Chapter 18

Primary Epithelial Cell Models for Cystic Fibrosis Research

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Abstract

When primary human airway epithelial (hAE) cells are grown in vitro on porous supports at an air–liquid interface (ALI), they recapitulate in vivo morphology and key physiologic processes. These cultures are useful for studying respiratory tract biology and diseases and for testing new cystic fibrosis (CF) therapies. This chapter gives protocols enabling creation of well-differentiated primary CF and non-CF airway epithelial cell cultures with non-proprietary reagents. We also discuss the production of retroviral and lentiviral vectors, the derivation of hAE cell lines, reporter gene assays, and the evolving science of gene overexpression and knockdown in ALI hAE cultures.

Key words: Respiratory tract, differentiation, physiology, pathogenesis, therapy, adenovirus, lentivirus.

1. Introduction

1.1. Primary Human Airway Epithelial (hAE) Cell Cultures

In cystic fibrosis (CF) lack of functioning CF transmembrane conductance regulator (*CFTR*) protein in airway epithelial cells impairs innate defense mechanisms, causing the infection diathesis. Human airway epithelial (hAE) cell cultures are key for basic and applied studies of airway biology, disease, and therapy related to CF. Heterologous *CFTR* expression systems and airway epithelial cell lines are important for CF research and development. However, well-differentiated primary hAE cultures grown on porous supports at an air–liquid interface (ALI) recapitulate the characteristic pseudostratified mucociliary morphology and key physiologic functions and are a quantum leap toward the

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in vivo biology. The cultures serve as a critical milestone test of biological relevance. Verification of efficacy in this model is a rational step for advancement of potential therapies, and peer reviewers for scientific journals and granting agencies often require its use.

Although primary hAE cultures have been created for over 25 years (1) and have been used for numerous studies, expense, technical complexity, and experimental limitations inhibit their full application. From 1984 to 2009, The University of North Carolina Cystic Fibrosis Center Tissue Procurement and Cell Culture Core has prepared cells from more than 6970 human tissue specimens, adopting new technologies and extending research capabilities. The current chapter distills and updates our prior detailed description (2), enabling others to employ this relevant cell culture model. The procedures detailed below are based on the original methods of Lechner and LaVeck (3) and Gray et al. (4), as currently employed in our laboratory.

1.2. Production of Retroviral and Lentiviral Vectors

Retroviral and lentiviral vectors are key components of the hAE research toolbox and their production by individual laboratories is within reach for many investigators. They are used extensively for creation of cell lines and for gene expression or knockdown as described in **Sections 1.3** and **1.5**. In **Sections 2.2** and **3.2** we describe production of retroviral and lentiviral vectors for infection of undifferentiated hAE cells on plastic culture dishes. Both vectors are produced in human 293T cells, which are easily transfected with the necessary plasmids.

1.3. Creation of Airway Epithelial Cell Lines

When employed properly, airway epithelial cell lines are a valuable complement to primary cultures. They have been derived from human lung cancers (5, 6), produced by mutagenesis (7), created by introduction of oncogenes, with (8, 9) or without (10, 11) co-introduction of human telomerase reverse transcriptase (*hTERT*) or by gene expression that suppresses senescence (12). Cell lines found useful for CF research were previously reviewed (13). We focus in **Sections 2.3** and **3.3** on two approaches for cell line creation (1) transformation with the potent viral oncogene Simian Virus 40 Early Region (SV40ER) in combination with *hTERT* and (2) growth extension using the mammalian oncogene *Bmi-1* plus *hTERT*. In our experience, the viral oncogene plus *hTERT* approach produces rapidly growing, immortal, and genetically unstable aneuploid cell lines that lose the ability to polarize or undergo mucociliary differentiation after multiple passages, while *Bmi-1* plus *hTERT* produces slowly dividing, growth-enhanced diploid cells that are not immortal, but are capable of polarizing and differentiating into mucous secretory and occasional ciliated cells up to passage 15 (8, 14).

1.4. Reporter Gene Assays in ALI hAE Cells

Reporter gene assays are widely employed and highly useful in modern cell biology, including high-throughput screening, mechanistic studies and promoter analysis. Typical assays rely on cellular expression of a reporter activity driven by a specific promoter indicative of pathway activation, which is normalized to a second, constitutively expressed reporter. There are multiple formats, including fluorescence imaging and biochemical assays of secreted or cell-associated enzymes. Changes in response to stimuli are typically measured as an increase in reporter activity and effects of chemical inhibitors/stimulators can be evaluated. Additionally, a gene, a dominant negative or small hairpin RNA (shRNA) construct, or a control can be co-transfected, in excess over the reporter, to evaluate effects on baseline or stimulated promoter activity.

When using appropriate protocols, tissue culture cells (3T3, 293, HeLa, A549, etc.) on plastic are easily transfectable with plasmid vectors. However, hAE cells even on plastic are transfection resistant and well-differentiated cells at an ALI are notoriously difficult. Replication-deficient adenoviral vectors are highly efficient for transient expression in tissue culture cells on plastic and, when coupled with permeabilization of the apical plasma membrane, are reasonably efficient in ALI hAE cells (15). Below (*see* **Sections 2.4** and **3.4**), we describe the use of an adenovirus expressing NF-κB-driven firefly luciferase (*fLuc*) to examine pathway activation in hAE cells at an ALI (**Fig. 18.1**). A constitutively expressed *LacZ gene* (β-galactosidase protein, β-gal) controls for transduction efficiency. In one example, co-transduction with dominant negative construct of the interleukin-1 receptor-associated kinase 1 indicated its key role in the ALI hAE cell response to *Pseudomonas aeruginosa* (16). Although time and expense can be substantial, replication-deficient adenoviruses are robust and adaptable and can be used for analysis of other pathways for which cognate promoters are available. Production of adenoviruses is beyond the scope of this chapter and can be subcontracted to a Vector Core (*see* **Note 1**) or created in the end user laboratory using the AdEasy system ((17); *see* also http://www.coloncancer.org/adeasy.htm).

1.5. Protein Expression or Knockdown in ALI hAE Cells

Experiments employing expression of a protein or a mutant version of the protein, or knockdown of a specific mRNA and thus protein, can provide valuable mechanistic and functional insights. Transgenic gene expression or knockout by homologous recombination is often used in mice or other organisms for this purpose. Tissue culture cells on plastic can be genetically manipulated by plasmids and/or siRNA oligonucleotides, but standard techniques for introduction of genetic material are not efficient in well-differentiated ALI hAE cells. In **Section 1.4.**, we illustrated

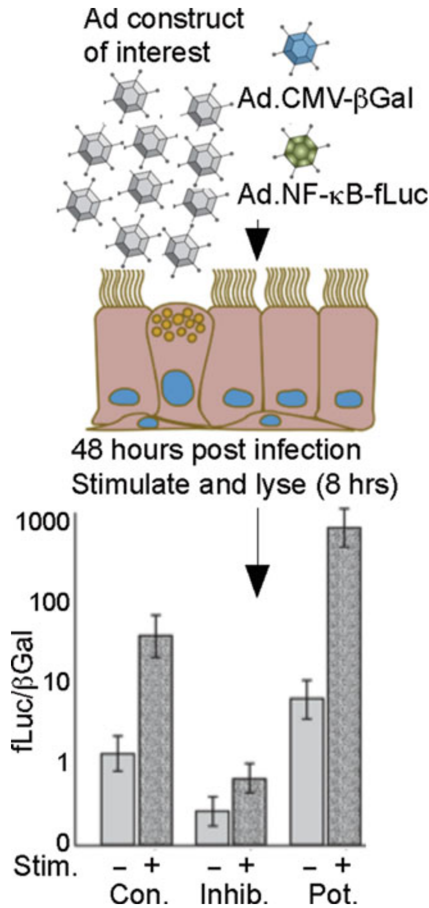

Fig. 18.1. Overview of the method for adenovirus (Ad) reporter gene assays in well-differentiated ALI hAE cells, employing co-transduction of a gene modifying the cell response. The panel below indicates the expected results with co-transduction of a control (Con.) or a construct that potentiates (Pot.) or inhibits (Inhib.) the baseline and stimulated reporter gene activity.

