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Gene Delivery to the Airway

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Abstract

This unit describes generation of and gene transfer to several commonly used airway models. Isolation and transduction of primary airway epithelial cells are first described. Next, the preparation of polarized airway epithelial monolayers is outlined. Transduction of these polarized cells is also described. Methods are presented for generation of tracheal xenografts as well as both ex vivo and in vivo gene transfer to these xenografts. Finally, a method for in vivo gene delivery to the lungs of rodents is included. Methods for evaluating transgene expression are given in the support protocols.

Model systems of differentiated airway epithelium have played a significant role in research pertaining to airway biology, pathophysiology, and gene therapy. The success of such systems is dependent on the ability to reconstitute the native cellular composition and architecture of the airway, within a setting that retains adequate flexibility for experimental manipulation. Furthermore, human airway models have provided significant advantages over other models, since the cell biology of the airway epithelium of humans can differ substantially in function and cellular composition from that of other species such as mice and rats. For example, the predominant secretory cell type in humans is the goblet cell, whereas in mice and rats it is the Clara and serous cell, respectively. Another important consideration is the marked variation observed in the tropism of recombinant viruses commonly used for gene therapy (e.g., adeno-associated virus) with recipient cells from different species.

One of the most widely used human systems to date consists of polarized monolayers of primary airway epithelial cells grown on permeable membrane supports (Karp et al., 2002; Randell et al., 2011; Yamaya et al., 1992). For many studies, this system has provided adequate differentiation when cells are grown at the air-liquid interface. However, the extent of mucociliary differentiation in this experimental model is often inadequate for studies related to in vivo airway epithelial functions. To circumvent these limitations of current airway models, tracheal xenograft models have been developed to study gene transfer and airway pathophysiology in human genetic diseases (Wilson, 1997). These airway xenograft models have proved extremely useful in studying host cell–vector interactions (Engelhardt et al., 1993b; Engelhardt et al., 1992; Goldman and Wilson, 1995) with human airway epithelium, as well as pathophysiology and gene therapy of the cystic fibrosis airways (Goldman et al., 1997; Zhang et al., 1995; Zhang and Engelhardt, 1999; Zhang et al., 1998; Zhang et al., 1996), and the identification of progenitor/stem cell targets for gene therapy in the human airway (Duan et al., 1998a; Engelhardt et al., 1995).

This unit describes generation of and gene transfer to several commonly used airway models. Isolation (see Basic Protocol 1) and transduction (see Basic Protocol 2) of primary airway epithelial cells are first described. Next, the preparation of polarized airway epithelial monolayers is outlined (see Basic Protocol 3). Transduction of these polarized cells by recombinant adenovirus, adeno-associated virus, retrovirus, or lentivirus is also described (see Basic Protocol 4). Methods are presented for generation of human and ferret tracheal xenografts (see Basic Protocol 5) as well as both ex vivo and in vivo gene transfer to these

xenografts (see Basic Protocol 6). Finally, a method for in vivo gene delivery to the lungs of rodents is included (see Basic Protocol 7).

Some methods for the evaluation of transgene expression are given in the support protocols. A method for harvesting xenografts for morphological analysis is described (see Support Protocol 1). The reporter gene β -galactosidase can be detected either histochemically (Support Protocol 2) or immunocytochemically (Support Protocol 3). If green fluorescent protein (GFP) is used as a reporter gene, it can be detected fluorescently (see Support Protocol 4). Finally, histochemical detection of alkaline phosphatase gene activity is described (see Support Protocol 5).

CAUTION: Radioactive, biological, and chemical substances require special handling; see *APPENDIX 2A* for guidelines.

STRATEGIC PLANNING

Choice of Airway Model System

The choice of an airway model system is dependent on the level of differentiation required to address the hypotheses at hand. Several model systems have been utilized that offer flexibility for genetic modification using recombinant vector systems. These model systems include: (1) proliferating cultures of primary airway epithelial cells, (2) polarized airway epithelial monolayers, (3) tracheal xenografts, and (4) intact lung in animal models. Since human airways are most suitable for addressing aspects of human genetic diseases and gene therapy, this unit has emphasized strategies for generating human airway models. For example, several of these model systems, including polarized epithelial monolayers and human tracheal xenografts, have been extremely useful in addressing issues pertaining to gene therapy of cystic fibrosis (CF), since airway tissue from CF patients can be used to generate epithelia. Several factors, listed in Table 13.9.1, may influence the choice of model systems for research, including the extent of epithelial differentiation and whether it is necessary to study human airway cells in the context of genetic diseases.

Choice of Vector System

The choice of vector system for genetically modifying airway model systems is an important consideration in the overall design of experiments aimed at addressing disease pathophysiology, airway biology, and gene therapy. Several vector systems have been successfully used in airway models including recombinant adenovirus (*UNIT 12.4*), recombinant retrovirus (*UNIT 12.5*), recombinant adeno-associated virus (*UNIT 12.9*), and cationic liposome/DNA complexes. Proliferating cultures of primary airway epithelial cells are more efficiently transduced with all of these vectors as compared to more differentiated models. In proliferating cultures of airway cells, typically, transduction with adenovirus is more efficient than transduction with retrovirus, which is more efficient than transduction with adeno-associated virus, which is, in turn, more efficient than transduction with liposomes. However, exceptions for certain applications should also be noted. For example, human tracheal xenografts can be genetically modified by an ex vivo approach where genes are transferred into proliferating airway cells prior to reconstitution of xenograft bronchial airways. In this instance retroviral and lentiviral vectors, which transduce cells by integrating recombinant transgenes, are preferred and are more efficient than recombinant adenovirus. However, in instances where gene transfer is performed in intact bronchial xenografts, recombinant adenovirus is by far the most efficient vector presently available. Additionally, recombinant adenoviral vectors may have a higher level of toxicity resulting from cryptic expression of viral genes. Hence, in some instances other less efficient gene transfer methods may be experimentally more preferable, depending on considerations of vector biology. Furthermore, the extent of gene transfer with some of the existing vector

systems is currently only limited by achievable titers. For example, new technologies for concentrating retroviral and lentiviral stocks have allowed for higher transduction efficiencies than were previously attainable in the airway. In this unit, the most commonly used viral vectors for gene transfer to the airway are described. The relative efficiency of each of these vector systems for use in the different model systems is outlined in Table 13.9.2.

Choosing a Reporter Gene

An important question when evaluating gene delivery to the airway is the efficiency of various vector systems for expressing encoded transgenes. Several reporter genes have been used historically—e.g., chloramphenicol acetyltransferase (CAT), firefly luciferase, renilla luciferase, human growth factor, β -glucuronidase, green fluorescent protein (GFP), β -galactosidase, and alkaline phosphatase. Additionally, a combination of two luminescent reporters, such as renilla luciferase and firefly luciferase, has been used to directly index the relative transduction efficiency in the airway from two viral vectors simultaneously (Flotte et al., 2010). For additional discussion of reporter genes, see CPMB Chapter 9. Since histologic evaluation of transgene expression is more effective in assessing gene transfer at the cellular level, the last three reporter genes mentioned above are the most commonly used. Green fluorescent protein (GFP) provides an additional advantage of allowing transgene expression to be assessed in viable cells. This is often very useful in evaluating the kinetics of gene delivery and transgene expression in a noninvasive manner. However, the application of this approach is most often limited to primary culture models. Another important factor in choosing an appropriate reporter gene is the level of endogenous enzymatic activity. Although experimental approaches have been designed to reduce the extent of background staining, both β -galactosidase and alkaline phosphatase have endogenous activities in airway epithelia. Approaches to minimizing complications of background staining include defined, short reaction times, inhibition and/or inactivation of endogenous enzymatic activities, and the stringent use of negative control vectors.

BASIC PROTOCOL 1 ISOLATION OF HUMAN PRIMARY AIRWAY EPITHELIAL CELLS

Gene transfer to human primary airway epithelial cell model systems has been extremely useful in testing gene therapy approaches and in studying aspects of pathophysiology in genetic diseases such as cystic fibrosis (CF). Although gene therapy for lung diseases is not conducive to ex vivo approaches, methods for generating airway models have been included in this unit because of their importance in gene therapy-related research. Human models of airway epithelium are based on the isolation of primary airway epithelial cells from human lung. A key aspect of this method is the expansion of multipotent airway epithelial cells using hormonally defined growth media under conditions capable of retaining the undifferentiated state of epithelial progenitors. These primary airway cultures can be studied directly or used to generate more differentiated model systems of the airway including polarized monolayers (see Basic Protocol 3) and tracheal xenografts (see Basic Protocol 5). Such models are extremely useful in evaluating complementation of genetic defects using gene therapy approaches—an important first step in the design of effective in vivo gene therapies.

Materials

- Human lung (keep on ice)
- Media A, B, and C (see recipes)

Medium A (see recipe) supplemented with 0.1% (w/v) protease type XIV (e.g., Sigma), 4°C

Fetal bovine serum (FBS; *APPENDIX 3G*)

Ham's F-12 medium (Life Technologies) with and without 10% FBS

0.1% trypsin/EDTA (Life Technologies)

Trypsin inhibitor buffer (see recipe)

Cryopreservation medium: medium C (see recipe) containing 10% DMSO and 10% FBS, 4°C

Dissecting equipment including forceps, scalpel, and hemostat

100- and 150-mm tissue culture dishes (uncoated plastic)

15- and 50-ml conical centrifuge tubes

Platform rocker

Tabletop centrifuge

3-cm² piece of 500-μm nichrome or copper wire mesh, sterile

2-ml cryovials (e.g., Nunc)

Additional reagents and equipment for culturing of mammalian cells and counting viable cells by trypan blue exclusion (*APPENDIX 3G*)

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

Isolate airway tissue

- 1 Dissect bronchial airways from the lung in a tissue culture hood on ice and place airways in medium A at 4°C.

Human airway tissue is best isolated from lung-transplant specimens that have been immediately placed on ice following removal from donors. However, post-mortem lungs have also been used with variable success. Typically in these instances, lungs should be harvested within 6 hr following death.

Dissection can be easily performed down to the fifth-order bronchus.

- 2 Place dissected airways into a 150-mm tissue culture dish with excess medium A. Remove excess adventitia from outside the airways and cut bronchial segments into 1- to 2-cm rings. Cut bronchial rings longitudinally to expose airway surface. Remove any excess mucous secretions.

Wash tissue

- 3 Place cleaned bronchial airway specimens into a 50-ml conical tube with 35 ml medium A and incubate with rocking at 4°C for 30 min.

Enough tissue can be added to bring the final volume of tissue and medium up to 45 ml total.

- 4 Decant medium, replace with fresh medium A, and again rock at 4°C for 30 min. Repeat for a total of six washes.

For tissue from CF patients it is important to wash samples well. If tissue is severely infected at time of harvest, it may be necessary to increase the number of washing steps for a total of 5 to 6 hr.

Dissociate airway epithelial cells

- 5 Place washed bronchial airway samples into a fresh 50-ml conical tube containing 35 ml medium A supplemented with 0.1% protease type XIV. Incubate at 4°C for 36 hr.

Enough tissue can be added to bring the final volume of tissue and medium up to 45 ml total.
- 6 Add FBS to a final concentration of 10% and shake gently for 30 sec to remove epithelial cells.

