.



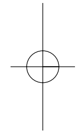
1. *LHC Basal Medium:* For small-scale production of BEGM or ALI, it is recommended to purchase the premade LHC basal medium (Biosource, Camarillo, CA, cat. no. P118-500). For large-scale production, LHC basal medium powder can be specially ordered from Sigma-Aldrich (*see* **Note 2**). In a 5-L volumetric flask, dissolve the 5 L prepackaged mixture in 4 L of dH_2O. Add 5 g $NaHCO_3$, 150 mL of 200 m*M* 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. *BEGM Medium:* BEGM medium 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 1000X stock solutions. For media made with homemade 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 1500*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. Large-scale media production requires a peristaltic pump system, such as a Mas-

terflex pump (Cole-Parmer Instruments, Vernon Hills, IL, cat. no. EW77910-20). Additives are dispensed into base media in a large flask. Masterflex tubing (Cole-Parmer, cat. no. 96400-17) is rinsed with 70% ETOH followed by dH_2O; and appropriate connections are made to filter-sterilize the media through a Gelman 0.45-µm filter cartridge (Fisher Scientific, cat. no. 28-146-179) into sterile 500-mL bottles. Store media at 4°C.

3. *ALI Medium:* ALI medium uses a 50:50 mixture of DMEM-H (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 filtered according to small- or large-scale production methods given in **Subheading 2.4.2.** Note that some additives are not 1000X stock solutions and that base ALI medium omits gentamicin and amphotericin. To prepare low endotoxin medium, use LHC basal medium (Biosource) DMEM (Fisher Scientific, cat. no. BW12-604F), BPE (Sigma-Aldrich), and a low endotoxin grade of BSA (Sigma-Aldrich, cat. no. A2058).

2.5. Antibiotics

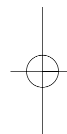
It is assumed that many primary human tissues contain yeasts, fungi, or bacteria. Media for primary cultures can be supplemented with gentamicin (50 µg/mL) and amphotericin (0.25 µg/mL). In our experience, fewer episodes of contamination will result from using amphotericin (Sigma-Aldrich, 1.25 µg/mL), ceftazidime (Fortaz®, GlaxoSmithKline, RTP, NC, 100 µg/mL), tobramycin (Nebcin®, Eli Lilly & Co., Indianapolis, IN, 80 µg/mL), and vancomycin (Vancocin®, Eli Lilly & Co., 100 µg/mL). When processing tissues that are chronically infected from CF patients, additional antibiotics are used for at least the first 3 d of culture. Supplemental antibiotics are chosen based on microbiology reports as described in a prior publication ***(18)***. In the event of repeated fungus or yeast contamination, nystatin (Sigma-Aldrich, final concentration of 100 U/mL, cat. no. N1638,) and Diflucan® (fluconazole for injection, Pfizer, NY, final concentration of 25 µg/mL) can be added for the first 3 d of primary cell culture. When antibiotics are obtained from the hospital pharmacy instead of suppliers of tissue-culture reagents, sterile liquids for injection may be added directly to media, whereas powders are weighed, dissolved in medium, and filter-sterilized. Antibiotics received from the pharmacy as powders contain a given amount of antibiotic and unknown quantities of salts and buffers. The purity of the antibiotic is determined by comparing the weight of the powder in the vial to the designated antibiotic content listed by the manufacturer. Once reconstituted, antibiotics from powders are stored at 4°C, and used within 1 d ***(18)***.



2.6. Cell-Culture Medias, Reagents, and Solutions

All solutions are filter-sterilized and stored at –20°C unless otherwise noted.

1. F-12 nutrient mixture (Ham) powder with 1 m*M* L-glutamine: To make 5 L of Ham's F-12 from powder (Gibco, cat. no. 21700075) add 4 L of dH_2O to a volumetric flask. Add 5 × 1 L packs to flask and supplement with 50 mL of 1.5 *M* HEPES (Sigma-Aldrich, cat. no. H3375), 100 mL of 0.714 *M* $NaHCO_3$, 4.0 mL gentamicin (Sigma-Aldrich, cat. no. G1397), and 5 mL of 1000X pen/strep (*see* **Subheading 2.3., item 13**). Adjust pH to 7.2. Bring total volume up to 5 L and store at 4°C.
2. Cell freezing solution: Combine 2 mL of 1.5 *M* HEPES, 10 mL of fetal bovine serum (Gibco, cat. no. 200-6140AJ), and 78 mL Ham's F-12. Gradually add 10 mL DMSO (Sigma-Aldrich, cat. no. D2650).
3. 1% Protease XIV with 0.01% DNase (10X stock): Dissolve Protease XIV (Sigma-Aldrich, cat. no. P5147) and DNase (Sigma-Aldrich, cat. no. DN-25) in desired volume of PBS and stir. A 1:9 dilution in minimum essential medium (MEM) (*see* **Subheading 2.6., item 6**) is used for cell dissociation.
4. Soybean trypsin inhibitor (1 mg/mL): Dissolve soybean trypsin inhibitor (Sigma-Aldrich, cat. no. T9128) in Ham's F-12, store at 4°C.
5. 0.1% trypsin with 1 m*M* ethylene diamine tetraacetic acid (EDTA) in PBS: Dissolve Trypsin Type III powder (Sigma-Aldrich, cat. no. T4799) in PBS. Add EDTA from concentrated stock for a final concentration of 0.1% trypsin with 1 m*M* EDTA. pH solution to 7.2–7.4.
6. MEM: Supplement 500 mL Joklik's MEM (Sigma-Aldrich, cat. no. M8028) with 5 mL L-glutamine (Sigma-Aldrich), 0.40 mL gentamicin (Sigma-Aldrich), and 0.50 mL 1000X pen/strep (*see* **Subheading 2.3., item 13**). Store at 4°C.