the strategy of adenovirus transduction and reporter gene assays. This approach typically results in expression of 5–30% of the total cells in an ALI hAE culture, thus limiting or precluding "whole culture" biochemical or functional analyses. However, the use of retro/lentiviral vectors, followed by selection and subculture, is a viable method for evaluating gene function in well-differentiated ALI hAE cells at the whole culture level (**Fig. 18.2**). This approach can be performed using viral vector constructs directing constitutive expression of proteins (18) or shRNA. Such vectors may not be feasible if the genetic manipulation inhibits cell growth and/or differentiation. In this case, inducible expression can be employed (19). Both of these approaches were used by our group to successfully knock down amiloride-sensitive epithelial sodium channel (ENaC) activity in passage 2, well-differentiated, ALI hAE cells by expressing shRNA targeting the alpha subunit gene (*SCNN1A*) (20) (*see* **Note 2**).

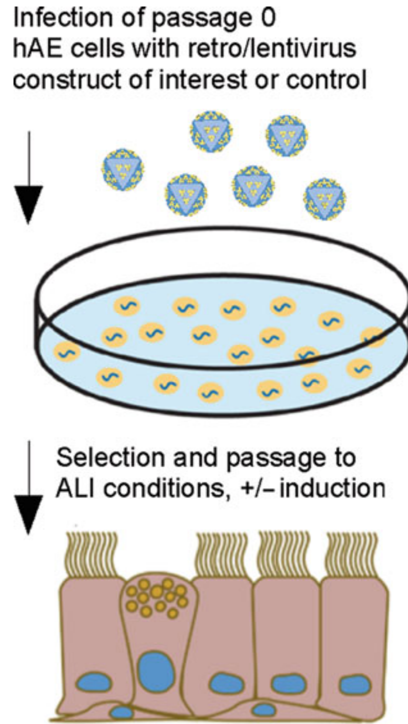

Fig. 18.2. Overview of the method for retroviral/lentiviral vector genetic manipulation of well-differentiated ALI hAE cells.

2. Materials

2.1. Primary Human Airway Epithelial (hAE) Cell Cultures

2.1.1. Tissue Procurement

Airway epithelial cells can be extracted from excess surgical pathology or autopsy specimens procured through cooperating surgeons and pathologists using protocols in accordance with relevant regulations. These include nasal turbinates or polyps not requiring histopathologic examination; lung tissue after lobectomy, pneumonectomy, or transplantation (surgical pathology); and trachea/lungs (autopsy) after examination and release by a pathologist. A useful source of normal tissue is the donor's lower trachea, carina, and mainstem bronchi left over after transplantation. These are transported to the laboratory in an appropriate container on wet ice in a physiologic solution (sterile saline, PBS, lactated Ringer's solution, or tissue culture medium). Lungs from potential organ donors are frequently unsuitable for transplantation but are useful for research. These can be obtained via establishing protocols with the agencies that normally oversee organ donation or from non-profit organizations that provide human biomaterials for research (e.g., in the USA – National Disease Research Interchange, www.ndri.com). Criteria for specimen acceptability are discussed in **Note 3**. Finally, non-CF hAE cells

are now available from a variety of commercial suppliers, circumventing the need for tissue procurement.

2.1.2. Media

Two closely related media are employed. Bronchial epithelial growth medium (BEGM) is used when plating initial cell harvests on type I/III collagen-coated plastic dishes or to expand passaged cells on plastic. Air–liquid interface (ALI) medium is used to support growth and differentiation on porous supports. Composition of BEGM and ALI medium is given in **Table 18.1** and the differences between BEGM and ALI medium are illustrated in **Table 18.2**. The base media (LHC basal; Invitrogen, Carlsbad, CA, Cat. #12677, and DMEM-H; Invitrogen, Cat. #11995-065) can be purchased commercially and additives are made as specified below.

2.1.3. Stock Additives for ALI Medium and BEGM

Additives are 0.2 μM filtered (unless all components are sterile) and aliquots are stored at –20°C for up to 3 months unless otherwise specified.

1. Bovine serum albumin 300× (150 mg/mL): Add PBS to BSA (Sigma-Aldrich, St. Louis, MO, Cat. #A7638) at a concentration of >150 mg/mL, gently rock or stir at 4°C for 2–3 h until dissolved, and adjust volume to yield 150 mg/mL.
2. Bovine pituitary extract (BPE) (dilution depends on lot, typically 125×): BPE is available from Sigma-Aldrich (Cat. #P1476) and is used at a final concentration of 10 μg/mL. Check the protein concentration per milliliter of the specific lot to determine the dilution factor.
3. Insulin 1000× (5 mg/mL; 0.87 mM): Dissolve insulin (Sigma-Aldrich, Cat. #I6634) in 0.9 N HCl.
4. Transferrin 1000× (10 mg/mL; 0.125 mM): Reconstitute human holo transferrin (Sigma-Aldrich, Cat. #T0665) in PBS.
5. Hydrocortisone 1000× (0.072 mg/mL; 0.21 mM): Reconstitute hydrocortisone (Sigma-Aldrich, Cat. #H0396) in distilled water (dH_2O).
6. Triiodothyronine 1000× (0.0067 mg/mL; 0.01 mM): Dissolve triiodothyronine (Sigma-Aldrich, Cat. #T6397) in 0.001 M NaOH.
7. Epinephrine 1000× (0.5 mg/mL; 2.7 mM): Dissolve epinephrine (Sigma-Aldrich, Cat. #E4250) in 0.01 N HCl.
8. Epidermal growth factor 1000× for BEGM, 50,000× for ALI medium (25 μg/mL; 4 μM):
   Dissolve human recombinant, culture-grade EGF (Invitrogen, Cat. #PHG0313) in PBS.

Table 18.1
BEGM and ALI medium composition

Additive		Final concentration in media	Company	Cat. #
Bovine serum albumin		0.5 mg/mL	Sigma-Aldrich	A7638
Bovine pituitary extract		10 μg/mL	Sigma-Aldrich	P1476
Insulin		0.87 μM	Sigma-Aldrich	I6634
Transferrin		0.125 μM	Sigma-Aldrich	T0665
Hydrocortisone		0.21 μM	Sigma-Aldrich	H0396
Triiodothyronine		0.01 μM	Sigma-Aldrich	T6397
Epinephrine		2.7 μM	Sigma-Aldrich	E4250
Epidermal growth factor		25 ng/mL – BEGM 0.50 ng/mL – ALI medium	Invitrogen	PHG0313
Retinoic acid		5×10^{-8} M	Sigma-Aldrich	R2625
Phosphorylethanolamine		0.5 μM	Sigma-Aldrich	P0503
Ethanolamine		0.5 μM	Sigma-Aldrich	E0135
Zinc sulfate		3.0 μM	Sigma-Aldrich	Z0251
Penicillin G sulfate		100 U/mL	Sigma-Aldrich	P3032
Streptomycin sulfate		100 μg/mL	Sigma-Aldrich	S9137
Gentamicin[a]		50 μg/mL	Sigma-Aldrich	G1397
Amphotericin[a]		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	30 nM	Sigma-Aldrich	S5261
	Manganese	1 nM	Sigma-Aldrich	M5005
	Silicone	500 nM	Sigma-Aldrich	S5904
	Molybdenum	1 nM	Sigma-Aldrich	M1019
	Vanadium	5 nM	Sigma-Aldrich	398128
	Nickel sulfate	1 nM	Sigma-Aldrich	N4882
	Tin	0.5 nM	Sigma-Aldrich	S9262