To ensure adequate agitation, vials should be no more than 75% full at time of shaking.
- 7 After shaking, allow 1 min for the airway tissue to settle to the bottom of the tube (liberated airway cells will remain in suspension). Pipet medium containing airway cells into a fresh tube on ice and place remaining tissue into a 100-mm tissue culture plate.
- 8 Using the blunt side of a scalpel, scrape surface of airways to remove remaining epithelial cells. Rinse airway samples with Ham's F-12 medium/10% FBS and combine washed cells into a 50-ml conical tube. Save a piece of airway tissue at -80°C for genotyping, if necessary.
- 9 Combine all supernatants containing epithelial cells and centrifuge 5 min at $\sim 300 \times g$ in a tabletop centrifuge, 4°C.
- 10 Decant medium and resuspend cells in a total of 10 ml of 4°C Ham's F-12 medium/10% FBS. Filter through a sterile 500- μ m wire mesh into a 15-ml conical tube to remove all the fibrous tissues.
- 11 Wash cells two more times in the same medium, each time by adding 4°C Ham's F-12 medium/10% FBS, centrifuging 5 min at $\sim 300 \times g$, 4°C, and removing the supernatant. Prior to the last washing, remove a 10- μ l aliquot of cell suspension and quantitate the total yield of viable isolated cells by trypan blue exclusion (APPENDIX 3G).

Care should be taken not to include red blood cells in the quantitation. Typical yield from one lobe of a lung is $\sim 2-4 \times 10^7$ cells.

Culture airway epithelial cells

- 12 Resuspend final cell pellets in 10 ml of 37°C medium B and plate at $\sim 1-2 \times 10^6$ cells per 100-mm uncoated plastic tissue culture dish (in 10 ml medium B per dish). Incubate 24 hr.

Medium B is a hormonally defined medium. If CF cells are used, antibiotic concentrations are doubled for the first 24 hr.
- 13 At end of 24-hr incubation, aspirate cells that have not adhered to the plate and wash the remaining adherent cells twice in 37°C Ham's F-12 medium to remove excess red blood cells. Feed cells with fresh medium B and incubate 48 hr.

IMPORTANT NOTE: The duration of exposure to medium B will be dependent on the degree of bacterial and fungal contamination in cultures. For heavily

contaminated cells, exposure should be continued for an additional 24 hr in fresh medium B (total exposure, 72 hr). However, amphotericin B is highly toxic to cells and time of exposure should be kept to a minimum unless problems are encountered with contamination. Fungal contamination often stems from tissue harvested in hospital pathology laboratories. Therefore, it is best, when possible, to acquire tissue directly in the transplantation operating room.

- 14 At end of 48-hr incubation (total 72 hr following plating), feed cells with medium C (at this point clones of expanding epithelial cells should be visible). Feed every 3 days with fresh medium C.

Typically, cells are ready for cryopreservation, passaging, or transplantation into xenograft models (see Basic Protocol 5) by 5 days post-plating (~80% confluency). Care should be taken not to allow cells to become more than 80% confluent or they will begin to differentiate and lose their capacity for subculturing.

Expand cells (optional)

- 15 Remove medium and incubate cells 1 to 3 min with 5 ml of 0.1% trypsin/EDTA at 37°C, while closely monitoring detachment of cells (see *APPENDIX 3G*). Neutralize by adding 5 ml trypsin inhibitor buffer. Harvest cells immediately once released by gentle tapping of the plate.

Typically, cells can be expanded one time without loss of ability to differentiate in a xenograft model.

- 16 Centrifuge cells 5 min at $300 \times g$, 4°C, to remove trypsin, then wash twice in medium C, each time by centrifuging at $300 \times g$. Plate cells at a 1:5 dilution and propagate in medium C as described in step 14.

Cryopreserve cells

- 17 Harvest cells as described in step 15. Centrifuge cells 5 min at $300 \times g$, 4°C, and resuspend pellets in 1 ml of 4°C cryopreservation medium for each 100-mm plate of cells. Divide into 1-ml aliquots in 2-ml cryogenic vials. Slow freeze vials overnight at -80°C, then transfer to liquid nitrogen (also see *APPENDIX 3G*).

Slow freezing can be performed using isopropanol-containing cryopreservation containers (Nalgene). Cells are then moved to liquid nitrogen storage. Typically, one subconfluent 100-mm plate of cells is frozen per vial in 1 ml of cryopreservation medium. Cryopreserved cells should be quick-thawed at 37°C and placed in 37°C growth medium immediately after thawing. It is not necessary to remove DMSO by washing cells, since each plug of cells is diluted into 50 ml of growth medium, then divided among five 100-mm plates.

BASIC PROTOCOL 2 TRANSDUCTION OF PRIMARY AIRWAY EPITHELIAL CELLS

Ex vivo gene transfer to proliferating cultures of primary airway epithelial cells has been useful in reconstituting genetically modified airway epithelium into bronchial xenografts. Because primary isolates of airway epithelial cells grown at subconfluency in hormonally defined medium lead to the expansion of progenitor cells, vectors of choice for manipulation at this stage include integrating recombinant viruses such as retroviruses and lentiviruses (*UNIT 12.5 and 12.10*) and adeno-associated virus (*UNIT 12.9*). Recombinant adenovirus (*UNIT 12.4*) is of somewhat less utility for gene transfer to proliferating airway epithelial cells

because the episomal viral genomes are significantly diluted following expansion of airway progenitor cells.

Materials

8 mg/ml polybrene (Sigma) in H₂O (store in aliquots at –20°C)

Primary airway epithelial cells (see Basic Protocol 1; freshly isolated cells are needed for retroviral infection; once-passaged cells may be used for adeno-associated virus infection)

Ham's F-12 medium (Life Technologies)

Medium C (see recipe)

0.45-µm filter syringe filter

100-mm tissue culture dishes

12,000- to 14,000-Da MWCO dialysis membrane (Life Technologies)

Additional reagents and equipment for culturing of mammalian cells and producing retroviral or lentivirus vectors (*UNIT 12.5, 12.10*) or producing and purifying adeno-associated viral vectors (*UNIT 12.9*)

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

To transduce with retrovirus and lentivirus

1a Harvest retroviral supernatants from confluent monolayers of viral producer cell lines or by other classical techniques including transient transfection (*UNIT 12.5*).

For clonal producer cells lines, 5 ml of DMEM/10% calf serum growth medium is placed onto a 100-mm confluent monolayer, 16 to 18 hr prior to harvesting.

2a Pass retroviral supernatant through 0.45-µm filter to remove cellular debris. Supplement with polybrene (from 8 mg/ml stock) to final concentration of 2 µg/ml.

3a Remove the medium from a 100-mm plate of freshly isolated primary airway epithelial cells on the second day post seeding (~10% confluency) and add 10 ml of retroviral supernatant. Incubate 2 hr.

4a Wash cells twice with Ham's F-12 medium, then add hormonally defined medium C and resume incubation.

Cells can be infected up to three times on sequential days.

Following infection cells can be used to generate genetically modified airway epithelium in tracheal xenografts (see Basic Protocol 6). Typically, retroviral titers of 1×10^6 cfu/ml are capable of transducing primary airway cells at an efficiency of 10% to 30% following three serial infections. Since infection with retroviral supernatants requires exposure to serum, it is best to work with freshly isolated airway epithelial cells to retain an adequate undifferentiated state for transplantation into xenografts. When using pseudotyped retroviruses or lentiviruses that are capable of being concentration, the use of serum can be

avoided and this is preferred. Using concentrated stocks of virus can also achieve higher transduction rates with a single infection.

To transduce with recombinant adeno-associated virus (rAAV)

- 1b** Prepare and purify rAAV (*UNIT 12.9*). Dialyze purified rAAV against 500 ml of Ham's F-12 medium at 4°C overnight using a 12,000- to 14,000-Da MWCO dialysis membrane, prior to application on primary airway epithelial cells.

Typically virus is purified through three rounds of equilibrium density centrifugation followed by heat inactivation at 58°C for 60 min to remove contaminating helper adenovirus. The Basic Protocol in *UNIT 12.9* details these techniques.
- 2b** Mix 5×10^9 DNA particles of rAAV (typically 5 to 100 μ l) with 1.5 ml medium C at room temperature.
- 3b** Remove growth medium from a 100-mm plate of primary airway epithelial cells containing $\sim 5 \times 10^5$ cells. Add 1.5 ml of the rAAV/medium C mixture and incubate 1 hr with rotation every 5 to 10 min.

To achieve optimal infection, primary airway epithelial cells are transduced at $\sim 5 \times 10^5$ cells/100-mm plate.

Because of the small volumes used for infection, plates must be rotated every 5 to 10 min to maintain an even distribution of virus during infection and to prevent cell drying.
- 4b** Add 5 ml of medium C and continue incubation for an additional 24 hr.

Typically infection of a proliferating culture of primary airway cells with 5×10^9 particles of rAAV gives an efficiency of 25% transgene-expressing cells following a single infection. Infections can be carried out on sequential days to improve the level of gene transfer. Since infection with purified rAAV does not require exposure to serum (in contrast to recombinant retrovirus), both freshly isolated and once-passaged primary cells can be used; both types of cells will retain their capacity for differentiation in xenograft models. However, it is important to recognize that rAAV does not integrate, so the transgene will be diluted as cells proliferate.

BASIC PROTOCOL 3 GENERATION OF POLARIZED PRIMARY AIRWAY EPITHELIAL MONOLAYERS

One of the major limitations in using proliferating primary airway epithelial cell cultures to study gene therapy of genetic diseases is that these cells are not representative of in vivo differentiated airways. To this end, polarized epithelial monolayers, which more closely model the biological characteristics of in vivo airways, have been developed (Karp et al., 2002; Randell et al., 2011; Yamaya et al., 1992). Maintenance of asymmetric polarity in differentiated airway epithelial cell models represents a critically fundamental requirement in studying the biological response to gene delivery by viral or nonviral vectors. Culturing of primary airway epithelial cells on permeable supports yields a model system that closely resembles the native cell morphology in the airway. Several different types of filter support membranes are available commercially and have been used under a variety of experimental settings to culture airway cells. These permeable support filters have different properties in terms of the materials they are made of (e.g., polycarbonate, polyethylene tetraphthalate, polyester, or hydrophilized PTFE biopore membrane) and the coating (e.g., collagen or fibronectin). Different species of airway cells may have different membrane and temperature

requirements for growth and differentiation (Liu et al., 2007). To generate fully differentiated ciliated epithelia on permeable supports, primary airway cells are seeded at high densities, which promotes differentiation, formation of tight junctions, and polarity. In the generation of these models, freshly isolated uncultured airway cells provide the highest level of differentiation. However, cells that have been passaged once can also yield good results depending on the quality of airway tissue sample used to generate primary cells.