3. Methods

3.1. Overview

AECs can be obtained from nasal or lung tissue specimens and can be seeded directly onto porous supports for primary ALI cultures or can be first grown on plastic for subculturing passage 1 or passage 2 cells to porous supports. An overview of the process is given in **Fig. 1**.

3.2. Type I and III Collagen Coating of Plastic Dishes

Primary and thawed cryopreserved cells are plated onto collagen-coated plastic dishes, whereas cells passaged without freezing do not require coated dishes. Add 2.5 mL of a 1:75 dilution of Vitrogen 100 (Cohesion, Palo Alto, CA) in dH_2O per 100-mm dish. Incubate for 2 h at 37°C. Aspirate remaining liquid and expose open dishes to UV in a laminar flow hood for 30 min. Plates can be stored for up to 6 wk at 4°C.

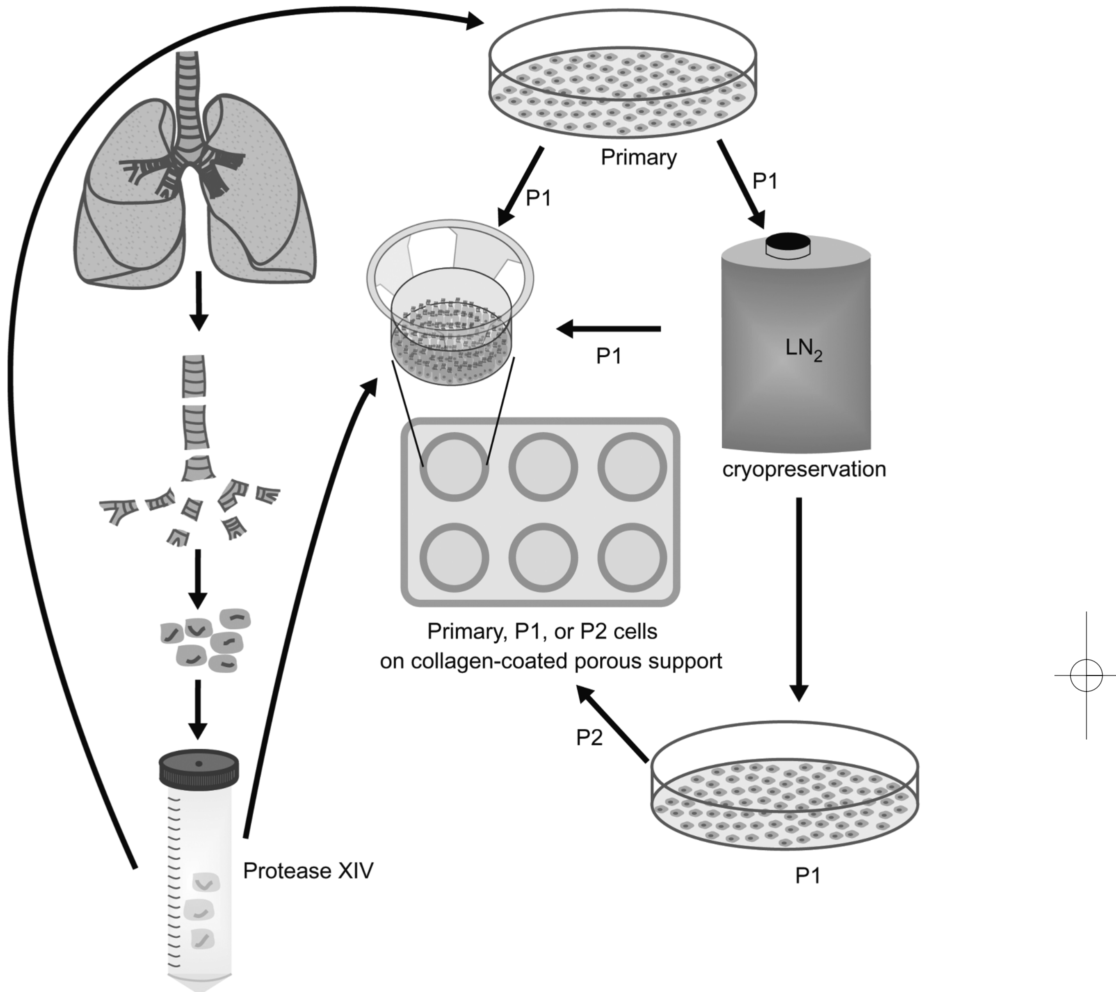


Fig. 1. Overview of the human AECC process. Primary cells may be plated directly on porous supports or on plastic dishes for subsequent cryopreservation and subpassage.

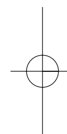
3.3. Type IV Collagen Coating of Porous Supports

A variety of porous supports are suitable for AECCs. Transwell®-COL PTFE membrane inserts, 12- or 24-mm diameter (Corning, Inc., Acton, MA, cat. nos. 3493 and 3491, respectively) are provided collagen precoated by the manufacturer. Transwell®-Clear (Corning, Inc., cat. nos. 3460 and 3450), Snapwell™ (Corning, Inc., cat. no. 3801), and Millicell-CM membranes (Millipore, Billerica, MA, cat. nos. PICM01250 and 03050) must be coated with type IV colla-

gen for successful long-term cultures. For unknown reasons, 0.4 μ*M*, rather than the 3.0 μ*M*, pore-size membranes consistently make superior cultures. To coat, first resuspend 10 mg collagen type IV (Sigma-Aldrich, cat. no. C7521) in 20 mL dH_2O and add 40 μL of concentrated acetic acid. Incubate for 15–30 min at 37°C until fully dissolved. Syringe filter (0.2 μm) and store aliquots at –20°C. Thaw frozen stock and dilute 1:10 with dH_2O. Add 150 μL per 12-mm Transwell Insert, Costar Snapwell Insert, or 12-mm Millicell-CM membrane or 400 μL per 24-mm Transwell Insert, Costar Snapwell Insert, or 30-mm Millicell-CM membrane. Allow to dry at room temperature in a laminar flowhood overnight. Expose to UV in a laminar flowhood for 30 min.