[a]Not in ALI medium

9. Retinoic acid (concentrated stock = 1×10^{-3} M in absolute ethanol, 1000× stock = 5×10^{-5} M in PBS with 1% BSA): Retinoic acid (RA) is soluble in ethanol and is light sensitive. Dissolve 0.3125 mg of RA (Sigma-Aldrich, Cat. #R2625) per mL in 100% ethanol. Store in foil-wrapped tubes at –70°C for up to 2 weeks. To prepare the 1000× stock, first confirm the RA concentration of

Table 18.2
Differences between ALI medium and BEGM

	ALI medium	BEGM
Base media	LHC basal:DMEM-H 50:50	LHC basal 100%
Base antibiotics	Penicillin/streptomycin (100 U/mL/100 μg/mL)	Penicillin/streptomycin (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 mM	0.11 mM

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, blanked on 100% ethanol. The molar extinction coefficient of RA in ethanol equals 44,300 $M^{-1}cm^{-1}$ at 350 nm. Thus, the absorbance of the diluted stock should equal 0.44. RA with absorbance readings below 0.18 should be discarded. If the absorbance equals 0.44, 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* step 1). For absorbance values less than 0.44, calculate the needed volume of ethanol stock as 1.35/absorbance unit and adjust the PBS volume appropriately.

10. Phosphorylethanolamine 1000× (0.07 mg/mL; 0.5 mM): Phosphorylethanolamine (Sigma-Aldrich, Cat. #P0503) is dissolved in PBS.
11. Ethanolamine 1000× (0.03 μL/mL; 0.5 mM): Dilute ethanolamine (Sigma-Aldrich, Cat. #E0135) in PBS.
12. Stock 11 1000× (0.863 mg/mL; 3 mM): Dissolve zinc sulfate (Sigma-Aldrich, Cat. #Z0251) in dH_2O. Store at room temperature.
13. Penicillin–streptomycin 1000× (100,000 U/mL and 100 mg/mL): Dissolve penicillin-G sodium (Sigma-Aldrich, Cat. #P3032) and streptomycin sulfate (Sigma-Aldrich, Cat. #S9137) in dH_2O for a final concentration of 100,000 U/mL and 100 mg/mL, respectively.
14. Gentamicin 1000× (50 mg/mL): Sigma-Aldrich, Cat. #G1397. Store at 4°C.
15. Amphotericin B 1000× (250 μg/mL): Sigma-Aldrich, Cat. #A2942.
16. Stock 4 1000×: Combine 0.42 g ferrous sulfate (Sigma-Aldrich, Cat. #F8048), 122.0 g magnesium chloride (J.T.

Table 18.3
Stock solutions for trace elements

Component	Sigma-Aldrich, Cat. #	Amount/100 mL	Molarity
Selenium ($NaSeO_3$)	S5261	520 mg	30.0 mM
Manganese ($MnCl_2 \bullet 4H_2O$)	M5005	20.0 mg	1.0 mM
Silicone ($Na_2SiO_3 \bullet 9H_2O$)	S5904	14.2 g	500 mM
Molybdenum [$(NH4)_6Mo_7O_{24} \bullet 4H_2O$]	M1019	124.0 mg	1.0 mM
Vanadium (NH_4VO_3)	398128	59.0 mg	5.0 mM
Nickel ($NiSO_4 \bullet 6H_2O$)	N4882	26.0 mg	1.0 mM
Tin ($SnCl_2 \bullet 2H_2O$)	S9262	11.0 mg	500 μM

Baker, Phillipsburg, NJ, Cat. #2444), 16.17 g calcium chloride dihydrate (Sigma-Aldrich, Cat. #C3881), and 800 mL dH_2O in a volumetric flask. Add 5.0 mL concentrated HCl. Stir to dissolve and bring volume to 1 L.

17. Trace elements 1000×: Prepare seven separate 100 mL stock solutions (*see* **Table 18.3**). Fill a 1-L volumetric flask 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 concentrated HCl. Store at room temperature.

2.1.4. BEGM and ALI Medium

We describe here production of 500 mL or 1 L batches, which are assembled in the reservoir of a 0.2-μm bottle top filter. Larger quantities (e.g., > 6 L) can be prepared in a volumetric flask and sterilized by peristaltic pumping (e.g., Masterflex pump; Cole-Parmer Instruments, Vernon Hills, IL, Cat. #EW77910-20) through a cartridge filter (Pall, Ann Arbor, MI, Cat. #12991). To clean tubing, rinse with dH_2O, then ethanol followed again by dH_2O.

1. BEGM: Dispense thawed additives into 100% LHC basal medium (Invitrogen, Cat. #12677) in a bottle top filter unit. Note that some additives are not 1000×. Add amphotericin after filtering. Store media at 4°C.
2. ALI medium: The ALI base is 50:50 DMEM-H (e.g., Invitrogen, Cat. #11995-065) and LHC basal (Invitrogen, Cat. #12677). Thaw and dispense additives as above. Note that some additives are not 1000×. ALI medium contains low EGF and omits gentamicin and amphotericin. To prepare low endotoxin medium, use low endotoxin BSA (Sigma-Aldrich, Cat. #A2058).

2.1.5. Antibiotics

Primary human tissues, even from non-CF sources, frequently contain yeast, fungi, or bacteria. Media for passage 0 cultures should be supplemented with at least gentamicin (50 μg/mL) and amphotericin (0.25 μg/mL) for the first 3–5 days. Less contamination will result by increasing the amphotericin concentration to 1.25 μg/mL and adding ceftazidime (100 μg/mL), tobramycin (80 μg/mL), and vancomycin (100 μg/mL). When processing tissues from CF patients, additional antibiotics are used as described in a prior publication (21). If no information is available, and assuming *P. aeruginosa* contamination, consider adding ciprofloxacin (20 μg/mL), meropenem (100 μg/mL), and colymycin (5 μg/mL). CF lungs infected with *Alcaligenes xylosoxidans, Burkholderia* sp., or *Stenotrophomonas maltophilia* may require a different spectrum of antibiotics including sulfamethoxazole/trimethoprim (80 μg/mL), chloramphenicol (5 μg/mL), minocycline (Sigma-Aldrich, Cat. #M9511, 4 μg/mL), tigecycline (2 μg/mL), or moxifloxacin (20 μg/mL). For fungus or yeast contamination, nystatin (Sigma-Aldrich, Cat. #N1638, 100 U/mL) and diflucan (25 μg/mL) can be added. Antibiotics listed above without sources are from the hospital pharmacy. Sterile liquids for injection may be added directly to media, whereas powders contain a given amount of antibiotic and unknown quantities of salts and buffers – purity of powders is determined by comparing the total vial powder weight to the designated antibiotic content and adjusting the micrograms per milliliter accordingly. A 25× concentrated antibiotic cocktail can be stored at 4°C and used within 1–2 days. Note that nystatin and amphotericin are suspensions and cannot be filter sterilized.