Materials

Glacial acetic acid

Collagen, Type IV, acid-soluble, from human placenta (Sigma Type VI)

Phosphate-buffered saline (PBS: *APPENDIX 2D*)

5% serum airway medium (see recipe)

Ussing chamber culture medium (see recipe)

0.2- μ m filter (Millipore Millex GS or equivalent Nalgene filter)

12-mm Millicell-HA culture plate inserts (Millipore)

24-well tissue culture plates

Additional reagents and equipment for culturing of mammalian cells and counting viable cells by trypan blue exclusion (*APPENDIX 3G*) and generating airway epithelial cells (see Basic Protocol 1)

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

Prepare permeable membrane supports

- 1 Add 50 μ l glacial acetic acid to 25 ml distilled water, then add 12.5 mg of Type IV human placenta collagen and dissolve by gently stirring for 30 min at 37°C.
It is important to screen different batches of collagen to find the one with the highest potency and lowest cell toxicity.
- 2 Dilute the collagen solution 1:10 with distilled water and pass through 0.2- μ m filter.
Final concentration is 50 μ g/ml. The collagen solution can be stored at 4°C for up to 1 month.
- 3 Coat 12-mm Millicell-HA culture plate inserts by placing 500 μ l of the diluted collagen from step 2 on the surface and incubating 18 hr (but <1 week) at room temperature.
Semipermeable Millicell-HA membranes have a 0.4- μ m pore size and a 0.6-cm² surface area.
- 4 Remove liquid collagen from coated surface just prior to the seeding of airway primary cells, then air dry for 3 to 5 min. Make sure all liquid collagen is removed, since it is toxic to airway epithelial cells. Rinse Millicell insert twice with PBS prior to the seeding of cells.

Culture primary airway epithelial cells at air-liquid interface

- 5 Generate primary airway epithelial cells (see Basic Protocol 1, steps 1 to 11).
Freshly isolated cells prepared as described in Basic Protocol 1, steps 1 to 11 work best; however, passage-1 cells (as prepared in Basic Protocol 1, steps 1 to 16, can also be used with some success). If passage-1 cells are used, proceed directly to step 9.
- 6 Resuspend cell pellet in 5% serum airway medium by gentle pipetting.
- 7 Remove fibroblasts by plating cell suspension in 100-mm tissue culture dishes and incubating 1 to 3 hr to allow fibroblasts to attach.
- 8 Collect the supernatant, containing nonattached airway primary epithelial cells. Count number of viable cells by trypan blue exclusion, then centrifuge 5 min at $300 \times g$ (APPENDIX 3G).
- 9 Place 500 μ l 5% serum airway media in each well of a 24-well tissue culture plate (which will be in the basolateral side of the chamber when the inserts are introduced), then place the collagen-coated Millicell inserts in the wells. Seed 3×10^5 airway primary cells (5×10^5 cells/cm²) in each insert (apical side of chamber) in a total volume of 100 to 500 μ l of 5% serum airway medium. Incubate 24 hr.
- 10 Remove medium on the apical (mucosal) side and replace 5% serum airway medium on the basal (basolateral) side with Ussing chamber culture medium. Continue to incubate cells with this air-liquid interface. Maintain CO₂ at 7.5% during the first week of culture to increase the efficiency of forming monolayer with higher cross-membrane resistance.

Figure 13.9.1 illustrates this setup. Well-differentiated monolayers of airway epithelia are obtained after 2 weeks in culture. Representative cultures demonstrate a transepithelial resistance of >1000 ohm cm².

ALTERNATE PROTOCOL 1 GENERATION OF POLARIZED AIRWAY EPITHELIAL MONOLAYERS FROM IMMORTALIZED CELL LINES

A limitation of using primary cells (Basic Protocol 3) to generate polarized monolayers is that the extent of differentiation, electrophysiological properties, and other biological properties may be different between donor individuals. Furthermore, if large quantities of cells are required for certain studies, sufficient tissue may not be readily available. Zabner and colleagues (Zabner et al., 2003) addressed these issues by using exogenously-expressed hTERT and human papillomavirus type 16 (HPV-16) E6 and E7 genes to generate immortal human airway epithelial cell lines from normal (NuLi) and cystic fibrosis (CuFi)-affected individuals. Polarized airway cultures from NuLi and CuFi cell lines exhibit a great deal of similar biology to primary lines, allowing for the generation of larger numbers of cultures with more reproducible properties.

Materials

- Glacial acetic acid
- Collagen, Type IV, acid-soluble, from human placenta (Sigma type VI)
- Phosphate-buffered saline (PBS: APPENDIX 2D)
- BEGM medium (see recipe)
- 0.25% Trypsin-EDTA (Gibco)

5% serum airway medium (see recipe)

Ussing chamber culture medium (see recipe)

0.2- μ m filter (Millipore Millex GS or equivalent Nalgene filter)

12-mm Millicell-HA culture plate inserts (Millipore)

24-well tissue culture plates

Additional reagents and equipment for culturing of mammalian cells and counting viable cells by trypan blue exclusion (*APPENDIX 3G*) and generating airway epithelial cells (see Basic Protocol 1)

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

Prepare collagen IV-coated plates and permeable membrane supports.

- 1 Prepare collagen solution as described in Basic Protocol 3 steps 1–2.
- 2 Coat 10-cm or 150-mm dishes by placing 5 ml or 10 ml, respectively, of the diluted collagen from step 2 on the surface and incubating 18 hr (but <1 week) at room temperature.
- 3 Coat 12-mm Millicell-HA culture plate inserts by placing 500 μ l of the diluted collagen from step 2 on the surface and incubating 18 hr (but <1 week) at room temperature.

Semipermeable Millicell-HA membranes have a 0.4- μ m pore size and a 0.6-cm² surface area.

- 4 Remove liquid collagen from coated surface just prior to the seeding of airway primary cells, then air dry for 3 to 5 min. Make sure all liquid collagen is removed, since it is toxic to airway epithelial cells. Rinse dish or Millicell insert twice with PBS immediately prior to the seeding of cells.

Collagen-coated plates and Millicell inserts can be stored in sealed bags at room temperature for several weeks.

Culturing NuLi and CuFi cell lines

- 5 Thaw frozen vial of NuLi or CuFi cells or pellet of previously-typsinized cells and dilute immediately into warm BEGM media.
- 6 Pellet cells by centrifugation at 300 $\times$ g for 5 min and resuspend in BEGM media. Subculture cells at 1:3 or 1:4. Feed every 2 days with fresh BEGM media.

It is important not to subculture NuLi and CuFi cells at a dilution greater than 1:4, otherwise, the cells may de-differentiate to an extent where it is difficult to polarize them. At a confluency of ~80%, the cells will be ready for culturing on to Millicell inserts or subculturing. With every passage, the ability of these cell lines to establish polarized monolayers diminishes, although Zabner et al. (Zabner et al., 2003) found that they could be polarized up to passage 25 with some measure of success.

Expanding cells

- 7 Remove medium and incubate cells 1 to 3 min with 5 ml of 0.25% trypsin/EDTA at 37°C. After 3 min, harvest cells immediately once released by gentle tapping of the plate, remove detached cells, and neutralize by adding 5 ml trypsin inhibitor buffer. Add a second 5 ml of trypsin/EDTA and repeat cell detachment 2–4 more times, or until all cells are dissociated.

Extensive trypsinization of cells can decrease their ability to propagate and polarize.
- 8 Centrifuge cells 5 min at $300 \times g$, 4°C, to remove trypsin, then wash twice in BEGM medium, each time by centrifuging at $300 \times g$. Plate cells at a 1:3 or 1:4 dilution and propagate in BEGM medium as described in step 5.

Cryopreserve cells

- 9 Harvest cells as described in step 7–8. Centrifuge cells 5 min at $300 \times g$, 4°C, and resuspend pellets in 1 ml of 4°C cryopreservation medium for each 100-mm plate of cells. Divide into 1-ml aliquots in 2-ml cryogenic vials. Slow freeze vials overnight at –80°C, then transfer to liquid nitrogen (also see *APPENDIX 3G*).

Slow freezing can be performed using isopropanol-containing cryopreservation containers (Nalgene). Cells are then moved to liquid nitrogen storage. Typically, one subconfluent 100-mm plate of cells is frozen per vial in 1 ml of cryopreservation medium. Cryopreserved cells should be quick-thawed at 37°C and placed in 37°C growth medium immediately after thawing. It is not necessary to remove DMSO by washing cells, since each plug of cells is diluted into 50 ml of growth medium, then divided among five 100-mm plates.

Culture NuLi and CuFi cells at air-liquid interface

- 10 Maintain and trypsinize cells as described in steps 5–7.
- 11 Resuspend cell pellet in 5% serum airway medium by gentle pipetting.
- 12 Count number of viable cells by trypan blue exclusion, then centrifuge 5 min at $300 \times g$ (*APPENDIX 3G*).
- 13 Place 400 μ l 5% serum airway media in each well of a 24-well tissue culture plate (which will be in the basolateral side of the chamber when the inserts are introduced), then place the collagen-coated Millicell inserts in the wells. Seed 2×10^5 NuLi or CuFi cells in each insert (apical side of chamber) in a total volume of 100 to 500 μ l of 5% serum airway medium. Incubate 24 hr.
- 14 Remove medium on the apical and basolateral sides and replace with Ussing chamber culture medium. Incubate 24 hr.
- 15 Remove apical medium and begin to incubate cells at air-liquid interface, removing apical media every day until none remains (usually <1 wk)
- 16 Continue to incubate cells at an air-liquid interface, feeding cells every 2 days for one week and 2–3 times per week every additional week.

See Figure 13.9.1 for this setup. Well-differentiated monolayers of airway epithelia are obtained with these cell line after 3–4 weeks in culture. Representative cultures of low-passage NuLi and CuFi cells demonstrate a peak transepithelial resistance of $>600 \text{ ohm cm}^2$.

BASIC PROTOCOL 4 GENE TRANSFER TO POLARIZED AIRWAY EPITHELIA

Multiple model systems have been used to study the efficiency of gene delivery to human airway epithelia. Although it has been demonstrated that both viral and nonviral systems can be used to introduce reporter and therapeutic genes to airway epithelial cells, there has been a lack of consistency in the efficiency of gene transfer between in vivo and in vitro models. To a large extent, these differences in gene transfer efficiency are due to variability in the extent of differentiation seen between various in vitro model systems. Polarized airway epithelial cultures are one in vitro model system that more closely resembles the native airway than proliferating cultures of epithelial cells. By 2 weeks post seeding, electron microscopic morphologic analysis demonstrates that polarized airway epithelia display many biological properties of the native airway epithelia such as the appearance of ciliated cells, goblet cells, and tight junctions. Gene transfer studies using this model system have been able to address many important questions closely related to clinical application of human gene-therapy protocols to airway. For example, polarity of the airway epithelial cells results in significant sidedness (i.e., apical versus basolateral) in gene-transfer efficiency (Duan et al., 1998b; Duan et al., 2000; Yan et al., 2012; Yan et al., 2006). In this protocol we describe delivering retrovirus, adeno-associated virus, and adenovirus to fully differentiated human airway epithelia grown at the air-liquid interface. Choice of vector is discussed in Strategic Planning, Background Information, and Table 13.9.2.