3.4. Isolating Primary AECs

Primary AECs originate from nasal turbinates, from nasal polyps, and from normal and diseased lungs. When handling human tissues, always follow standard safety precautions to prevent exposure to potential bloodborne pathogens, including gloves, lab coat, and eye protection. Tissue is transported to the laboratory in sterile containers containing sterile chilled lactated Ringer's (LR) solution (Abbot Laboratory, North Chicago, IL, cat. no. 7953), MEM, or another physiologic solution. Nasal tissue samples are usually processed without further dissection but whole lungs require significant dissection as described below.



1. Assemble the following on a clean countertop or in a laminar flowhood.
   a. Absorbent bench covering (3M Health Care, St. Paul, MN, cat. no. 1072).
   b. Large plastic sterile drape (3M Health Care, cat. no. 1010).
   c. Ice bucket containing sterile specimen cups (Tyco Health Care Group LP, Mansfield, MA, cat. no. V2200) filled with LR solution.
   d. Use instrument sterilizer (Fine Science Tools, Foster City, CA, cat. no. 18000-45) or preautoclaved instruments. Suggested tools include curve-tipped scissors, delicate 4.5″ (Fisher Scientific, cat. no. 08-951-10); heavy scissors, straight, sharp, 11.5 cm (Fine Science Tools, cat. no. 14058-11); forceps, blunt-pointed, straight, 15 cm (Fine Science Tools, cat. no. 11008-15); rat-tooth forceps 1 × 2, 15.5 cm (Fine Science Tools, cat. no. 11021-15); scalpels, #10 (Bard-Parker™, Becton Dickinson and Co., Hancock, NY, cat. no. 371610); sterile covered sponges, 4″ × 4″ (Tyco Health Care Group LP, cat. no. 2913).
2. Dissect airways by removing all excess connective tissue and cutting into 5–10 cm segments. Clean tissue segments, removing any additional connective tissue and lymph nodes and rinsing in LR solution. Slit segments longitudinally and cut into 1 × 2 cm portions with a scalpel. Transfer to specimen cup containing chilled LR solution. *See* **Fig. 2A–D** for dissection process.
3. Because human tissue samples are likely to contain yeasts, bacteria, or fungi, we begin antibiotic exposure as soon as possible. Prepare 250 mL of J-MEM plus desired antibiotics (*see* **Subheading 2.5.**), named Wash Media. Aspirate LR solu-

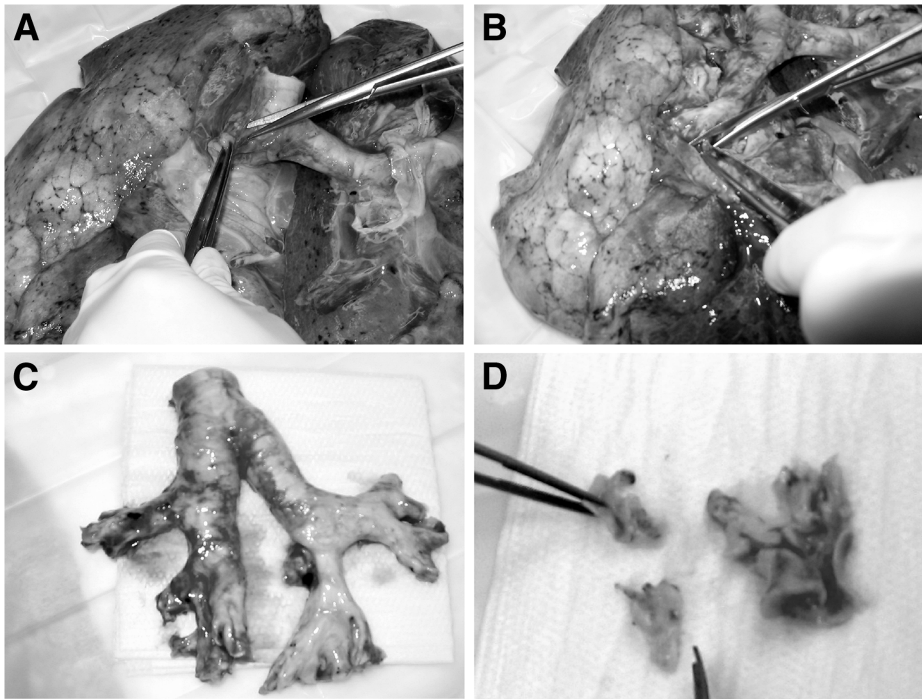


Fig. 2. Dissection process for human lungs. Adherent structures are removed from proximal airways **(A)** and distal airways are freed from lung tissue **(B)**. The airway tree is excised **(C)**, cut into portions and adherent structures are removed. Airways are slit open and cut into segments **(D)**. Segments are placed in specimen cups (not shown) and processed as described in the text.

tion from tissue and add Wash Media, swirl, and replace wash media three times. Transfer washed tissue segments into 50-mL conical tubes containing 30 mL Wash Media plus 4 mL Protease/DNase solution. (Approximate tissue to fluid ratio of 1:10, final volume = 40 mL.) Place tubes on a rocking platform in a cold room at 4°C, selecting 50–60 cycles/min.