2.1.6. Assorted Reagents and Solutions

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

1. Ham's F-12 medium with 1 mM L-glutamine: Mediatech, Manassas, VA, Cat. #10-080. Store at 4°C.
2. Cell freezing solution: Combine 2 mL of 1.5 M HEPES (pH 7.2), 10 mL of fetal bovine serum (Sigma-Aldrich, Cat. #F6178), and 78 mL Ham's F-12 medium. Gradually add 10 mL DMSO (Sigma-Aldrich, Cat. #D2650).
3. 1% Protease XIV with 0.01% DNase (10× stock): Dissolve protease XIV (Sigma-Aldrich, Cat. #P5147) and DNase (Sigma-Aldrich, Cat. #DN25) in desired volume of PBS and stir. A 1:9 dilution in JMEM (*see* step 6) is used for cell dissociation.
4. Soybean trypsin inhibitor (1 mg/mL): Dissolve soybean trypsin inhibitor (Sigma-Aldrich, Cat. #T9128) in Ham's F-12. Store at 4°C.

5. 0.1% Trypsin with 1 mM EDTA in PBS: Dissolve trypsin type III powder (Sigma-Aldrich, Cat. #T4799) in PBS. Add EDTA from concentrated stock for a final concentration of 0.1% trypsin with 1 mM EDTA. Adjust pH of the solution to 7.2–7.4.
6. Joklik minimum essential medium (JMEM): Sigma-Aldrich, Cat. #M8028. Store at 4°C.
7. Type I/III collagen: Purecol® (Advanced BioMatrix, San Diego, CA, Cat. #5005). Store at 4°C.

2.1.7. Porous Supports

There are multiple porous support options for ALI cultures. Ideal supports are optically clear, facilitate attachment and long-term growth, and are amenable to downstream analyses. However, in our experience, there have been problems with membrane consistency and quality control. We strongly recommend coating all porous supports with human type IV placental collagen (*see* **Section 3.1.3**).

1. Recommended: Transwell-Clear®, 0.4 μM pore size (Corning, Inc., Cat. #'s 3450, 3460, and 3470) or Snapwell® (Corning, Inc., Cat. #3801) membranes.
2. We have also had recent success with Millipore Isopore™ membrane (polycarbonate) (Millipore Corporation, Billerica, MA, Cat. #PIHP01250) but these are not optically clear and thus not amenable to direct visualization on an inverted microscope.

2.2. Materials for Production of Retroviral and Lentiviral Vectors

1. Human 293T embryonic kidney cells (ATCC, Manassas, VA, Cat. #CRL-11268, *see* **Note 4**) are cultured in DMEM with 4500 mg/L glucose, sodium pyruvate, and L-glutamine (Invitrogen, Cat. #11995-065) supplemented with 10% FBS.
2. Both retroviral and lentiviral vectors are produced using three-plasmid co-transfection. For production of HIV-1-based lentiviral vectors, pCMVdeltaR8.74 (22) and pCI-VSV-G (23), which encode HIV-1 Gag-Pol and the VSV-G envelope, respectively, provide necessary helper functions. A third plasmid, which carries the transgene of interest (promoter plus the transgene open reading frame), also contains HIV-1 sequences necessary for encapsidation and a single round of replication. For production of murine leukemia virus (MLV)-based retrovirus vectors, the necessary helper functions are provided for by the pCI-GPZ Gag-Pol expression vector (23) and pCI-VSV-G (23). As before, a third plasmid encodes the gene of interest as well as MLV sequences permitting encapsidation and replication. Plasmid DNA is purified using an endotoxin-free plasmid purifica-

tion kit (Qiagen, Valencia, CA, Cat. #12362). The DNA concentration for transfection is determined by agarose gel electrophoresis and comparing the supercoiled DNA band to a mass standard run in a parallel lane (High DNA Mass Ladder; Invitrogen, Cat. #10496-016) (*see* **Note 5**).

3. 2× HBS (HEPES buffered saline, 500 mL): Dissolve 6 g HEPES (4-{2-hydroxyethyl}-1-piperazine ethanesulfonic acid) (Boehringer Mannheim, Indianapolis, IN) and 7.3 g NaCl in dH_2O to a final volume of 480 mL. Add 5 mL of 150 mM Na_2PO_4. Adjust pH to 7.10 ± 0.03 with 3 N NaOH and filter (0.2 μm) (*see* **Note 6**).
4. 2 M $CaCl_2$: Dissolve 29.4 g $CaCl_2{\bullet}2H_2O$ in dH_2O to a final volume of 0.1 L and filter (0.2 μm).
5. 150 mM Na_2HPO_4: Dissolve 4.02 g $Na_2HPO_4{\bullet}7H_2O$ in dH_2O to a final volume of 100 mL and filter (0.2 μm).
6. 500 mM Sodium butyrate: Dissolve 0.55 g (Alfa Aesar, Ward Hill, MA, Cat. #A11079-22) in dH_2O to a final volume of 10 mL and filter (0.2 μm). Store at –20°C.
7. PBS: Mediatech, Cat. #21-040.

2.3. Materials for Creation of Airway Epithelial Cell Lines

1. Frozen aliquots of retro- or lentiviruses expressing SV40ER, Bmi-1, and hTERT as prepared in **Section 3.2**. The Bmi-1, hTERT, and SV40ER containing HIV-1-based gene transfer vectors were obtained from Patrick Salmon (24). We typically use undiluted producer cell line supernatants instead of concentrated or purified virus. For purified virus, dilutions must be determined empirically. A multiplicity of infection (MOI) of 1–5 is typical.
2. Polybrene (Sigma-Aldrich, Cat. #H9268, 4.0 mg/mL in dH_2O). Filtered (0.2 μm) and aliquots stored at –20°C.
3. Passage 0 or 1 primary hAE cells on plastic at less than 40% confluence (our example is for cells growing on 100-mm tissue culture dishes – mathematically adjust volume for other formats).
4. Selection agent as appropriate for viral construct (geneticin, 100 μg/mL; puromycin, 1.0 μg/mL; hygromycin, 0.1 μg/mL; *see* **Note 7**).

2.4. Materials for Reporter Gene Assays in ALI hAE Cells

Reagents are stored at room temperature unless otherwise specified:

1. Adenovirus constitutively expressing LacZ (Ad.CMV-lacZ (25), aliquots stored at –80°C) (*see* **Note 1**).
2. Adenovirus expressing NF-κB-responsive firefly luciferase (Ad.NF-κB-fLuc (25), aliquots stored at −80°C) or other promoter-fLuc reporter construct of interest (*see* **Note 1**).