Materials

Hanks' balanced salt solution (HBSS; *APPENDIX 2D*)

25% and 40% (w/v) sucrose in HBSS

Lactose storage buffer (see recipe)

8 mg/ml polybrene in H₂O (store in aliquots at –20°C)

Ussing chamber culture medium (see recipe)

Polarized airway epithelial cells grown on Millicell-HA culture plate inserts (see Basic Protocol 3)

Ham's F-12 medium (Life Technologies)

Phosphate-buffered saline (PBS; *APPENDIX 2D*)

0.45-µm filter (Millipore)

Sorvall RC-26 Plus centrifuge with SS-34 rotor (or equivalent) and centrifuge tubes accommodating 40 ml

Beckman ultracentrifuge with SW 41 rotor (or equivalent) and SW 41 centrifuge tubes

Filtron 100K concentrator (PALL)

58°C water bath

12,000- to 14,000-Da MWCO dialysis membrane (Life Technologies)

Additional reagents and equipment for generating and/or purifying recombinant retrovirus and lentivirus (*UNIT 12.5 and 12.10*), adeno-associated virus (*UNIT 12.9*), or adenovirus (*UNIT 12.4*)

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

To transduce with recombinant retrovirus and lentivirus

- 1a** Generate retroviral or lentiviral supernatants to be used for gene-transfer studies (*UNIT 12.5, 12.10*). For higher-titer preparations, remove cell debris to clarify retrovirus-containing culture medium by filtering through a 0.45- μ m filter.

Several modifications to the procedures for generating recombinant retroviruses and lentiviruses have significantly improved the efficiency of transduction (Unit 2.5). Predominately these include the ability to concentrate viral stocks to higher titers. Typically, titers of recombinant retrovirus and lentivirus should be $> 10^8$ cfu/ml for these studies.

- 2a** Centrifuge viral supernatant at 40 ml per tube for 16 hr at $7000 \times g$ in a Sorvall RC-26 Plus centrifuge with a SS-34 rotor (40 ml per tube) at 4°C. After centrifugation, remove the supernatant and resuspend the viral pellet in 0.5 ml HBSS.
- 3a** Generate a 25% to 40% sucrose gradient by placing 4 ml of 40% sucrose in the bottom of an SW 41 centrifuge tube and layering an equal volume of 25% sucrose on top by slow pipetting. Layer viral suspension on top of the gradient and centrifuge 1.5 hr at $265,000 \times g$ (40,000 rpm in SW 41 rotor), 4°C.
- 4a** Collect virus from the interface between the two layers of sucrose and exchange buffer to lactose storage buffer by diafiltration through a Filtron 100K concentrator according to the manufacturer's instructions. Titer viral stock (*UNIT 12.5*) and store in aliquots at -80°C .
- 5a** Prior to infection of polarized epithelial monolayers, add polybrene (from 8 mg/ml stock) to retroviral stock to a final concentration of 8 $\mu\text{g/ml}$.
- 6a** Mix retroviral stock with an equal volume of Ussing chamber culture medium and apply 100 μl to the apical surface of primary airway epithelial cells grown on Millicell-HA culture plate insert. Alternatively, apply virus to the basal side of polarized airway epithelia using 250 μl of virus.
- 7a** Incubate for a total of 4 hr. Following infection, remove virus-containing medium and return the epithelial monolayers to their normal culturing conditions (air on the apical surface and Ussing chamber culture medium on the basolateral surface; see Fig. 13.9.1).
- 8a** Analyze cultures functionally and/or examine transgene expression.
- Typically, retroviral transduction from the apical side of a polarized culture gives little gene transfer, while infection from the basolateral side is ~1% to 5% efficient (Wang et al., 1998). Several modifications have been recently described that improve the level of retroviral transduction to polarized airway epithelial cultures (Wang et al., 1999). Stimulating airway epithelial cells to divide by the addition of keratinocyte growth factor (KGF; Amgen) at 50 ng/ml for 36 hr prior to retroviral infection from the basal side results in an ~20% transduction efficiency. This augmentation in transduction efficiency is not seen when virus is applied to the apical surface. Transient disruption of tight junctions with a hypotonic EGTA solution (1.5 to 3 mM EGTA in water, applied to the apical surface for 10 min) prior to infection from the mucosal surface (apical side) dramatically improves transduction efficiency. Impressive transduction efficiencies approaching 60% can be achieved by a combination of

KGF and EGTA treatments when virus is applied to either the apical or basolateral sides of polarized airway epithelia.

To transduce with recombinant adeno-associated virus (rAAV)

- 1b** Purify rAAV through three rounds of CsCl density ultracentrifugation or other chromatography methods (*UNIT 12.9*).
- 2b** Heat purify the virus at 58°C for 60 min to inactivate contaminating helper adenovirus used in the preparation of rAAV.
- 3b** If rAAV is purified by CsCl density ultracentrifugation, dialyze rAAV against 500 ml of PBS at 4°C overnight using a 12,000- to 14,000-Da MWCO dialysis membrane to remove CsCl, prior to application onto polarized airway epithelia.
- 4b** Mix 5 to 20 μ l rAAV ($\sim 1 \times 10^{10}$ particles) with each 100 μ l of Ussing chamber culture medium.
- 5b** Perform infection from apical surface of polarized monolayers by applying the 100 μ l of virus/medium mixture directly to the apical surface of the airway cells on the Millicell insert.
- 6b** Alternatively, perform gene transfer to the basolateral side by applying 250 μ l virus/media mixture to the bottom of the supporting filter membrane.

Infection can be performed from the apical or basolateral side of epithelia. With AAV serotype 2 (AAV2), basolateral infection provides ~100-fold higher efficiency than apical infection. Other serotypes exhibit different polarity (AAV1 is equally efficient from the apical and basolateral membranes).

- 7b** Incubate for a total of 24 hr. Following infection, remove virus-containing medium and return the epithelial monolayers to their normal culturing conditions (air on the apical surface and Ussing chamber culture medium in the basolateral compartment; see Fig. 13.9.1).
- 8b** Analyze cultures functionally and/or examine transgene expression.

Unlike rAAV infection in proliferating primary airway epithelial cells, expression of transgenes in polarized airway epithelia requires a significantly longer period of time. Transgene expression in polarized epithelia begins at 4 days and reaches a peak at 40 days post infection. This phenomenon resembles the transduction kinetics of rAAV in tissues such as muscle. Infections with rAAV2 from the apical side performed at 10^{10} particles/well—i.e., at multiplicity of infection (MOI) = ~1000—yields little transgene expression in the absence stimuli such as proteasome inhibitors (40 μ M LLnL and 5 μ M doxorubicin), while infection from the basolateral surface will result in ~1% transgene-expressing cells by 30 to 40 days post infection in the absence of stimuli. Higher initial titers of infection will yield higher levels of transduction only from the basolateral surface in the absence of proteasome inhibitors. Proteasome inhibitors are generally useful to induce rAAV transduction following apical infection for many serotypes (Duan et al., 2000; Yan et al., 2012; Yan et al., 2006).

To transduce with adenovirus

- 1c** Generate recombinant adenovirus (*UNIT 12.4*).
- 2c** Prior to infection, dilute adenoviral stock in Ussing chamber media to 1×10^9 pfu/250 μ l.

- 3c** Perform infection by applying 250 μ l Ussing chamber media containing 1×10^9 pfu (MOI of ~ 1000 pfu/cell) onto the basolateral surface of epithelia. Incubate 12 hr.
- Alternatively efficient transduction can be achieved by applying the virus apically following treatment with 5 mM EGTA for 10 min to break down the tight junctions. Apical transduction by adenovirus alone is highly inefficient.
- 4c** Following infection remove virus-containing medium and wash basolateral surface twice with Ussing chamber culture medium.
- 5c** Return the chamber inserts to their normal culturing conditions (air on the apical surface and Ussing chamber culture medium on the basolateral compartment; see Fig. 13.9.1).
- Transgene expression peaks by 3 days post infection.
- 6c** Analyze cultures functionally and/or examine transgene expression.
- Adenovirus infection in poorly differentiated epithelia (within 3 days of seeding) will result in $\sim 50\%$ to 60% transgene-expressing cells, which is ~ 3 - to 4 -fold more effective than infection of well-differentiated epithelia with a cilia lawn (Zabner et al., 1997). Infection efficiency is generally saturated after 12 hr of incubation (Zabner et al., 1996). Recently, it was also found that conjugation of recombinant adenovirus with polycationic polymers and cationic lipids increases adenoviral-mediated gene transfer from the apical side. Conjugation of adenovirus with poly-L-lysine (PLL) at a ratio of 200:1 (PLL molecules:viral particles) can increase efficiency 5- to 10-fold (Fasbender et al., 1997).

BASIC PROTOCOL 5 GENERATION OF HUMAN TRACHEAL XENOGRAPTS

Human tracheal xenografts represent another model of the airway, which has some advantages over in vitro-polarized epithelial cultures. These advantages include a higher level of epithelial differentiation that closely mirrors the composition of cell types found in the primate airway (Engelhardt et al., 1993b). Human proximal airway xenografts can be generated from multiple tissue sources, including nasal, tracheal, and bronchial epithelium. However, xenograft airways generated from different regions of the lung can have different biologic properties and/or cell-type compositions. This protocol will cover the most widely used type of xenograft generated from tracheobronchial airways. Trachea xenografts are generated from primary airway epithelial cultures, which are seeded into denuded rat tracheas (this process is summarized in Fig. 13.9.2). This is followed by the ligation of flexible plastic tubing to both ends of the explant (Fig. 13.9.3). These “open-ended” xenografts are then transplanted subcutaneously into athymic mice, in such a way that both ends of the tubing exit through the back of the neck (Fig. 13.9.4). This modification has provided a significant improvement in the level of epithelial differentiation over previous methodologies employing “close-ended” xenografts (Wilson, 1997). Furthermore, luminal access to differentiated airways allows for assessment of in vivo gene transfer and subsequent functional in vivo studies. For xenografts generated from cystic fibrosis airway tissue, such functional measurements include assessment of secretory products, transepithelial potential difference measurements, and fluid transport (Goldman and Wilson, 1995; Zhang et al., 1995; Zhang and Engelhardt, 1999; Zhang et al., 1998; Zhang et al., 1996).

Materials

Fisher 344 rats, 200 to 250 g, male (Harlan Bioproducts for Science or Charles River Labs)

70% ethanol
 MEM medium (Life Technologies), 4°C
 Medium C (see recipe)
 Primary airway epithelial cells (see Basic Protocol 1) at ~80% confluency
nu/nu athymic mice, 20 to 25 g, male (Harlan Bioproducts for Science)
 Ketamine
 Xylazine
 Phosphate-buffered saline (PBS; *APPENDIX 2D*)
 Povidone-iodine
 Ham's F-12 medium (Life Technologies)
 Silastic tubing (0.030 in. i.d. × 0.065 in. o.d.; Dow Corning)
 Teflon tubing (0.031 in. i.d. × 0.063 in. o.d.; Thomas)
 Gas sterilization pouch (M.D. Industries) and gas sterilization apparatus
 2-ml screw-cap tubes (Sarstedt)
 Adapter (0.8-mm barb-to-barb connector; Bio-Rad)
 2–0 braided silk suture (e.g., Ethicon)
 0.035 in. diameter Chromel A steel wire (Hoskins Mfg.)
 Hemostat
 100-mm tissue culture plates
 Small airtight transfer chamber to equilibrate xenograft cassettes in 5% CO₂
 Sterile surgical drapes for mouse surgery
 Self-sealable autoclave sterilization pouch, (M.D. Industries)
 Small forceps (2 per mouse) and sharp scissors (1 per mouse)
 Disposable skin stapler 35R (American Cyanamid)
 Sterile cages for mice
 21-G × 0.75-in. Surflo winged infusion set (Terumo Medical)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

Prepare xenograft cassette

- 1** Cut tubing and adapters used for generating xenograft cassettes to length and assemble as outlined in Figure 13.9.3A.
- 2** Place the assembled parts of the xenograft cassette (Fig. 13.9.3A) in a 100-mm tissue culture plate. Seal in a sterilization pouch and gas sterilize.