4. Tissues from chronically infected patients or any specimens containing abundant secretions are treated to remove mucus and other debris. The tissues are soaked in a solution containing supplemental antibiotics (*see* **Subheading 2.5.**), dithiothreitol (DTT) (Sigma-Aldrich, cat. no. D0632), and DNase (Sigma-Aldrich, cat. no. DN-25). To prepare this Soak Solution, add 65 mg DTT and 1.25 mg DNase to 125 mL of Wash Media and filter sterilize. The final concentration of DTT and DNase are 0.5 mg/mL and 10 µg/mL, respectively. Aspirate LR solution from tissue and add 60 mL Soak Solution, swirl, and soak 5 min. Repeat Soak Solution step. Next, rinse tissue three times in Wash Media to remove DTT/DNase. Transfer tissue to 50-mL tubes containing 30 mL Wash Media plus 4 mL protease/

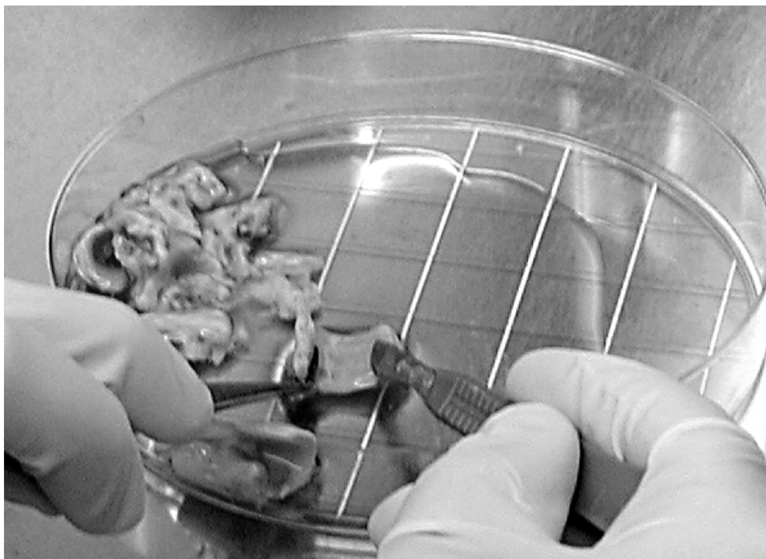
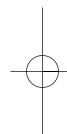


Fig. 3. Epithelial cell removal after protease dissociation. The concave, luminal airway surface is gently scraped with a convex scalpel blade to remove adherent cells.



DNase (final volume = 40 mL) and place tissue on platform rocker at 4°C, 50–60 cycles/min for 48 h.

5. Nasal turbinates, polyps, and small bronchial specimens undergo the same procedure, except that these tissues can be dissociated in 24 h in 15-mL tubes containing 8 mL Wash Media plus 1 mL protease solution.

3.5. Harvesting Cells

Follow standard sterile tissue-culture techniques under a laminar flow hood.

1. End dissociation of tissue by pouring contents of 50-mL tubes into a 150-mm tissue-culture dish; add FBS (Gibco) to a final concentration of 10% (v/v) to neutralize protease.
2. Scrape epithelial surface with a convex surgical scalpel blade #10 as illustrated in **Fig. 3**. Rinse tissue surfaces and collection dish with PBS and pool solutions containing dissociated cells into 50-mL conical tubes.
3. Centrifuge at 500*g* for 5 min at 4°C. Wash cells once in media, resuspend in a volume calculated to be approx 5×10^6 cells/mL, and count using a hemocytometer.

3.6. Plating Cells

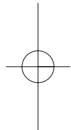
Primary AECs may be cultured directly on porous supports in ALI medium at a density of $0.1–0.25 \times 10^6$-cells per cm^2, which is equivalent to $0.8–2.0 \times 10^5$ cells per 12 mm support or $0.7–1.75 \times 10^6$ cells per 24–30 mm

support (*see* **Note 3**). Alternatively, to generate P1 or P2 cells for subculture to porous supports, primary cells can be plated in BEGM on collagen-coated plastic dishes at a density of 2–6 × 10^6 per 100-mm dish. Primary cell media should be supplemented with additional antibiotics (*see* **Subheading 2.5.**) for the first 3 d after plating, and should be changed every 2–3 d or as needed to prevent acidification (*see* **Subheading 3.7.3.**).

3.7. Cell Culture Maintenance

3.7.1. Primary Cells on Plastic

Assess attachment to plastic dishes 24 h after plating primary cells. If the cells attached well and the dish contains few clumps of floating epithelial cells, wash the cells with PBS and feed with BEGM plus antibiotics (*see* **Subheading 2.5.**). Large floating clumps of cells can be "rescued" to increase cell yield. Harvest the media in 50-mL conical tubes. Gently wash dishes with PBS and add to harvested clumps. Pellet cells at 500*g* for 5 min. Aspirate the supernatant and add 10–15 mL of freshly prepared declumping solution containing 2 m*M* EDTA, 0.5 mg/mL DTT, 0.25 mg/mL collagenase (Sigma-Aldrich, cat. no. C6885), and 10 μg/mL DNase in PBS. Incubate 15 min to 1 h at 37°C while visually monitoring clump dissociation. Add FBS to 10% (v/v), centrifuge at 500*g* for 5 min, remove supernatant, and resuspend pellet in BEGM for counting. Plate at a density of 2–6 × 10^6 per 100-mm collagen coated dish. Medium is changed every 2–3 d.



3.7.2. Passaging Primary Cells on Plastic

When primary cultures reach 70–90% confluence, they are ready for passage. We believe it is important to harvest hard-to-detach cells while minimizing trypsin exposure of cells that release quickly. Thus, we use a double-trypsinization process. Rinse cells with PBS, add 2 mL of trypsin/EDTA per 100-mm dish and incubate 5–10 min at 37°C. Gently tap dish to detach cells. Rinse cells with PBS and harvest into 50-mL conical tube containing 20 mL STI solution on ice. Add another 2 mL of trypsin/EDTA to dishes and repeat, visually monitoring detachment. Pool harvested cells and centrifuge at 500*g* for 5 min. Aspirate supernatant and resuspend cells in desired volume of media for counting.