3. Control, overexpression, dominant negative, or shRNA construct of interest cloned into a suitable shuttle vector and made into adenovirus by a vector core or using the AdEasy system (aliquots stored at −80°C).
4. 30 mM Sodium caprate (C10, capric acid, sodium decanoate; Sigma-Aldrich, Cat. #C4151) in PBS, filter sterilized, and aliquots stored at −20°C.
5. Passive lysis buffer, 5× (Promega, Madison, WI Cat. #E194A); aliquots stored at −20°C.
6. Luciferase assay buffer, stored in single-use aliquots at −20°C: to make 25 mL, combine 2.5 mL of 0.25 M glycylglycine (Sigma-Aldrich, Cat. #G1002, 25 mM final) in dH_2O, pH 7.8; 3.75 mL of 0.1 M potassium phosphate buffer (15 mM final), pH 7.8; 3.75 mL of 0.1 M $MgSO_4$ (15 mM final) in dH_2O; 1.0 mL of 0.1 M EGTA in dH_2O (pH 8.0 to dissolve, 4 mM final); 0.5 mL of 0.1 M ATP (Sigma-Aldrich, Cat. #A3377, 2 mM final) in 5 mM Tris, pH 7.5 (stored in single-use aliquots at −20°C); 0.25 mL of 0.1 M dithiothreitol in dH_2O (1 mM final, stored in single-use aliquots at −20°C); and 13.5 mL dH_2O.
7. D-Luciferin solution, stored in single-use aliquots at −80°C (protect from light): To make 90 mL, add 5.0 mg D-luciferin (Sigma-Aldrich, Cat. #L9504, 0.2 mM final); 9.0 mL 0.25 M glycylglycine (Sigma-Aldrich, Cat. #G1002, 25 mM final) in dH_2O, pH 7.8; 9.0 mL of 0.1 M dithiothreitol in dH_2O (10 mM final, stored in single-use aliquots at −20°C) to 72 mL dH_2O.
8. β-gal assay buffer, make fresh: To 20 mL PBS, add 10 μL of 0.5 M chlorophenol red-β-D-galactoside (CPRG, Sigma-Aldrich, Cat. #59767, 0.25 mM final) in dH_2O, aliquots stored at −20°C, 0.2 mL of 0.1 M $MgSO_4$ (1.0 mM final) in dH_2O, and 27 μL β-mercaptoethanol (19.3 mM final).
9. Luminometer (procedure illustrated is for a Turner BioSystems Veritas 96-well luminometer).
10. 96-Well optical plate reader.

2.5. Materials for Protein Expression or Knockdown in ALI hAE Cells

1. Frozen aliquots of retro- or lentiviruses expressing the constitutive or inducible protein, mutant protein, shRNA (*see* **Note 2**), or control (*see* **Notes 5, 8,** and **9**) construct of interest prepared as described below in **Section 3.2**. We have used pSIREN- (http://www.clontech.com) and pSLIK (19)-based constructs for constitutive and inducible expression, respectively. Undiluted virus producer cell line supernatants or empirically determined dilutions of concentrated or purified virus, enabling adequate cell survival after selection, are necessary.

2. Polybrene (*see* **Section 2.3, step 2**).
3. Passage 0 or 1 primary hAE cells on plastic (*see* **Section 3.1.6**). Enough cells should be available to accommodate the experimental treatment and all necessary controls.
4. Selection agent (*see* **Section 3.3, step 11**).
5. Induction agent, depending on vector and construct (we use 1 μg/mL doxycycline in media, filter sterilized, for the pSLIK vector system).

3. Methods

3.1. Primary Human Airway Epithelial (hAE) Cell Cultures

3.1.1. Primary Cell Culture Overview

Primary hAE cells can be obtained from nasal, tracheal, or lung tissue specimens and can be seeded directly onto porous supports for passage 0 air–liquid interface cultures or can be first grown on plastic for cryopreservation and/or sub-culture of passage 1 or passage 2 cells to porous supports (**Fig. 18.3**).

3.1.2. Type I/III Collagen Coating of Plastic Dishes

Passage 0 and freshly thawed, cryopreserved cells are plated on collagen-coated plastic dishes, whereas cells passaged without freezing do not require coated dishes. Add 3.0 mL of 1:75 dilution of Purecol® (*see* **Section 2.1.6, step** 7) in dH_2O per 100-mm dish. Incubate for 2–24 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 8 weeks at 4°C.

3.1.3. Type IV Collagen Coating of Porous Supports

There are multiple porous support options for ALI cultures. *See* **Section 2.1.7** for a description of the various porous supports for collagen coating:

1. Re-suspend 10 mg of collagen powder (Sigma-Aldrich Type VI, Cat. #C7521) in 20 mL dH_2O and add 50 μL of concentrated acetic acid. Incubate for 30 min at 37°C until dissolved. Filter the solution using a syringe filter (0.2 μm) (Pall, Cat. #PN4192) and store aliquots at –20°C.
2. Thaw frozen stock and dilute 1:10 with dH_2O. Add 100 or 400 μL per 12- and 24-mm insert, respectively, and dry in a laminar flow hood overnight. Expose to UV in a laminar flow hood for 30 min, wrap dishes with parafilm, and store at 4°C for up to 1 month.

3.1.4. Isolating Primary hAE Cells

Primary hAE cells originate from nasal turbinates, nasal polyps, trachea, and bronchi. When handling human tissues, always follow locally prescribed safety precautions to prevent potential blood-borne pathogen exposure. Tissue is transported to the

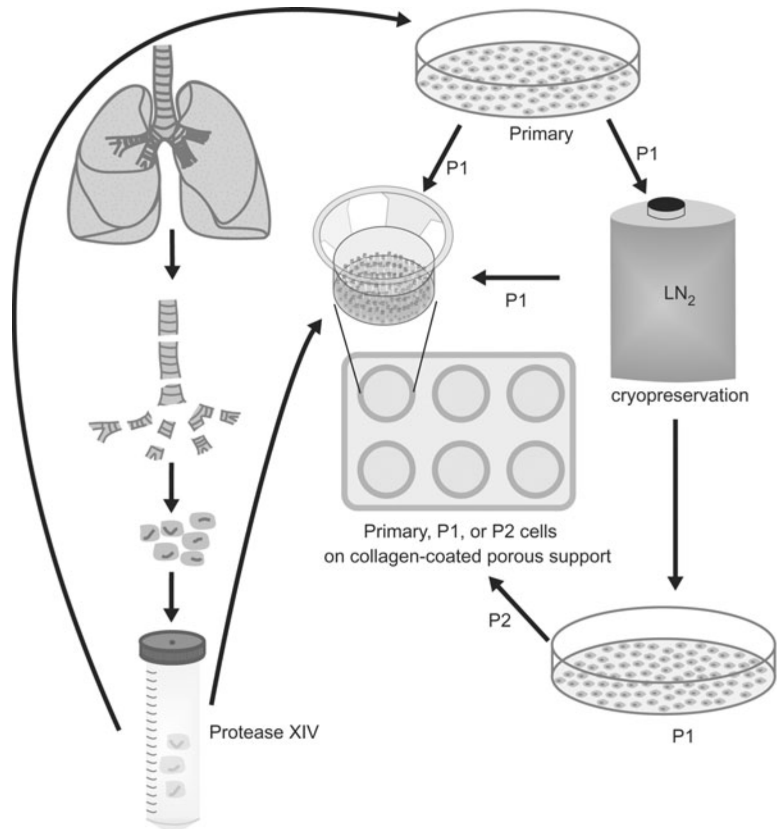

Fig. 18.3. Overview of the process for creating well-differentiated air–liquid interface (ALI) cultures of primary human airway epithelial (hAE) cells. P, passage.

laboratory in sterile containers containing sterile-chilled lactated Ringer's (LR) solution, JMEM, F12, or another physiologic solution. Nasal tissue samples are usually processed without further dissection but whole lungs require dissection as described below:

1. Assemble in a laminar flow hood:
   a. Absorbent bench covering (many suppliers).
   b. Large plastic sterile drape (3M Health Care, St. Paul, MN, Cat. #1010).
   c. Ice bucket containing sterile specimen cups (many suppliers) filled with LR solution.
   d. Use instrument sterilizer (Fine Science Tools, Foster City, CA, Cat. #18000-45) or preautoclaved instruments. Suggested tools include curve-tipped scissors, delicate 4.5 in. (Fisher Scientific, Cat. #08-951-10); heavy scissors, straight, sharp, 11.5 cm (Fine Science Tools, Cat. #14058-11); forceps, blunt-pointed, straight, 15 cm (Fine Science Tools, Cat. #11008-15); rat-tooth forceps 1 × 2, 15.5 cm (Fine Science Tools, Cat. #11021-15);

scalpels, #10 (many suppliers); sterile 4-in. × 4-in. cover sponges (many suppliers).