Isolate rat tracheas

- 3** Euthanize rats by CO₂ asphyxiation and pin to a Styrofoam bed.

- 4 Clean neck and chest of euthanized rats with excess 70% ethanol and excise trachea from the pharynx to the carina (bifurcation of bronchi at the end of the trachea).
Take care not to nick arteries, which will cause excessive bleeding and technical difficulties in surgery. Harvesting is best done in a laminar flow hood to prevent contamination of tracheas. However, under good clean conditions, tracheas can be harvested on an open bench.
- 5 Immediately following excision, place each trachea in a separate 2-ml screw cap tube and keep on ice until all tracheas have been harvested.
- 6 Denude tracheas of all viable epithelium by three rounds of freeze/thawing each consisting of freezing at -80°C and thawing at room temperature.
- 7 Following the third round of freeze/thawing, clean tracheas of excessive fat and cut to size (typically at the first and thirteenth tracheal ring). Rinse each tracheal lumen with 10 ml of 4°C MEM.
All procedures are performed in a laminar flow hood under sterile conditions.
- 8 Pair tracheas for length, and combine pairs in the same tube. Store at -80°C .

Seed primary airway epithelial cells

- 9 Using 2–0 braided silk sutures, ligate rat tracheas to the adapter (“e”) attached to tubing “b” as shown in Figure 13.9.3. Securely tie sutures with triple knots and loop them around the tubing and trachea a total of three times.
- 10 Inject $1\text{--}2 \times 10^6$ primary bronchial epithelial cells in 20 μl of 4°C medium C under sterile conditions into the open end of the rat trachea using an 20- μl micropipettor. Insert the pipet tip as deeply as possible into the trachea and slowly withdraw it as the cells are injected into the trachea.
Primary airway epithelial cells are ready for transplantation into bronchial xenografts at ~80% confluence.
- 11 With care not to allow cells to leak out, ligate the remaining open end of the rat trachea to the adapter (“e”) attached to tubing c as shown in Figure 13.9.3, using the suturing technique described in step 9.
- 12 Secure the length of the rat trachea by stretching it to physiologic length and clamping tubing b and tubing a with a hemostat. Tie the remaining two sutures as shown in Figure 13.9.3B to secure adapters to tubing d.
Tubing d, which is made of rigid Teflon, serves as a stent to retain the proper length of the trachea.
- 13 Place xenograft cassettes with seeded cells (from step 10) into a 100-mm tissue culture plate with 1 to 2 ml medium C overlaid on top of the trachea to keep it moist. Incubate plates 1 to 2 hr in a humidified 37°C , 5% CO_2 incubator to equilibrate pH before proceeding to transplantation into mice. For transport, use a small airtight sterile container equilibrated in the incubator with the dishes of xenografts to maintain CO_2 prior to transplantation.

Transplant xenografts into nu/nu mice

- 14 Anesthetize male *nu/nu* athymic mouse by intraperitoneal injection of 100 mg/kg ketamine and 20 mg/kg xylazine in PBS. Once mice are anesthetized, place

them onto a sterile drape and remove sterile surgical instruments from autoclave pouches.

Each surgery requires two small forceps and one pair of sharp small scissors. Care should be taken to maintain sterility during surgery using autoclaved surgical instruments and sterile fields. Additionally, efforts should be taken to prevent hypothermia of mice during surgery by placing mice on a warm water bottle during and just after surgery prior to recovery. Coligan et al. (1998) provides details on intraperitoneal injection and anesthesia of mice.

- 15 Clean sites of surgical incisions with povidone-iodine followed by alcohol. Make four incisions as shown in Figure 13.9.4 (panel B). Make very small incisions on the neck of the mouse (0.16 cm) with just enough width to pass the tubing. Make two incisions on the flanks of the mouse, ~1 cm long. Separate skin from muscle by blunt dissection and place the xenograft cassette subcutaneously, tunneling the distal end of tubing a out of the incision behind the neck using forceps to guide the xenograft tubing (Fig. 13.9.4, panels A and C).
- 16 Following placement of xenografts, use 2 or 3 staples to close each of the largest incisions. Use an additional staple to anchor each xenograft to the skin at the loop of tubing “c.” Take care not to puncture the xenograft tubing while inserting this staple (see Fig. 13.9.4, panel C, solid arrowheads).
- 17 After transplantation, transfer the mouse to a sterile cage and monitor until it is awake.

Mice harboring xenografts must be housed separately since they may chew each others tubing. All caging, food, and water are autoclaved prior to cage changing. Since mice are immunocompromised, this is a very important aspect of preventing infection and death. Pathogen-free housing is critical to the success of this model system. Athymic mice are extremely susceptible to infection, which is further enhanced by the extensive surgery. Efforts to maintain mice in nonsterile caging or under non-pathogen-free housing will lead to significant mortality. Coligan et al. (1998) provides details on maintaining immunocompromised animals.

- 18 For the first 3 weeks after transplantation, irrigate xenografts weekly with 1 ml of Ham's F-12 medium using a Surflo winged infusion set with an 0.75-in., 21-G needle, to remove excess mucous secretions, using the following technique.
 - a. Insert a 21-G needle attached to a 1-ml syringe (with the plunger removed) that has been filled with room temperature Ham's F-12 medium, into the tubing from one end of the xenograft.
 - b. Insert a winged 21-G infusion needle attached to a second syringe (with the plunger attached and pushed all the way in), into the opposite end of the xenograft.
 - c. Apply negative pressure by withdrawing the plunger of the second syringe to aspirate the liquid through the lumen of the xenograft. Finally, aspirate air into the lumen of the xenograft.

By using this strategy, which applies negative pressure to irrigate the xenografts, fewer problems with mucus plugging will occur, which may lead to subcutaneous leaks in xenografts. If animals are restless, it may be necessary to anesthetize with ketamine/xylazine as in step 14. However, it is advised that the dose be reduced to 25% to 50% of what

is stated in that step, as frequent anesthesia can lead to increased mortality.

19 After 3 weeks post transplantation, irrigate xenografts twice per week.

ALTERNATE PROTOCOL 2 GENERATION OF NEWBORN FERRET TRACHEAL XENOGRAFTS

In addition to human tracheal xenografts (Basic Protocol 5), ferret tracheas from animals up to 15 days in age can be direct engrafted on to *nu/nu* mice without the need for a rat tracheal support. These xenografts develop a fully differentiated airway epithelium and fully differentiated submucosal glands (Dajani et al., 2005; Fisher et al., 2011; Sun et al., 2010; Wang et al., 2001), making them an excellent model for studying several innate immune components of the airway. In addition, the generation of an animal model for cystic fibrosis in the ferret (Sun et al., 2010), allows for the direct study of several aspects of pathophysiology without the need for extensive rearing protocols of diseased ferrets.

Materials

Newborn ferrets, 1–15 days old (Marshall Farms)
 70% ethanol
 MEM medium (Life Technologies), 4°C
 Medium D (see recipe)
nu/nu athymic mice, 20 to 25 g, male (Harlan Bioproducts for Science)
 Ketamine
 Xylazine
 Phosphate-buffered saline (PBS; *APPENDIX 2D*)
 Povidone-iodine
 Ham's F-12 medium (Life Technologies)
 Silastic tubing (0.030 in. i.d. × 0.065 in. o.d.; Dow Corning)
 Teflon tubing (0.031 in. i.d. × 0.063 in. o.d.; Thomas)
 Gas sterilization pouch (M.D. Industries) and gas sterilization apparatus
 2-ml screw-cap tubes (Sarstedt)
 Adapter (0.8-mm barb-to-barb connector; Bio-Rad)
 2–0 braided silk suture (e.g., Ethicon)
 0.035 in. diameter Chromel A steel wire (Hoskins Mfg.)
 Hemostat
 100-mm tissue culture plates
 Small airtight transfer chamber to equilibrate xenograft cassettes in 5% CO₂
 Sterile surgical drapes for mouse surgery
 Self-sealable autoclave sterilization pouch, (M.D. Industries)
 Small forceps (2 per mouse) and sharp scissors (1 per mouse)

Disposable skin stapler 35R (American Cyanamid)

Sterile cages for mice

21-G × 0.75-in. Surflo winged infusion set (Terumo Medical)

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

Prepare xenograft cassette as in Basic Protocol 5.

Isolate ferret tracheas

- 1 Euthanize ferrets by injection of pentobarbatol.
- 2 Clean neck and chest of euthanized ferrets with excess 70% ethanol and excise trachea from the pharynx to the carina (bifurcation of bronchi at the end of the trachea).

Take care not to nick arteries, which will cause excessive bleeding and technical difficulties in surgery. Harvesting is best done in a laminar flow hood to prevent contamination of tracheas. However, under good clean conditions, tracheas can be harvested on an open bench.
- 3 Immediately following excision, place each trachea in a separate 15-ml conical tube containing 10 ml Medium D and keep on ice until all tracheas have been harvested.
- 4 Incubate tracheas on a rotator at 4°C overnight.

This step is necessary to ensure sterility of tracheas for engraftment.
- 5 Clean tracheas of excessive muscle and fat tissue and cut to size. Rinse each tracheal lumen with 10 ml of 4°C Medium D.

All procedures are performed in a laminar flow hood under sterile conditions.
- 6 Pair tracheas for length, and combine pairs in the same tube containing MEM the day of engraftment.

Ligate trachea to tubing

- 7 Using 2–0 braided silk sutures, ligate ferret tracheas to the adapter (“e”) attached to tubing “b” as shown in Figure 13.9.3. Securely tie sutures with triple knots and loop them around the tubing and trachea a total of three times.

When using newborn ferret trachea, adapter (“e”) as shown in Figure 13.9.3 is replaced with 1mm PE tubing.
- 8 Ligate the remaining open end of the ferret trachea to the adapter (“e”) attached to tubing c as shown in Figure 13.9.3, using the suturing technique described in step 7.
- 9 Secure the length of the ferret trachea by stretching it to physiologic length and clamping tubing b and tubing a with a hemostat. Tie the remaining two sutures as shown in Figure 13.9.3B to secure adapters to tubing d.

Tubing d, which is made of rigid Teflon, serves as a stent to retain the proper length of the trachea.
- 10 Place xenograft cassettes with ferret trachea into a 100-mm tissue culture plate with 1 to 2 ml medium C overlaid on top of the trachea to keep it moist.

Incubate plates 1 to 2 hr in a humidified 37°C, 5% CO₂ incubator to equilibrate pH before proceeding to transplantation into mice. For transport, use a small airtight sterile container equilibrated in the incubator with the dishes of xenografts to maintain CO₂ prior to transplantation.

Transplant xenografts into nu/nu mice as described in Basic Protocols 5.