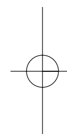
3.7.3. Media Change in ALI Cultures

Primary, passage 1, or passage 2 AECs may be grown on collagen-coated porous supports. Remove media on the top with a Pasteur pipet attached to a vacuum, and rinse the apical surface with PBS. Prior to confluence, replace media in the apical and basolateral compartment but after confluence do not

add media to the apical compartment. The volume of media added to the apical and basolateral chambers depends on the specific porous support. Transwell insert and Costar Snapwell inserts hang in 12- or 6-well plates. Millicell CM membranes stand on legs and can be kept in a variety of dishes. During periods of rapid cell growth, cells on Transwell insert in the standard configuration will yellow the media rapidly and will require daily media changes. We have devised Teflon adapters to enable 12-mm Transwell inserts to be kept in six-well plates with a larger, 2.5-mL, basolateral reservoir, which decreases the media change frequency; 24-mm Transwell Insert may be kept in Deep Well Plates (Collaborative Biomedical Products, Bedford, MA, cat. no. 01-05467) with 12.5 mL media. Typically 6 × 12 mm or 2 × 30 mm Millicell CM inserts are kept in 10 mL of media in a 100-mm dish.

3.8. Cryopreservation of Cells

1. Primary AECs are trypsinized from plastic dishes (now P1 cells) and cryopreserved for long-term storage in liquid nitrogen. Cells are resuspended in Ham's F-12 media at a concentration of 2–6 × 10^6 cells/mL.
2. Keep cells on ice and slowly add an equal amount of freezing media (*see* **Subheading 2.6., item 2**) to the cell suspension.
3. Place cryovials in Nalgene Cryo Freezing container (VWR, cat. no. 5100-0001) and place in –80°C freezer for 4–24 h. An insulated box can be used as a substitute.
4. Transfer vial(s) from the –80°C freezer to liquid N_2 (–196°C) for long-term storage.



3.9. Thawing Cells

1. Warm Ham's F-12 and plating media to 37°C. Note: Warm media must be added gradually so that the DMSO concentration gradient is not too steep.
2. Thaw the cryovial in a beaker of 37°C water. Remove cryovial and wipe outside with 70% ethanol. Transfer cells to a 15-mL conical tube.
3. Dilute the cell suspension by slowly filling the tube with warm Ham's F-12. Centrifuge at 600*g* for 5 min at 4°C.
4. Gently resuspend cells in the appropriate plating media, count cells, and assess viability.

3.10. Histological Methods

Histologic assessment of ALI cultures is a useful experimental tool but the thin, pliable membrane poses unique challenges. To maintain the integrity of cells grown on membranes for morphologic analysis, cultures are generally processed without removing the membrane from the support, and then re-embedded to enable production of cross sections. This applies to cultures processed for frozen, paraffin, or plastic sectioning, and for transmission electron microscopy. Representative examples of frozen, paraffin, and plastic sections are shown in **Fig. 4A–C**.

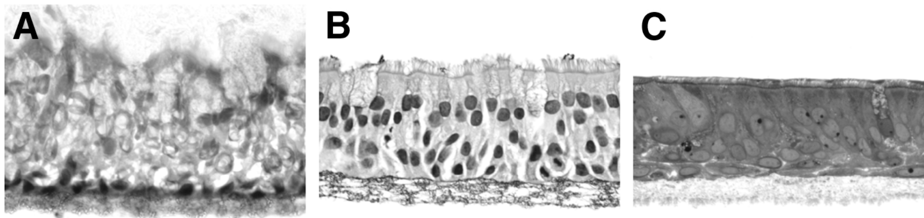
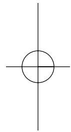


Fig. 4. Histologic assessment of AECCs. Frozen **(A)**, paraffin **(B)**, and plastic **(C)** sections of well-differentiated cultures. Original magnification = 500X, A and B = H&E stain, C = Richardsons stain.

3.10.1. Frozen Sections

To obtain frozen sections of cells grown on membranes, media is removed and, if desired, the cells are rinsed with PBS. Excess fluid is blotted and the culture is sandwiched between two layers of embedding media (Fisher Scientific, cat. no. NC9418069), using a weigh boat to support the bottom layer. The sandwiched membrane is then frozen and removed from the support with a scalpel and cut into slices within a chilled cryostat chamber. The slices are placed on edge in a disposable embedding mold that is then filled with embedding media and frozen to create a tissue block for sectioning on a cryostat ***(19)***. When performed carefully, the tissue remains frozen throughout the double-embedding process and cross sections of the epithelium are produced when the final block is sectioned.



3.10.2. Paraffin and Plastic Sections

Paraffin sections of cells grown on membranes are obtained by fixing, dehydrating to 100% ethanol, and clearing in Slide Brite (Sasco, Albany, GA) before infiltration with paraffin embedding media ***(20)***. For plastic embedding, the sample is transferred from 100% ethanol to 50:50 solutions of plastic embedding media before infiltration with 100% plastic. For both paraffin and plastic, the membrane is sandwiched between two layers of embedding media, then hardened as usual and cut from the support and into slices. The slices are placed on edge in an embedding mold, covered with embedding media, and again hardened as usual to create a tissue block resulting in cross sections.

3.10.3. Transmission Electron Microscopy Sections

Cultures for transmission electron microscopy are treated similarly as those processed for paraffin sections except using glutaraldehyde fixation and osmium tetroxide post fixation. The cultures are dehydrated into 100% ethanol and then infiltrated with graded mixtures of resin and ethanol and finally pure

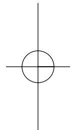
resin **(21)**, avoiding propylene oxide which will dissolve the membrane support. A flat wafer of the culture is then polymerized, cut into slices, and re-embedded.

3.10.4. Scanning Electron Microscopy

Samples are processed for scanning electron microscopy by fixing in glutaraldehyde and postfixing in osmium tetroxide followed by dehydration to 100% ethanol. While still in the support, the culture is critical point dried and mounted using a carbon conductive tab (Ted Pella, Redding, CA, cat. no. 16084-1). The membrane is removed from the support with a scalpel and is coated with gold for viewing in a scanning electron microscope **(22)**.