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 rinse by "dipping" in LR solution. Slit segments longitudinally and cut into 1 cm × 2 cm portions. Transfer to specimen cup containing chilled LR solution.

3. Since human tissue samples are likely to contain yeasts, bacteria, or fungi, begin antibiotic exposure as soon as possible. Prepare 250 mL of "wash media" – JMEM plus desired antibiotics (*see* **Section 2.1.5**). Aspirate LR solution and add "wash media," swirl, and repeat three times. Transfer washed tissue to 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 platform rocker (50–60 cycles/min) at 4°C for 24 h.

4. CF tissues or others with abundant mucus are soaked in dithiothreitol (DTT, 0.5 mg/mL; Sigma-Aldrich, Cat. #D0632) and DNase (10 μg/mL; Sigma-Aldrich, Cat. #DN25) plus supplemental antibiotics (*see* **Section 2.1.5**). To prepare "soak solution," add 65 mg DTT and 1.25 mg DNase to 125 mL of "wash media" and filter sterilize. Aspirate LR solution, add 40 mL "soak solution," swirl, soak for 5 min, repeat, then rinse tissue three times in "wash media." Transfer tissue to 50-mL tubes containing 30 mL "wash media" plus 4 mL protease/DNase (final volume 40 mL). Place tubes on platform rocker (50–60 cycles/min) at 4°C for 24 h.

5. Nasal turbinates, polyps, and small bronchial specimens can be dissociated in 4–24 h, depending on the size of the tissue, in 15-mL tube containing 9 mL "wash media" plus 1 mL protease solution.

3.1.5. Harvesting Cells

Follow standard sterile tissue culture techniques in a laminar flow hood:

1. End dissociation by pouring contents of 50-mL tubes into a 150-mm tissue culture dish; add fetal bovine serum (Sigma-Aldrich) to a final concentration of 10% (v/v).

2. Gently scrape epithelial surface with a #10 scalpel blade. Rinse tissue and plate surface with PBS, collect and pool solutions, and distribute into 50-mL conical tubes.

3. Centrifuge at 500×g for 5 min at 4°C. Aspirate supernatant and add 12 mL of declumping solution (2 mM EDTA, 0.05 mg/mL DTT (Sigma-Aldrich, Cat. #D0632), 0.25 mg/mL collagenase (Sigma-Aldrich, Cat. #C6885),

0.75 mg/mL calcium chloride (Sigma-Aldrich, Cat. #C3881), 1 mg/mL magnesium chloride (Sigma-Aldrich, Cat. #M8266), and 10 μg/mL DNase in PBS). Incubate for 15 min to 1 h at 37°C, visually monitoring clump dissociation. Add FBS to a final concentration of 10% (v/v), centrifuge at 500×g for 5 min, remove supernatant, and re-suspend pellet in F12 for counting using a hemocytometer.

3.1.6. Plating Cells

Culture dissociated P0 hAE cells directly on porous supports in ALI medium with additional antibiotics (*see* **Section 2.1.5**) at a density of 0.1–0.25 × 10^6 cells per cm^2 (0.8–2.0 × 10^5 cells per 12-mm support or 0.7–1.75 × 10^6 cells per 24-mm support, *see* **Note 10**). To generate P1 or P2 cells, plate cells in antibiotic-supplemented BEGM on type I/III collagen-coated plastic dishes at a concentration of 2–6 × 10^6 cells per 100-mm dish. Change media at 24 h and every 2–3 days as needed to prevent acidification.

3.1.7. Cell Culture Maintenance

1. *P0 cells on plastic:* Assess attachment after 24 h of plating P0 cells; if few clumps of floating cells are present, wash with PBS and feed with BEGM plus antibiotics (*see* **Section 2.1.5**). Rescue floating clumps of cells by washing dishes with PBS, harvesting into 50-mL conical tubes, pelleting at 500×g for 5 min, and repeating the "declumping" procedure (*see* **Section 3.1.5, step 3**).
2. *Passaging primary cells on plastic*: Passage primary cultures at 70–90% confluence. Harvest hard to detach cells, while minimizing trypsin exposure of cells that release quickly using "double trypsinization." Rinse cells with PBS, add 3 mL of trypsin/EDTA per 100-mm dish, and incubate for 5–10 min at 37°C. Gently tap dish to detach cells, rinse with PBS, and harvest into 50-mL conical tube containing 3 mL STI solution on ice. Add another 3 mL of trypsin/EDTA to the dish and repeat, visually monitoring detachment. Pool harvested cells and centrifuge at 500×g for 5 min at 4°C. Aspirate supernatant and re-suspend cells in media for counting.
3. M*edia change in ALI cultures:* For P0, P1, or P2 hAE cells grown on collagen-coated porous supports, remove apical media and rinse the apical surface with PBS. Prior to confluence, replace apical and basolateral media volumes as specified for the porous support, but after confluence, do not add media apically. During periods of rapid cell growth, cells on Transwell® inserts in the standard configuration will acidify the media rapidly and require daily changes. We have devised Teflon adapters to enable 12-mm Transwell® inserts to be kept in six-well plates with a 2.5-mL basolateral reservoir, which decreases media change frequency. The 24-mm Transwell® insert may be kept in "Deep-Well Plates" (BD

Bioscience, Bedford, MA, Cat. #355467) with 12.5 mL media.

3.1.8. Cryopreservation of Cells

1. Trypsinize P0 hAE cells from plastic dishes (now P1 cells) and cryopreserve for long-term storage in liquid nitrogen. Re-suspend cells 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* **Section 2.1.6, step 2**) to the cell suspension.
3. Place cryovials in Nalgene cryofreezing container (Nalgene® Labware, Rochester, NY, Cat. #5100) and place in –80°C freezer for 4–24 h.
4. Transfer vial(s) from the –80°C freezer to liquid N_2 (–196°C) for long-term storage.

3.1.9. Thawing Cells

1. Thaw the cryovial at 37°C and wipe outside with 70% ethanol. Transfer cells to a 15-mL conical tube.
2. Dilute the cell suspension by slowly filling the tube with Ham's F-12. Centrifuge at 500×g for 5 min at 4°C.
3. Gently re-suspend cells in media, count, and assess viability.

3.2. Production of Retroviral and Lentiviral Vectors

1. Culture human 293T embryonic kidney cells in DMEM with 4500 mg/L glucose, sodium pyruvate, and L-glutamine supplemented with 10% FBS. Grow cells on plastic dishes or flasks at 37°C in a 5% CO_2 incubator and split 1:8 every 3 days. For routine splitting (100-mm plates), remove and discard media, rinse cell layer briefly with PBS, and add 3 mL of trypsin/EDTA solution to plates until the cell layer is dispersed (usually within 5 min). Add 6–8 mL of complete growth medium and aspirate cells by gently pipetting. Add appropriate aliquots of cells to new culture vessels.
2. Seed 293T cells at 4.5 × 10^6 cells/100-mm tissue culture dish to obtain ~80% confluence the next day. Incubate overnight at 37°C in a humidified incubator with 5% CO_2.
3. In polystyrene tubes, mix 15 μg gene transfer vector, 15 μg Gag-Pol expression vector, and 9 μg VSV-G expression vector to give a final volume of 262.5 μL. Add 37.5 μL of 2 M $CaCl_2$.
4. For each transfection, aliquot 300 μL of 2× HBS solution into a polystyrene tube. To this add the 300 μL DNA/$CaCl_2$ mixture dropwise (but somewhat rapidly) and then mix gently. Incubate for 10 min at room temperature.
5. Remove medium from cells and replace with 6 mL fresh growth medium. Next add 600 μL plasmid sample, prepared

as above, to the cells dropwise. Swirl the plate gently to mix and incubate overnight at 37°C.