BASIC PROTOCOL 6 GENE TRANSFER TO HUMAN TRACHEAL XENOGRAFTS

Several gene-transfer applications in human bronchial xenografts have been used to address aspects of airway biology and gene therapy. Most notably, these include the use of recombinant retroviruses or lentiviruses to address aspects of progenitor-progeny relationships in the airway, and the use of recombinant adenoviruses to address aspects of gene therapy for cystic fibrosis using functional measurements of complementation. The two most successful approaches to genetic modification of the human airway epithelia of xenografts have been: (1) ex vivo gene transfer with recombinant retroviruses to primary airway epithelial cells prior to reconstitution into xenografts; and (2) in vivo gene transfer with recombinant adenovirus to fully differentiated xenograft airway epithelia. This protocol describes the application of these two main approaches.

Materials

Primary proliferating human airway cells (for ex vivo transduction with retrovirus; see Basic Protocol 1) *or* mouse with fully differentiated xenograft (for in vivo transduction with adenovirus; see Basic Protocol 5)

Retroviral supernatant (*UNIT 12.5*) *or* purified recombinant adenovirus (*UNIT 12.4*)

Ham's F-12 medium (Life Technologies)

5mM EGTA, sterile filtered

Additional reagents and equipment for transduction of proliferating human airway cells (see Basic Protocol 2) and generation/maintenance of xenografts (see Basic Protocol 5)

For ex vivo transduction with retrovirus or lentivirus

- 1a** Transduce primary proliferating human airway cells with recombinant retrovirus (see Basic Protocol 2).

For best transduction, cells are infected three times.

- 2a** Typically on the fifth day post seeding, trypsinize primary airway epithelial cells (see Basic Protocol 1, steps 15 and 16) and seed into xenograft cassettes (see Basic Protocol 5).

- 3a** Transplant xenograft cassettes into *nu/nu* mice and maintain xenografts (see Basic Protocol 5).

- 4a** Following differentiation (which typically takes 4 weeks), either use grafts for functional measurements or harvest them for histologic assessment of transgene expression (see Support Protocols 1 to 3).

Typically, the efficiency of transduction in primary cells is maintained in the final reconstituted xenografts. For example, if 30% of cells express a marker gene in the primary airway cells, xenografts reconstituted with these cells will also have 30% transgene expression (Engelhardt et al., 1995).

For in vivo transduction with adenovirus

- 1b** Generate recombinant adenovirus (*UNIT 12.4*).
Virus is normally purified through two rounds of CsCl density centrifugation followed by desalting with by Sephadex G-50 filtration into Ham's F-12 medium, prior to infection into fully differentiated xenografts.
- 2b** Flush xenograft with 1 ml of Ham's F12 media.
- 3b** Flush xenograft with 5mM EGTA and leave solution in the tubing. Seal graft and incubate 10 min.
Apical transduction of epithelial cells by adenovirus is highly inefficient. Treatment with EGTA breaks down epithelial tight junctions and allows access of the virus to the basolateral membrane, through which transduction is highly efficient.
- 4b** Flush xenograft with 1 ml Ham's F12 to remove EGTA.
- 2b** Instill 100 to 150 μ l of purified virus into the lumen of one end of the fully differentiated xenograft (4 weeks post transplantation) and close the ends of the xenograft by insertion of Chromel wire plugs (see Basic Protocol 5).
- 3b** Infect xenografts for 16 hr, then irrigate with 1 ml of Ham's F-12 medium, followed by air, on the following day to remove excess virus (see Basic Protocol 5, step 18).
- 4b** Typically, if functional measurements are to be performed on xenograft airways, allow xenografts to recover from acute toxicity of viral infection for 48 to 72 hr.
Infections can be carried out by either of two methods depending on the level of infection required for studies. A single infection with 5×10^{10} pfu, accomplished by instillation of 100 μ l of purified virus for 16 hr, provides ~10% infection efficiency as assessed by transgene expression. Alternatively three sequential 16-hr infections every other day with 5×10^{10} pfu provides >95% infection efficiencies (Zhang et al., 1998). The addition of EGTA at the time of infection can improve transduction with a single dose of virus (Fisher et al., 2012; Fisher et al., 2011).
Very high titers of recombinant adenovirus are needed to achieve efficient infection in xenografts. At these titers, precipitation of virus can occur shortly after desalting by gel filtration. Hence, virus should be used immediately following purification and desalting to avoid precipitation, which will result in significant losses in activity. Dialysis should be avoided as a method for desalting because precipitation is more prominent under conditions of slow salt removal.

BASIC PROTOCOL 7 IN VIVO GENE DELIVERY TO THE LUNG

Multiple model systems have been used to study the delivery of recombinant viruses to the lung including: (1) transtracheal instillation; (2) selective intubation of subsegments of the lung followed by instillation of virus; (3) nasal inhalation; and (4) aerosolization. In this protocol, the most widely used method of transtracheal administration will be reviewed, as applied to small-rodent animal models such as the mouse and cotton rat. Delivery of recombinant adenovirus will be used as an example because it has been most extensively studied.

The lungs from animals infected with E1 deleted recombinant *lacZ* adenovirus typically express the highest levels of transgene activity between 3 and 7 days post infection. Several

methods of tissue preparation have been employed in an attempt to analyze multiple experimental endpoints including: (1) histopathology using formalin inflation–fixed lungs; (2) localization of *lacZ* transgene activity by in situ histochemical staining of lungs by inflation; and (3) preparation of lungs for multiple histologic endpoints such as immunocytochemistry, histochemistry, and in situ hybridization in lungs inflated with OCT for frozen sectioning. In this review we will summarize the latter technique, frozen sectioning, which is the most flexible.

Materials

Cotton rats (80–120 g; Virion Systems)

Isoflurane

Purified recombinant adenovirus (*UNIT 12.4*)

PBS (*APPENDIX 2D*)

Pentobarbital

100% optimal cutting temperature (OCT) medium (Baxter) and 50% (v/v) OCT medium in PBS

Pulverized dry ice/isopentane slurry

Gauze pads

50-ml conical centrifuge tubes

Isopropyl alcohol swabs

Scalpel, forceps (2), and small sharp scissors

Disposable skin stapler 35R (American Cyanamid)

1-ml syringe with 30-G needle

18-G AngioCath (Becton Dickinson) and 3-ml syringe

Plastic embedding blocks (Baxter)

Cryostat

Deliver recombinant adenovirus to lung of rats

- 1 Anesthetize 80- to 120-g cotton rats with isoflurane by placing an isoflurane-soaked gauze pad into the bottom of a 50-ml conical tube and positioning the tube over the nose of the rat.

The depth of anesthesia is judged by noting the respiration rate and controlled by manipulating the distance of the conical tube from the nose of the animal.
- 2 Clean neck with an isopropyl alcohol swab and make a 1-cm midline incision with a sharp scalpel.
- 3 Expose the trachea by blunt dissection and make a small (3-mm) incision through the muscle surrounding the trachea. Instill $1\text{--}5 \times 10^{10}$ infectious particles in a total volume of 100 to 150 μl PBS through a 1-ml syringe with a 30-G needle, directly through the tracheal wall without an incision, followed by 300 μl of air.
- 4 Close incision with two staples and return animal to cage.

Similar procedures can be performed on mice by reducing the total dose and volume of virus according to total kg weight of the animal. Typically a 25-g mouse tolerates a dose of 2×10^9 pfu of recombinant virus instilled in a total volume of 25 to 35 μ l.

The lungs from animals infected with E1-deleted recombinant lacZ adenovirus typically express the highest levels of transgene activity between 3 and 7 days post-infection.

The volumes stated in this protocol will give gene transfer to both airways and alveolar regions. To enhance gene transfer to the airways and to limit alveolar gene transfer, smaller inoculum volumes can be used with the same dose of virus (50 μ l total for rats; 15 μ l for mice).

Harvest lungs for histologic analysis

- 5 Euthanize animals by an overdose (200 mg/kg) of pentobarbital, administered intraperitoneally.
- 6 Open the thorax and expose the proximal end of the trachea. Attach an 18-G AngioCath to a 3-ml syringe filled with a 37°C 1:1 mixture of OCT embedding medium and PBS. Insert the end of the AngioCath tubing into the proximal end of the trachea and ligate with a triple-knotted suture.

The authors have adopted this method of in situ inflation to avoid accidental nicking of the lungs upon removal, which will result in insufficient inflation pressures.
- 7 Apply gentle pressure to the syringe plunger until lungs are visibly inflated within the thorax. Do not overinflate.

Typically inflation of a cotton rat lung requires 2 ml of OCT/PBS.
- 8 Once inflated, remove the heart-lung cassette and quickly cool in ice-cold PBS for 10 min.

Once the lungs have cooled, they will become more rigid and can be easily cut into tissue blocks for embedding.
- 9 Remove the heart-lung cassette from the ice-cold PBS, place on an ice-cooled surface, and trim away non-lung tissue.
- 10 Cut the lung into large (~1-cm) wedges and place in plastic embedding blocks filled with 100% OCT embedding medium, so that the cut face is in contact with the bottom of the block.
- 11 Freeze blocks in a pulverized dry ice/isopentane slurry.

Since some air may be retained within the lung tissue, care must be taken to manipulate tissue samples during freezing so as to retain placement on the bottom of the block.
- 12 Cut 6- μ m frozen sections using a cryostat and process for multiple histologic analyses such as immunocytochemistry, histochemistry, and in situ hybridization.

Other, similar methods of lung inflation can be used to retain better morphology useful in histopathologic evaluation—e.g., by inflation with buffered formalin followed by embedding in paraffin for sectioning. Alternatively, lungs can also be stained in situ with Xgal by inflation fixation in 0.5% glutaraldehyde/PBS for

10 min, followed by two sequential 15-min lavages with 1 mM MgCl/PBS and staining in Xgal staining solution (see recipe) for 1 to 4 hr at 37°C. Following staining in Xgal, lungs should be washed with PBS twice by lavage and instilled with buffered formalin/1% glutaraldehyde, then post-fixed overnight before processing in paraffin for sectioning.

SUPPORT PROTOCOL 1 HARVESTING OF HUMAN TRACHEAL XENOGRAPTS FOR MORPHOLOGIC ANALYSIS TO EVALUATE TRANSGENE EXPRESSION

Adequate tissue preservation is essential in the evaluation of gene transfer to epithelia of human tracheal xenografts. Several of the reporter proteins described in this unit (see Support Protocols 2 to 5) require different fixation protocols to maximize sensitivity and reduce the potential of background from endogenous proteins with similar enzymatic activities. This support protocol provides essential steps and precautions in harvesting human bronchial xenografts for histologic analysis of reporter gene expression when using fresh-frozen tissue sections.

Materials

Mice harboring xenografts (see Basic Protocols 5 and 6)

PBS (*APPENDIX 2D*)

Optimal cutting temperature (OCT) medium (Baxter)

Pulverized dry ice/isopentane slurry

Dissecting equipment: small sharp scissors, forceps, and razor blades

Plastic embedding block (Baxter)

Cryostat

1. Euthanize mice harboring xenografts, by CO₂ asphyxiation, carefully excise the xenograft cassette, and place on a flat surface lined with Parafilm. Using two razor blades, carefully slice the xenograft trachea into 2-mm rings by opposing strokes of the cutting edge of the razor blades.
2. Rinse the rings of tissue in PBS and blot on Kimwipes to remove excess mucus from the lumen.