3.11. Electrophysiologic Assessment of AECCs

Cystic fibrosis is the most common fatal genetic disorder of the Caucasian population **(23)**. The cloning of the CF gene *(CFTR)* marked a new era in our understanding of the pathophysiology of CF **(24–26)**. Heterologous expression and bilayer reconstitution studies showed *CFTR* to be a cAMP-regulated Cl^- channel **(27,28)**. Mutations in *CFTR* also result in defective regulation of the epithelial Na^+ channel, ENaC **(29)**, and alter the function of an epithelial Ca^+-activated Cl^- channel, CaCC **(30,31)**. Thus, the CF epithelium is characterized by the absence of cAMP-mediated Cl^- conductance, and hyperactiviation of ENaC and CaCC. Human AECCs, mounted in Ussing chambers, have been used to characterize ion transport properties of CF and normal tissues and for testing of potential pharmacologic or genetic therapies.



For study in Ussing chambers, CF and normal AECCs are plated onto Costar Snapwell (Corning, Inc., cat. no. 3801) tissue-culture inserts precoated with collagen type IV (*see* **Subheading 3.2.**). Cells are visually evaluated for confluence, development of cilia, and maintenance of an ALI. Transepithelial resistance (R_T) and potential difference (PD) are measured using an EVOM device (World Precision Instruments, Sarasota, FL), as per manufacturer's instructions. Monolayers generating at least a 1-mV PD and a 150 Ω • cm² R_T are used for Ussing chamber studies, which typically occurs 10–14 d after plating. Transepithelial voltage (V_T), R_T, and short-circuit current (I_{SC}), are measured using Ussing chambers specifically designed for Snapwell inserts (Physiologic Instruments, La Jolla, CA, cat. no. P2300). Cells are typically bathed in Krebs Bicarbonate Ringer's solution (KBR) on the basolateral side and a modified KBR, high K^+, low Cl^- solution (HKLC), on the apical side, to enable focusing on apical Cl^- secretion **(30–32)**. All bathing solutions are bubbled with 95% O_2, 5% CO_2 and maintained at 37°C. Voltage is clamped to zero, and pulsed to 10 mV for 0.5-s duration every minute. Electrometer output is digitized online

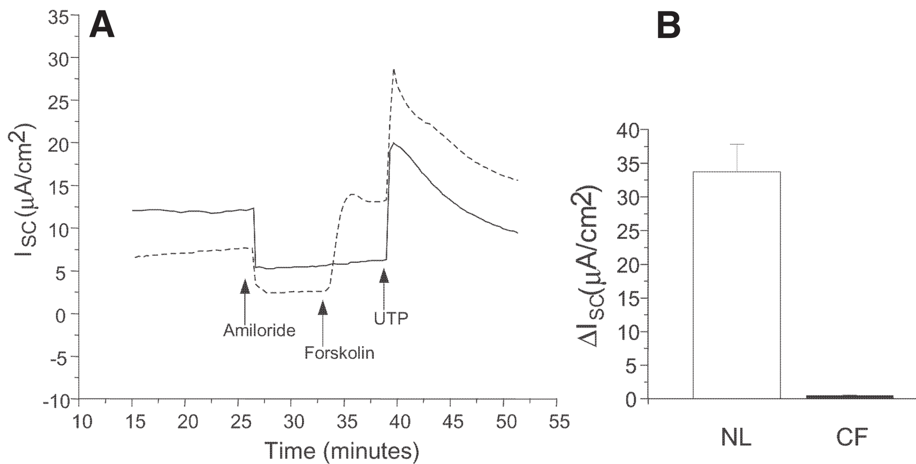
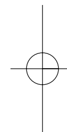


Fig. 5. Electrophysiologic assessment of AECCs. Representative I_{SC} traces of normal (dashed) and CF (solid) human airway epithelial cultures studied in Ussing chambers as described in the text. Amiloride (10^{-4} *M*), forskolin (10^{-5} *M*) and uridine triphosphate (UTP) (10^{-4} *M*) are added to determine the presence of ENaC, CFTR, and CaCC, respectively **(A)**. Summary I_{SC} values from normal (open bars, $n = 18$) and CF (filled bar, $n = 19$) human bronchial epithelial cultures in response to bilateral addition of forskolin (10^{-5} *M*) **(B)**.



and I_{SC}, R_T, and calculated V_T are displayed on a video monitor and stored on a computer hard drive. Drugs are added from concentrated stock solutions to either lumenal and/or serosal sides of the tissue.

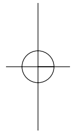
Representative tracings of both normal and CF airway epithelial cultures are shown in **Fig. 5A**. The most reliable and reproducible difference between CF and normal epithelial cultures is the absence of a cAMP-mediated I_{SC} response in CF cultures (*see* **Fig. 5B**). Other ion transport properties used to distinguish CF from normal cultures include greater percentage inhibition of the basal current by amiloride (10^{-4} *M*) in CF cultures, and an elevated response of the calcium-activated Cl^- conductance as measured by the I_{SC} response to purinergic receptor activation by uridine triphosphate.

4. Notes

1. To protect the safety of research personnel, we do not accept specimens from individuals with known infection with human immunodeficiency virus, hepatitis B, hepatitis C, or tuberculosis. Samples from individuals on immunosuppressive therapy, especially long-term, may pose increased risk. All human tissue samples must be treated as potentially biohazardous and handled using standard precau-

tions. It is a research team decision, related to the scientific goals, whether to accept specimens from individuals with an extensive smoking history. AECs can be procured successfully from lungs with acute lung injury or pneumonia. The latter can usually be cultured successfully by selecting appropriate antibiotics (*see* **Subheading 2.5.**). A range of clinical data (laboratory values including blood gases, X-ray, or bronchoscopy findings) can be used to help determine lung acceptability. Owing to the lack of systematic studies, there are no hard and fast rules guiding the relationship between physiologic function and successful cell culture, but an arterial P_{O2} of greater than 150 mmHg on 100% inspired oxygen is a reasonable lower limit.