6. Remove the medium and replace with 6 mL of fresh growth medium per plate containing 10 mM sodium butyrate (*see* **Note 11**). Incubate cells with sodium butyrate for 8–10 h. Remove medium and replace with 7 mL fresh medium (without sodium butyrate) containing 2% FBS and return cells to incubator. Incubate for 16–24 h.
7. The next day, swirl the plates gently, then remove virus, and filter through a 0.2-μm polyethersulfone (PES) filter. Store virus in 1 mL aliquots at –80°C. Just prior to use, thaw the virus in a water bath at 37°C.

3.3. Creation of Airway Epithelial Cell Lines

1. Employ specified personal protective equipment (lab coat, double gloves, safety glasses, and particle mask) and secure the cell culture area (post notice and close the door to limit non-essential access). Experiments involving human cells and recombinant viral vectors require Biological Safety Level 2 containment. Consult your local Institutional Biosafety Committee for advice on safe working practices when using these materials.
2. Prepare 20% chlorine bleach solution in a suitable beaker and place in tissue culture hood (all virus-contaminated items are to be bleached for at least 30 min).
3. Transfer cells (one cell type at a time, *see* **Note 12**) to hood, aspirate media, and add room-temperature PBS.
4. Thaw polybrene (need 2 μL/mL of virus-containing media).
5. Rapidly thaw virus by swirling vial in 37°C water, record vial identification on culture dish lid and notebook, and clean outside of vial with 70% ethanol.
6. Aspirate PBS.
7. Add 1.5 mL of SV40ER or *Bmi-1* virus supernatant and 1.5 mL of *hTERT* virus supernatant per 100-mm diameter dish (i.e., one oncogene plus *hTERT*, in 3 mL total).
8. Add 6 μL of polybrene (*see* **Section 2.3, step 2**) and gently swirl in a figure eight pattern to distribute evenly.
9. Incubate for 3 h at 37°C and gently swirl every hour.
10. Remove virus-containing media, wash with PBS, and add growth media.
11. Initiate appropriate selection (if desired; *see* **Note 13**) for 7 days beginning 48 h after infection and change media every 2–3 days, preventing acidification.

12. When cells reach 70–90% confluence, trypsinize and cryopreserve an aliquot (*see* **Sections 3.1.7, step 2** and **3.1.8**). Passage the remainder for expansion.
13. Cryopreserve cells at regular intervals during passaging.

3.4. Reporter Gene Assays in ALI hAE Cells

1. Use well-differentiated ALI hAE cultures in replicates of 3–4 wells per experimental group (*see* **Note 14**).
2. Soak apical surface with PBS (100 μL for each 10–12-mm insert) for 20 min 24 h prior to adenovirus exposure and rinse one more time with PBS to remove excess mucus.
3. On the day of adenovirus exposure, wash the apical surface once with PBS and aspirate the residual liquid.
4. Prepare adenovirus solution in ALI media (room temp.). Optimal concentrations should be determined by titration but are typically in the range of 0.5×10^7 CFU/mL for the reporter viruses (10× greater for co-transduction with test constructs of interest when used).
5. Add 30 mM sodium caprate in PBS to the apical surface *only*, 50 μL per 10–12-mm insert, for 3 min. Aspirate and wash the apical surface once with PBS (*see* **Note 15**).
6. Infect cells by applying adenovirus to both the apical and the basolateral surfaces in volumes needed for the specific culture format (50 μL apically and 1 mL basolaterally for a 10–12-mm insert in a 12-well plate). Perform equivalent maneuvers, but without virus in duplicate control wells. Place in 37°C, 5% CO_2 incubator for 2 h.
7. Aspirate apical and basolateral media.
8. Replace basolateral media without virus; cells are ready for challenge and assay 24–48 h later.
9. Eight hours after challenge (if needed, as per the experimental design), lyse cells by removing media, transferring to a Petri dish, and adding 100 μL of 1× passive lysis buffer while pressing down on the insert and scraping cells with a rubber policeman. Repeat 1× per well for a total of 200 μL pooled sample. Assay fLuc and β-gal on the day of harvest or freeze in aliquots and store at –20°C, avoiding repeated freeze–thaw cycles. Handle all samples to be directly compared identically (including assays below).
10. Assay fLuc as per the luminometer format. Samples may need dilution, determined empirically, if very high activity. For automated 96-well format (e.g., Turner Veritas luminometer), add 90 μL luciferase assay buffer per well of an opaque 96-well plate and add 20 μL of lysate supernatant (14,000×g spin for 2 min) per well. Program the luminometer: one injector, no plate read before injection, 40 μL of luciferin solution per well, 0 s delay time, and

5 s integration time. Prime the injector with luciferin solution (requires 500 μL). Perform reading. Reverse purge the injector 3X dH_2O, 3X 70% ethanol, 3X dH_2O, 3X air.

11. Assay β-gal by adding 180 μL of β-gal assay buffer per well of 96-well flat-bottomed clear plate. Add 20 μL of lysate supernatant (14,000×g spin for 2 min) per well, using non-transfected cell lysate as a control. Use adhesive 96-well plate tape and shake for 5 min. Incubate at 37°C until color develops, typically 10 min to 3 h. Read at 575 nm; values from 0.12 to 1.8 are within the linear range.
12. Express data as fLuc light units (arbitrary units) divided by β-gal OD 575 (- blank).

3.5. Protein Expression or Knockdown in ALI hAE Cells

1. Using protocols described in **Sections 1.2** and **3.2**, primary hAE cells on plastic are transduced with retroviral or lentiviral vectors and selected.
2. Selected cells are trypsinized, counted, and passaged to porous supports as in **Sections 3.1.6** and **3.1.7**, *see* **Note 16**.
3. For most studies, cultures are allowed to grow and differentiate at an ALI. Studies of protein function can be conducted at any time if a constitutive promoter is being used, but generally, differentiated cells are preferred (~ 3 weeks).
4. If induction is required, the relevant induction agent is added once the cells have reached the preferred differentiation state. In a 48–72 h time frame after induction (or longer), changes in mRNA and/or protein expression can be determined by quantitative RT-PCR or Western blots, respectively, using routine protocols which are beyond the scope of this chapter (*see* **Note 17**). Functional changes induced by the genetic manipulation are examined as per the experimental purpose.

4. Notes

1. The Ad.CMV-*lacZ* and Ad.NF-κB-*fLuc* viruses described herein (25) were originally created in the University of Iowa Vector Core (http://www.uiowa.edu/~gene/) and amplified in the University of North Carolina Vector Core (http://genetherapy.unc.edu/jvl.htm); these cores and others (e.g., http://www.med.upenn.edu/gtp/vector_core.shtml) provide services for external investigators.
2. Although results will vary across genes and shRNA sequences, we have obtained 60–70% mRNA knockdown in well-differentiated hAE cells with effective shRNA

sequences, employing either constitutive or inducible expression systems with a corresponding, or somewhat greater, decrease in protein expression and function.