Care should be taken not to clamp the epithelial surface with forceps. Additionally, the ends of the xenograft tissue where tubing is attached should be discarded.

The tissue can now either be directly histochemically stained or fresh-frozen in OCT embedding medium as described in the following steps.
3. Slowly lower the xenograft rings, with the cut face down, into an OCT-filled plastic embedding block. Take care to allow OCT to enter the lumen of the xenograft ring, or frozen sections will be difficult to cut.
4. Push the rings to the bottom of the block with blunt forceps.

Care should be taken not to enter the lumen of the xenograft while positioning sample. Up to six rings can be embedded into one block.
5. Place tissue blocks into a pulverized dry ice/isopentane slurry and store at –80°C until sectioning.

Fast rates of freezing are critical for retaining optimal tissue morphology in frozen sections. The quickest freezing is obtained when a very fine powder of dry ice is mixed with a minimum amount of isopentane sufficient to make a slurry.

6. Section tissue blocks into 6- μ m frozen sections on a cryostat and proceed to detection (see Support Protocols 2, 3, and 5).

Care should be taken to avoid repeated freeze/thawing of freshly cut sections before processing for histochemical staining. Hence, slides are precooled prior to sectioning and sections only thawed once for placement onto slides. Once sections are placed on slides they should be kept within the cryostat (-18°C) until staining and not be air dried. Alternatively slides can be stored at -80°C for several weeks without substantial loss of β -galactosidase activity.

SUPPORT PROTOCOL 2 HISTOCHEMICAL DETECTION OF β -GALACTOSIDASE TRANSGENE ACTIVITY

The bacterial β -galactosidase (*lacZ*) gene is one of the most widely used genetic reporter gene. The deep blue color generated by hydrolysis of Xgal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) facilitates the localization of the delivered transgene. Endogenous background staining is seen in ciliated cells when long incubation times are used (>6 to 8 hr). Since endogenous β -galactosidase enzymatic activity is greatest under acidic conditions, neutral pH is critical to reduce background.

Materials

6- μ m frozen sections of tracheal xenograft (see Support Protocol 1)

0.5% (v/v) glutaraldehyde in PBS (prepare fresh)

1 mM MgCl_2 in PBS

1% (v/v) glutaraldehyde in buffered formalin (prepare fresh)

Xgal staining solution (see recipe)

Optimal cutting temperature medium (OCT; Baxter)

Pulverized dry ice/isopentane slurry

Hematoxylin or neutral red stain

1. Fix 6- μ m frozen sections by immersing in 0.5% glutaraldehyde/PBS for 10 min.
2. Wash sections twice, each time by immersing for 15 min in 1 mM MgCl_2 /PBS at room temperature.
3. Place sections in Xgal staining solution for 4 to 6 hr at 37°C , then wash twice in PBS and post-fix in buffered formalin containing 1% glutaraldehyde for 15 min at room temperature.

This method of post-fixation helps to minimize leaching of Xgal precipitate in the organics used for dehydration of tissue sections before coverslipping.

4. Counterstain sections briefly in hematoxylin (without blueing).

Alternatively, sections stained in neutral red also provide good contrast to the blue Xgal precipitate.

SUPPORT PROTOCOL 3 IMMUNOHISTOCHEMICAL DETECTION OF β -GALACTOSIDASE TRANSGENE ACTIVITY

The immunocytochemical detection of β -galactosidase reporter-gene expression is mostly used when other endogenous epithelial markers need to be colocalized in transgene-expressing cells. Such an experimental design affords the flexibility to directly monitor cell phenotype of transgene-expressing cells. Additionally, if background staining is high, immunocytochemical localization can provide a second confirmatory method to demonstrate the specificity of transgene expression.

Materials

6- μ m frozen sections of tracheal xenograft (see Support Protocol 1)

Methanol, -20°C

20% (v/v) goat serum in PBS, filtered through 0.45- μ m filter (prepare fresh)

1.5% (v/v) goat serum/PBS containing 25 $\mu\text{g/ml}$ rabbit anti- β -galactosidase antibody (purchase from 5 Prime 3 Prime), freshly prepared

Fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit antibody (or other fluorescently labeled goat anti-rabbit secondary antibody)

Citifluor antifade mounting medium (Ted Pella)

Humidified (moist) chamber (*UNIT 4.3*)

Coverslips

Fluorescence microscope

1. Post-fix 6- μ m frozen sections by immersing in -20°C methanol for 10 min, then air dry.
2. Block sections by placing 500 μl of 20% goat serum/PBS over section and incubating for 30 min at room temperature in a humidified chamber.
3. Remove blocking solution without allowing section to dry (e.g., by tilting on to a Kimwipe). Replace blocking solution with 25 $\mu\text{g/ml}$ rabbit anti- β -galactosidase in 1.5% goat serum/PBS and incubate 90 min at room temperature in a humidified chamber.
4. Wash sections three times, each time by immersing 8 min in 1.5 % goat serum/PBS at room temperature.
5. Incubate 30 min at room temperature with 25 $\mu\text{g/ml}$ FITC-labeled goat anti-rabbit antibody in 1.5% goat serum/PBS in a humidified chamber.
6. Wash sections three times as in step 4.
7. Coverslip sections in antifade mounting medium (Citifluor) and visualize by fluorescence microscopy.

SUPPORT PROTOCOL 4 FLUORESCENT DETECTION OF GREEN FLUORESCENT PROTEIN TO EVALUATE TRANSGENE EXPRESSION

The green fluorescent protein (GFP) gene provides the added attraction of allowing for direct, noninvasive assessment of transgene expression in living cells. This gene is most useful in primary cell culture models (i.e., polarized epithelial cultures and primary monolayers). However, since the gene is small, it can also easily be incorporated into viral

vectors with therapeutic transgenes. It is important to note the relatively high solubility of GFP when evaluating GFP expression in xenograft sections. Therefore, in the evaluation of nonviable cells (such as tissue sections), prefixing of tissue is required prior to sectioning.

Materials

Tissue samples incorporating GFP reporter gene

4% (w/v) paraformaldehyde in PBS, pH 7.4 to 7.6, 4°C

10%, 20%, and 30% sucrose in PBS, 4°C

PBS (*APPENDIX 2D*), cold

Fluorescence microscope with 450- to 500-nm FITC filter sets

Additional reagents and equipment for freezing and cryosectioning xenograft samples (see Support Protocol 1)

- 1** Pre-fix tissues 6 hr to overnight in 4% paraformaldehyde/PBS, pH 7.4 to 7.6 at 4°C.

Tissue must be prefixed prior to sectioning of tissue otherwise GFP will diffuse out of the tissue sample and significantly reduce sensitivity of detection.

- 2** Immerse tissue samples successively for 4 hr in 10%, 20%, and 30% sucrose at 4°C.

The samples are cryoprotected by equilibrating tissue in increasing concentrations of sucrose. Typically the first two equilibration steps are performed for 4 to 6 hr and the last step into 30% sucrose is performed overnight.

- 3** Freeze cryoprotected samples in OCT and cut into 6- to 10- μ m frozen sections (see Support Protocol 1).

- 4** To view GFP fluorescence, place a small drop of cold PBS on the 6- to 10- μ m frozen section and apply coverslip. Assess slides immediately by indirect fluorescent microscopy.

Alternatively, section can be stored at -80°C up to 2 to 3 weeks. It is important not to freeze/thaw frozen sections repeatedly or allow sections to completely dry, as this may lead to significant decreases in GFP signal. Caution should also be taken in choosing coverslip sealer since GFP is very sensitive to nail polishes, which are used in some laboratories to seal coverslips.

Direct detection of GFP reporter-gene expression in viable tissue culture cells and polarized airway epithelia can be obtained by viewing cells under direct fluorescence microscopy. Since GFP has similar absorption-emission characteristics to fluorescein, a FITC filter set that allows transmission of light at 450 to 500 nm is used for GFP detection.

To reduce background autofluorescence of the medium when viewing GFP in viable cells, the authors recommend replacing culture medium with PBS at the time of examination as described above. Also, double-barrier filters may be used to change the color of autofluorescent wavelengths to red-orange, which allows for more accurate assessment of GFP emission. For example, instead of using a High Q FITC filter set (Leica), a FITC/Texas red filter set (also from Leica) may be used, which differentiates true GFP signal from autofluorescence background.

SUPPORT PROTOCOL 5 HISTOCHEMICAL DETECTION OF ALKALINE PHOSPHATASE TRANSGENE ACTIVITY

Cleavage of the phosphate group from 5-bromo-4-chloro-3-indolyl phosphate (BCIP) by heat-stable placental alkaline phosphatase and subsequent reduction of NBT results in a blue-purple color in cells transduced with the alkaline phosphatase reporter gene. Alkaline phosphatase works best at the pH of 9.0 to 9.5. The background produced by endogenous, nonspecific alkaline phosphatase can be minimized by heating samples at 65°C for 30 min and by chemical inhibitors (such as levamisole). When more than one reporter gene is required for analysis, the combination of alkaline phosphatase and β -galactosidase may be attractive (Engelhardt et al., 1995).

Materials

6- μ m frozen sections of tracheal xenograft (see Support Protocol 1)

0.5% (v/v) glutaraldehyde in PBS (prepare fresh)

1 mM MgCl₂ in PBS

PBS (*APPENDIX 2D*)

Alkaline phosphatase prestaining buffer (see recipe)

Alkaline phosphatase staining solution (see recipe)

Neutral red stain

Aqueous mounting medium

65°C waterbath or oven

Coverslips

- 1 Post-fix 6- μ m frozen sections by immersing for 10 min in 0.5% glutaraldehyde/PBS for 10 min at room temperature.

Cultured cells in a tissue culture plate are immediately fixed in 0.5% glutaraldehyde in PBS for 10 min at room temperature after aspiration of the culture medium from the plate.
- 2 Wash samples by immersing in 1 mM MgCl₂/PBS at room temperature for 10 min.

If cells or tissue sections are to be double-stained for alkaline phosphatase and β -galactosidase, stain for β -galactosidase first at this point. Following β -galactosidase staining, rinse cells in 1 mM MgCl₂/PBS once, then proceed to the next step.
- 3 Inactivate endogenous heat-labile alkaline phosphatase by incubating samples, immersed in 1 mM MgCl₂/PBS that has been preequilibrated to 65°C, in a 65°C water bath for 30 min.

Care should be taken to make sure samples are submerged during the entire heat-inactivation process.
- 4 Rinse sample by immersing in PBS for 1 min, then wash sample in alkaline phosphatase prestaining buffer by immersing for 5 min at room temperature.
- 5 Place sample in alkaline phosphatase staining solution and incubate 4 to 6 hr at 37°C.

To minimize background staining, levamisole (Sigma) may be added to the staining solution (as 24 mg/ml stock) to a final concentration of 0.24 mg/ml.

- 6 Briefly counterstain samples in neutral red and coverslip with an aqueous mounting media.

It is important not to put alkaline phosphatase-stained samples in organic solvent, which will dissolve alkaline phosphatase. Tissue culture cells can be kept in PBS at 4°C for up to 1 week.

Alkaline phosphatase reporter-gene expression in ciliated airway epithelial cells is localized to the apical membrane. This peculiar apical expression should not be confused with the lack of transgene expression. In goblet and basal cells alkaline phosphatase expression is localized throughout the cytoplasm.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2d; for suppliers, see SUPPLIERS APPENDIX.