2. If anticipated usage of LHC basal medium exceeds 550 L per year, powdered stock can be custom ordered from Sigma-Aldrich. The composition is given in **Table 4**.
3. Seeding densities. Primary and passaged primary human airway epithelial cells are mortal and their growth characteristics depend on a sufficient seeding density. Furthermore, attachment and growth of cells from different individuals and preparations may vary. Thus, generous seeding densities of primary cells on porous supports are required to consistently obtain confluent cultures that differentiate and maintain a long-lasting, patent ALI. Although it is tempting to expand primary cells on plastic to geometrically increase cell number, growth capacity of mortal cells is finite, and "overexpansion" should be avoided. The seeding guidelines herein will generally enable successful differentiated cultures persisting for at least 45 d. Primary cells to be first grown on plastic dishes should be seeded at no less than 1×10^6, and preferably $2–6 \times 10^6$, cells per 100-mm collagen coated dish. Seeding densities for smaller or larger dishes should be calculated mathematically based on surface area. Under these conditions the cells should grow to >70% confluence within 7–10 d. If longer periods are required to reach >70% confluence, subsequent growth may be impaired. Cells at >70% confluence, but not >95% confluence, should be trypsinized for cryopreservation or subpassaged to a porous support. Alternatively, the cells can be can be expanded one more round at a seeding density of 1×10^6 cells per uncoated 100-mm tissue-culture dish for expansion to passage 2. Seeding densities for primary, passage 1, and passage 2 cells on porous supports should be in the range of 1.5×10^5 cells/cm^2. Thus, 12-mm Millicell CM or 12-mm Transwell membranes are typically seeded with approx 125,000 cells each, whereas 30-mm Millicell CM or 24-mm Transwell membranes are seeded with approx 1×10^6 cells. This seeding density will result in confluence, or near confluence, within 1–3 d after seeding, at which point an ALI should be established. Lower seeding densities may be fully successful with some specimens, which can be determined empirically using aliquots of passage 1 or 2 cells. Unfortunately, prescreening is not possible when plating primary cells, and greater variability is anticipated between different patient preparations.



Acknowledgment

The authors wish to acknowledge Lisa Brown for editing and graphic design.

Table 4
LHC Basal Media Formula

Component	Sigma-Aldrich Cat. no.	Formula g/L	g/550L Batch
L-Arginine HCl	A6969	0.421	231.55
L-Alanine	A7469	0.009	4.95
L-Asparagine•H_2O	A7094	0.015	8.25
L-Aspartic acid	A7219	0.004	2.2
L-Cysteine HCl monohydrate	C6852	0.042	23.1
L-Glutamic acid	G8415	0.0148	8.14
Glycine free base	G8790	0.0076	4.18
L-Histidine HCl	H5659	0.0336	18.48
L-Isoleucine	I7403	0.004	2.2
L-Lysine monohydrochloride	L8662	0.0366	20.13
L-Leucine	L8912	0.1312	72.16
L-Methionine	M5308	0.009	4.95
L-Proline	P5607	0.0346	19.03
L-Phenylalanine	P5482	0.01	5.5
L-Serine	S4311	0.1262	69.41
L-Tryptophan	T8941	0.0062	3.41
L-Tyrosine disodium	T2269	0.00756	4.158
L-Threonine	T8441	0.0238	13.09
L-Valine	V0513	0.0702	38.61
Adenine HCl	A8751	0.03088	16.984
D-Biotin	B4501	0.00002	0.011
Choline chloride	C1879	0.028	15.4
Folic acid	F7876	0.00079	0.4345
D-(+)-glucose	G8270	0.5405	297.275
Myoinositol	I5125	0.018	9.9
Niacinamide	N3376	0.00004	0.022
D-Pantothenic acid hemicalcium	P2250	0.00026	0.143
Putrescine•$_2$HCl	P7505	0.00016	0.088
Pyridoxine hydrochloride	P9755	0.00006	0.033
Riboflavin	R4500	0.00004	0.022
Thiamine hydrochloride	T4625	0.00034	0.187
Thymidine	T9441	0.00073	0.4015
Vitamin B_{12}	V2876	0.00041	0.2255
Cupric sulfate pentahydrate	C7631	0.000002	0.0011
Potassium chloride	P4504	0.112	61.6
Sodium phosphate dibasic	S0876	0.284088	156.2484
Sodium chloride	S9625	6.084	3346.2
D-(+)-glucose	G8270	0.27025	148.6375

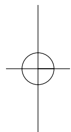
(continued)

Table 4 *(continued)*

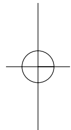
Component	Sigma-Aldrich Cat. no.	Formula g/L	g/550L Batch
Pyruvic acid sodium	P2256	0.055	30.25
Phenol red sodium	P4758	0.00124	0.682
Sodium acetate anhydrous	S8750	0.301	165.55
D-(+)-glucose	G8270	0.27025	148.6375
Dl-6,8-Thioctic acid	T5625	0.00021	0.1155
HEPES free acid	H3375	5.4	2970