3. In our experience, autopsy specimens must be procured within approximately 8 h of time of death, but surgical pathology specimens can be stored for up to 3 days at 4°C. To protect personnel, do not accept specimens posing a known infection risk for HIV, Hep B and C, or tuberculosis. Samples from individuals on long-term immunosuppressive therapy may pose increased risk. All human tissue samples must be treated as a potential biohazard and handled using standard precautions. Steps for specimen procurement may breach sterility and/or tissues (especially CF) are likely infected with bacteria or fungi, thus appropriate antibiotics are necessary (*see* **Section 2.1.5**). A range of clinical data (laboratory values including blood gases, X-ray, and bronchoscopy findings) can guide acceptability of sub-transplant quality donor lungs. There are no hard and fast rules, but an arterial PO_2 of greater than 100 mmHg on 100% inspired oxygen is a reasonable lower limit for acceptance.
4. 293T cells obtained from different sources have variable properties and optimal cell numbers for maximum vector production may need to be determined empirically. We have found it useful to single-cell clone 293T cells from a trusted source and to test clonal cell lines for their ability to be transfected at high efficiency (95–100%) and their ability to produce vectors at high titer (>10^6 infectious units/mL) (23). Clonally derived cells with the desired properties can be cryopreserved and thawed when needed. After thawing, cells usually retain their vector-producing properties for at least 1–2 months before they need to be replaced.
5. Retroviral/lentiviral gene transfer vectors are available from a number of sources. For constitutive expression of a target gene as well as an antibiotic selection marker, we have had good success using the murine leukemia virus-based Retro-X Q Vectors from Clontech. The pSIREN-RetroQ gene transfer vector is a murine leukemia virus-based vector (Clontech, Cat. #PT3737-5) used for constitutively expressing small hairpin RNA (shRNA). The pSLIK vectors (ATCC, Cat. #MBA-268) are HIV-1-based gene transfer vectors used for inducible shRNA expression (19). On occasion, plasmids purified using the Qiagen methods are contaminated with insoluble material (most likely residue from the column). In such cases, the plasmid DNA is clarified by centrifugation (10,000×g, 5 min), and the DNA in the supernatant is transferred to a new tube.

6. The pH of the 2× HBS solution is critical for obtaining efficient transfection. We usually make up several 2× HBS solutions that vary in pH between 7.05 and 7.2 and then test to see which 2× HBS solution yields the best vector production. 2× HBS can be stored for several months at room temperature.
7. The selection agent concentrations are based on our experience with passage 0–2 primary hAE cells. Densely growing cells are more resistant to selection agents. Maintaining selection for at least 7 days and passaging dense cells into media with selection agent (if necessary) is recommended.
8. Screening short RNA sequences for effective knockdown is critical. We typically screen four commercially supplied siRNA sequences in cell lines that express the protein of interest (16HBE (13) or UNCN3T (14) cells) followed by quantitative RT-PCR, or Western blot if antibodies are available. We have had good results with the Amaxa cell transfection system (Lonza, Walkersville, MD). Effective sequences (typically 1–2 out of 4 are found) are cloned into the vector of choice using appropriate methods. Screening with siRNAs may not be possible for genes/proteins not expressed in cell lines on plastic (e.g., ciliated cell-specific genes), in which case multiple shRNA vectors and empirical testing on the hAE cells will be necessary to identify active sequences.
9. Appropriate controls are essential, especially for shRNAs potentially having off-target and/or non-target effects (e.g., interferon response). Empty vector, scrambled shRNA, and/or shRNAs to irrelevant genes are good choices. Rescue by expression of an shRNA-resistant point mutant protein is the gold standard (26).
10. Seeding densities: Primary human airway epithelial cells are mortal and require sufficient seeding density. Attachment and growth of cells from different individuals and preparations may vary. Generous seeding densities of passage 0 cells on porous supports (>1.5×10^5 cells/cm^2) are required to obtain consistent, confluent, well-differentiated ALI cultures. Although it is tempting to expand primary cells on plastic, "overexpansion" should be avoided. Passage 0 cells first grown on plastic dishes should be seeded at not less than 1×10^6, and preferably $2–6 \times 10^6$, cells per 100-mm collagen-coated dish (or as calculated mathematically for other dish sizes). Under these conditions, the cells should grow to >70% confluence within 5–7 days – if a longer period is required, subsequent growth may be impaired. Cells at >70% confluence, but not >95%

confluence, should be trypsinized for cryopreservation or subpassage to a porous support or expanded one more round to passage 2 by seeding >1 × 10^6 cells per 100 mm tissue culture dish. Passage 1 and 2 cells seeded on porous supports at ~1.5 × 10^5 cells/cm^2 (~170,000 and ~0.7 × 10^6 cells per 12- and 24-mm Transwell® membranes, respectively) should result in confluence within 3–5 days 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 with aliquots of frozen cells, but is not possible when plating passage 0 cells, and greater variability is anticipated between different preparations.

11. Production of retroviral/lentiviral vectors from most sources of 293T cells is enhanced up to fivefold by treatment with sodium butyrate. An alternate method of treatment is to leave the sodium butyrate-containing medium with 2% FBS on the cells overnight prior to harvesting the virus the next day. The brief exposure of hAE cells to sodium butyrate in the resulting virus stock does not appear to affect their growth and differentiation properties.
12. Cross-contamination of cell lines is an important concern and it is a good policy to work with only one cell line at a time in the culture hood.
13. The decision to employ a vector system enabling selection is up to the investigator. Selection ensures more uniform and higher level transgene expression in the cell population. However, resistance to selection agents may limit cell downstream utility or options, e.g., in gene expression or knockdown experiments requiring selection. When seeded at the recommended density, the selected cells should be 70–90% confluent by day 6 or 7 but may take longer if the efficiency of infection is low as indicated by abundant cell death occurring during selection. If the cells are not confluent within 10–12 days, they may not be able to differentiate well at an ALI. To achieve adequate infection efficiencies, it is recommended that the minimum titer of retroviral/lentiviral vectors is 2 × 10^5 infectious units/mL.
14. It is important that ALI cultures are confluent and healthy in order to withstand caprate permeabilization. Access of caprate to the basolateral solution in non-confluent cultures will result in cell exfoliation.
15. We recommend performing pilot experiments with 30 mM sodium caprate exposure to verify that it will not cause excess cell cytotoxicity and cell exfoliation of a given set of wells, and reducing the exposure time if necessary.

16. Having nearly equivalent viral titers for control and experimental vectors and equivalent survival during selection (determined by cell counting) is an important factor. If cell survival differs greatly, then cell growth and differentiation after subculture to an ALI may be different, causing differences in expression or function independent of the specifically introduced changes, thus confounding the interpretation. As always, replication of studies is necessary to reduce the likelihood of misinterpretation.
17. Although we have demonstrated functional knockdown of ENaC using the pSLIK inducible system, GFP-reporter gene expression using this system was not uniform in different cell types in well-differentiated ALI hAE cultures and was predominantly found in columnar, non-ciliated, non-basal cells. Variations in expression among cells likely result from different levels of construct integration as well as the potential for cell-type-specific preferential expression or silencing. Evidently, the CMV enhancer promoters in the pSLIK vector are silenced in ciliated cells. At this point, further studies are needed to comprehensively determine vector backbone and promoter elements resulting in uniform expression in well-differentiated ALI hAE cells. Thus, initial experiments with the vector system to be employed, using a reporter gene such as *GFP*, are strongly recommended to determine whether there is appropriate expression in the differentiated cell types of interest.

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