References

1. Lechner, J. F., Haugen, A., McLendon, I. A., and Pettis, E. W. (1982) Clonal growth of normal adult human bronchial epithelial cells in a serum-free medium. *In Vitro* **18,** 633–642.
2. Gruenert, D. C., Finkbeiner, W. E., and Widdicombe, J. H. (1995) Culture and transformation of human airway epithelial cells. *Am. J. Physiol.* **268,** L347–L360.
3. Terzaghi, M., Nettesheim, P., and Williams, M. L. (1978) Repopulation of denuded tracheal grafts with normal, preneoplastic, and neoplastic epithelial cell populations. *Cancer Res.* **38,** 4546–4553.
4. Yankaskas, J. R., Knowles, M. R., Gatzy, J. T., and Boucher, R. C. (1985) Persistence of abnormal chloride ion permeability in cystic fibrosis nasal epithelial cells in heterologous culture. *Lancet* **1,** 954–956.
5. Jorissen, M., Van Der Schueren, B., van den Berghe, H., and Cassiman, J. J. (1989) The preservation and regeneration of cilia on human nasal epithelial cells cultured *in vitro*. *Arch. Otorhinolaryngol.* **246,** 308–314.
6. Wu, R., Yankaskas, J., Cheng, E., Knowles, M. R., and Boucher, R. (1985) Growth and differentiation of human nasal epithelial cells in culture. Serum-free, hormone-supplemented medium and proteoglycan synthesis. *Am. Rev. Respir. Dis.* **132,** 311–320.
7. Benali, R., Tournier, J. M., Chevillard, M., et al. (1993) Tubule formation by human surface respiratory epithelial cells cultured in a three-dimensional collagen lattice. *Am. J. Physiol.* **264,** L183–L192.
8. Whitcutt, M. J., Adler, K., and 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.
9. Matsui, H., Grubb, B. R., Tarran, R., et al. (1998) Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* **95,** 1005–1015.
10. Matsui, H., Randell, S. H., Peretti, S. W., Davis, C. W., and Boucher, R. C. (1998) Coordinated clearance of periciliary liquid and mucus from airway surfaces. *J. Clin. Invest.* **102,** 1125–1131.



11. Pickles, R. J., McCarty, D., Matsui, H., Hart, P. J., Randell, S. H., and Boucher, R. C. (1998) Limited entry of adenoviral vectors into well differentiated airway epithelium is responsible for inefficient gene transfer. *J. Virol.* **72,** 6014–6023.
12. Zhang, L., Peeples, M. E., Boucher, R. C., Collins, P. L., and Pickles, R. J. (2002) Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. *J. Virol.* **76,** 5654–5666.
13. Bernacki, S. H., Nelson, A. L., Abdullah, L., et al. (1999) Mucin gene expression during differentiation of human airway epithelia *in vitro*. Muc4 and muc5b are strongly induced. *Am. J. Respir. Cell Mol. Biol.* **20,** 595–604.
14. Zhang, Y. J., O'Neal, W. K., Randell, S. H., et al. (2002) Identification of dynein heavy chain 7 as an inner arm component of human cilia that is synthesized but not assembled in a case of primary ciliary dyskinesia. *J. Biol. Chem.* **277,** 17,906–17,915.
15. Yoon, J. H., Koo, J. S., Norford, D., Guzman, K., Gray, T., and Nettesheim, P. (1999) Lysozyme expression during metaplastic squamous differentiation of retinoic acid-deficient human tracheobronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **20,** 573–581.
16. Gray, T. E., Guzman, K., Davis, C. W., Abdullah, L. H., and Nettesheim, P. (1996) Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **14,** 104–112.



17. Lechner, J. F. and LaVeck, M. A. (1985) A serum-free method for culturing normal human bronchial epithelial cells at clonal density. *J. Tiss. Cult. Meth.* **9,** 43–48.
18. Randell, S. H., Walstad, L., Schwab, U. E., Grubb, B. R., and Yankaskas, J. R. (2001) Isolation and culture of airway epithelial cells from chronically infected human lungs. *In Vitro Cell Dev. Biol. Anim.* **37,** 480–489.
19. Bancroft, J. and Stevens, A. (1996) *Theory and Practice of Histological Techniques*, Battle Press, Columbus, OH, pp. 69–80.
20. Sheehan, D. and Hrapchak, B. (1980) *Theory and Practice of Histotechnology*, Battle Press, Columbus, OH, Vol. **2,** 59–66.
21. Hayat, M. A. (1989) *Principles and Techniques of Electron Microscopy* **3,** 79–92.
22. Goldstein, J. (1984) *Scanning Electron Microscopy and X-ray Microanalysis*, pp. 495–540.
23. Boat, T. F., Welsh, M. J., and Beaudet, A. L. (1989) Cystic fibrosis, in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Stansbury, J. B., Wyngaarden, J. B., and Frederickson, D. S., eds.), 2649–2680.
24. Rommens, J. M., Iannuzzi, M. C., Kerem, B.-T., et al. (1989) Identification of the cystic fibrosis gene: Chromosome walking and jumping. *Science* **245,** 1059–1065.
25. Riordan, J. R., Rommens, J. M., Kerem, B.-T., et al. (1989) Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. *Science* **245,** 1066–1073.
26. Kerem, B., Rommens, J. M., Buchanan, J. A., et al. (1989) Identification of the cystic fibrosis gene: Genetic analysis. *Science* **245,** 1073–1080.

27. Anderson, M. P., Rich, D. P., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1991) Generation of cAMP-activated chloride currents by expression of CFTR. *Science* **251,** 679–682.
28. Kartner, N., Hanrahan, J. W., Jensen, T. J., et al. (1991) Expression of the cystic fibrosis gene in non-epithelial invertebrate cells produces a regulated anion conductance. *Cell* **64,** 681–691.
29. Stutts, M. J., Canessa, C. M., Olsen, J. C., et al. (1995) CFTR as a cAMP-dependent regulator of sodium channels. *Science* **269,** 847–850.
30. Gabriel, S. E., Makhlina, M., Martsen, E., Thomas, E. J., Lethem, M. I., and Boucher, R. C. (2000) Permeabilization via the P2X7 purinoceptor reveals the presence of a Ca^{2+}-activated Cl^- conductance in the apical membrane of murine tracheal epithelial cells. *J. Biol. Chem.* **275,** 35,028–35,033.
31. Tarran, R., Loewen, M. E., Paradiso, A. M., et al. (2002) Regulation of murine airway surface liquid volume by CFTR and Ca^{2+}-activated Cl^- conductances. *J. Gen. Physiol.* **120,** 407–418.
32. Donaldson, S. H., Hirsh, A., Li, D. C., et al. (2002) Regulation of the epithelial sodium channel by serine proteases in human airways. *J. Biol. Chem.* **277,** 8338–8345.