Alkaline phosphatase prestaining buffer

100 mM Tris-Cl, pH 9.5

50 mM MgCl₂

100 mM NaCl

Store up to 6 months at room temperature

Alkaline phosphatase staining solution

10 ml alkaline phosphatase prestaining buffer (see recipe)

44 µl nitroblue tetrazolium chloride (NBT; Life Technologies; 75 mg/ml final)

33 µl 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP; Life Technologies; 50 mg/ml final)

0.1 ml 24 mg/ml (100×) levamisole (Sigma; store stock solution up to 6 months at −20°C; optional)

Prepare fresh

BEGM

To formulate BEGM (complete bronchial epithelial growth medium), add the following components of the BEGM SingleQuot kit (Clonetics) to 250 ml of Ham's F12 media and 250 mL of DMEM. The hormonal SingleQuot supplements can be purchased in a BEGM BulletKit from Clonetics):

2 ml of 13 mg/ml bovine pituitary extract (BPE; 0.052 mg/ml final)

0.5 ml of 0.5 mg/ml hydrocortisone (0.0005 mg/ml final)

0.5 ml of 0.5 µg/ml human recombinant epidermal growth factor (hEGF; 0.0005 µg/ml final)

0.5 ml of 0.5 mg/ml epinephrine (0.0005 mg/ml final)

0.5 ml of 10 mg/ml transferrin (0.01 mg/ml final)

0.5 ml of 5 mg/ml insulin (0.005 mg/ml final)

0.5 ml of 0.1 µg/ml retinoic acid (0.0001 µg/ml final)

0.5 ml of 6.5 µg/ml triiodothyronine (0.0065 µg/ml final)

All hormonal supplements are obtained from Clonetics in the specific-sized aliquots and concentrations tabulated above; final concentrations are also given for reference. Some variability is seen in the quality of the bovine pituitary extract (BPE) from Clonetics. It may be necessary to prescreen BPE batches for those that provide optimal growth performance.

Lactose storage buffer

25 mM Tris·Cl, pH 7.4 (*APPENDIX 2D*)

60 mM NaCl

1 mg/ml arginine

50 mg/ml lactose

Filter-sterilize using 0.2 µm filter

Store up to 6 months at –20°C

Medium A

Modified Eagle medium (MEM; Life Technologies) supplemented with:

50 U/ml penicillin

50 µg/ml streptomycin

80 µg/ml tobramycin (Eli Lilly)

100 µg/ml ceftazidime (Eli Lilly)

100 µg/ml Primaxin (Merck)

5.0 µg/ml amphotericin B

10 µg/ml DNase I (Type II-S; from bovine pancreas; Sigma)

0.5 mg/ml dithiothreitol

Prepare fresh and keep at 4°C

Medium B

BEGM (see recipe) supplemented with:

50 U/ml penicillin

50 µg/ml streptomycin

80 µg/ml tobramycin

100 µg/ml ceftazidime

100 µg/ml Primaxin

5.0 µg/ml amphotericin B

Store up to 2 weeks at 4°C

Medium C

BEGM (see recipe) supplemented with:

50 U/ml penicillin
50 µg/ml streptomycin
40 µg/ml tobramycin
50 µg/ml ceftazidime
50 µg/ml Primaxin
Store up to 2 weeks at 4°C

Medium D

Modified Eagle medium (MEM; Life Technologies) supplemented with:

50 U/ml penicillin
50 µg/ml streptomycin
100 µg/ml ceftazidime (Eli Lilly)
100 µg/ml gentamicin
100 µg/ml Primaxin (Merck)
5.0 µg/ml amphotericin B
Store up to 2 weeks at 4°C

Serum airway medium, 5%

279 ml DMEM (high-glucose formulation; Life Technologies)
279 ml Ham's F-12 nutrient mixture (Life Technologies)
30 ml fetal bovine serum (FBS; *APPENDIX 3G*)
100 U/ml penicillin
100 µg/ml streptomycin
Store up to 2 weeks at 4°C

Trypsin inhibitor buffer

Prepare 1 mg/ml trypsin inhibitor (Type I-S, from soybean; Sigma) in Ham's F-12 medium (Life Technologies). Filter through 0.2-µm filter and store in aliquots up to 6 months at -20°C.

Ussing chamber culture medium

49% (v/v) DMEM
49% (v/v) Ham's F-12 nutrient mixture (Life Technologies)
2% (v/v) Ultraser G (Sepracor)
100 U/ml penicillin
100 µg/ml streptomycin
5.0 µg/ml amphotericin B
Store up to 2 weeks at 4°C

Xgal staining solution

PBS, pH 7.4 (*APPENDIX 2d*), containing:

1 mg/ml Xgal (5-bromo-4-chloro-3-indolyl- β -galactopyranoside)

5 mM $K_3Fe(CN)_6$

5 mM $K_4Fe(CN)_6$

1 mM $MgCl_2$

Prepare fresh from 10 $\times$ PBS and 20 $\times$ stocks of $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, and $MgCl_2$. Add Xgal just prior to use.

COMMENTARY

Background Information

This unit describes several model systems that have been useful for studying gene delivery to the airway. Potential therapeutic applications of gene therapy to the lung include treatment of inherited disorders such as cystic fibrosis (CF) and α_1 -antitrypsin deficiency (α_1 -AT), as well as of acquired disorders such as bronchitis, asthma, emphysema, and respiratory malignancies. The most widely studied application to date has involved gene therapy of CF lung disease. In this context the airway model systems and gene-transfer vectors described in this unit have led to a better understanding of airway biology, CF pathophysiology, and molecular aspects of gene transfer that are relevant to generating successful gene-therapy approaches for this disease (Driskell and Engelhardt, 2003). The success of current airway model systems in reconstituting the complex cellular organization of the airway is a necessary requirement for continued research in this area.

The cellular architecture of cartilaginous airways (trachea and bronchi) predominantly includes five cell types: basal, goblet, intermediate, nonciliated columnar, and ciliated cells (Breeze and Wheeldon, 1977). Identification of these cell types is predominately based on morphologic criteria. Ciliated cells are identified by the presence of apical cilia. Basal cells are cuboidal in appearance with high nuclear to cytoplasmic ratio and nuclei in the lowest layer of epithelium, showing direct contact with the basal lamina and no luminal contact. Goblet cells are distinguished by the presence of mucous granules. Nonciliated columnar cells are marked by the absence of cilia and secretory granules, but have an apical boundary at the luminal surface. Intermediate cells are cells adjacent to the basal lamina with cytoplasm extending upward into the epithelium but not contacting the luminal surface. Although it is generally accepted that both goblet and basal cells have the capacity for self-renewal, the multipotent capacity of these cell types to give rise to ciliated and intermediate cells is currently debated (Engelhardt et al., 1995; Liu et al., 2006). Such characteristics are relevant to obtaining persistent gene transfer with integrating vectors. More distal regions of the conducting airway epithelium include the bronchioles, which are composed of at least two cell types, Clara and ciliated cells. The progenitor cell of this region has traditionally been thought to be the Clara cell (Kauffman, 1980). Strategies for prolonged gene therapy to the lung of CF patients will likely need to target progenitor cells of the airway which give rise to cystic fibrosis transmembrane regulator (CFTR)-expressing cellular targets. However, since the turnover rate of respiratory epithelial cells in the nondiseased airway is >100 days (Donnelly et al., 1982; Rawlins and Hogan, 2008), substantial lengths of correction can also be achieved by directly targeting transiently lived, fully differentiated cell types.

Numerous vector systems have been used to target cells of the lung, including recombinant adenovirus, adeno-associated virus, retrovirus, lentivirus, and liposomes (Flotte, 2005; Flotte

and Carter, 1995; Flotte et al., 2007; Gill et al., 2010; Rosenecker et al., 2006; Wagner and Gardner, 1997; Wilson, 1995). Many of these vector systems have been evaluated in several animal models, including mice (Hyde et al., 1993; Kaplan et al., 1997; Liu et al., 2009), rats (Engelhardt et al., 1994; Rosenfeld et al., 1992), human tracheal xenografts (Engelhardt et al., 1993b; Fisher et al., 2012; Goldman et al., 1997; Keswani et al., 2012; Zhang et al., 1998), rabbits (Flotte et al., 1993; Sinn et al., 2005), and nonhuman primates (Afione et al., 1996; Engelhardt et al., 1993a; Flotte et al., 2010; Zabner et al., 1994)). The largest obstacle in evaluating the ability of various gene-transfer systems to reverse CF airway pathophysiology is the lack of an animal model reconstituting the pathologic progression of CF lung disease. Although genetically defined CFTR-deficient mice have been useful for evaluating nasal epithelial defects in electrolyte transport, analogous studies evaluating lung pathophysiology have been disappointing due to the differences in cell biology between mouse and human lungs. The human bronchial xenograft model has proven useful for evaluating specific cellular defects found in the CF airway; however, this model system also lacks immune responses and whole-lung physiology. Newborn ferret tracheal xenografts, however, develop mature submucosal glands in addition to airway epithelia, and therefore demonstrate usefulness in evaluating different mechanisms of innate immunity. New CF animal models in the pig (Rogers et al., 2008; Welsh et al., 2009) and ferret (Sun et al., 2010; Sun et al., 2008) have shown very similar lung pathology to humans with the disease, and hold great promise for elucidating disease mechanisms and evaluating gene therapies (Keiser and Engelhardt, 2011).

Recombinant Adenovirus—Recombinant adenoviruses (*UNIT 12.4*) have been the most extensively studied vehicles for gene transfer to the lung (Brunetti-Pierri and Ng, 2006). These vectors, which consist of a double-stranded, linear 36-kb DNA genome, have the attractive advantage of high attainable titers together with the ability to infect nondividing cells (Yeh and Perricaudet, 1997). This is a particular asset for targeting of fully differentiated adult airways since the rate of cell division is quite low. Multiple animal models, including the cotton rat, mouse, human tracheal xenografts, and nonhuman primates have been used for evaluating the efficiency of recombinant adenoviral gene transfer to the lung. Each animal model possesses several advantages in the analysis of host-vector interactions and efficiency of gene transfer. A commonly used model involves the cotton rat, which has been shown to be permissive for adenoviral infection and provides a pathophysiologically relevant correlate to human adenoviral infections in the lung (Ginsberg et al., 1990). Although the mouse has traditionally been thought to be less permissive for adenoviral infection, because genetically defined mouse strains are readily available mice provide additional advantages in evaluating host-vector inflammatory responses and generating new viral vectors with increased persistence and decreased inflammatory responses (Yang et al., 1994). The human bronchial xenograft, although immune-incompetent with respect to T cells, provides the ideal system for the analysis of cellular interactions between human epithelium and adenoviral vectors. This system has been useful in addressing the capacity of these vectors to replicate and express viral genes within the relevant target human airway epithelium. Lastly, nonhuman primates have allowed for the evaluation of safety and efficacy within an animal model that is close in evolutionary terms to humans (Flotte et al., 2010).

Despite initial successes in achieving high-level gene transfer throughout the mouse airway (Kaplan et al., 1997; Yang et al., 1994) and cotton rat airway (Engelhardt et al., 1994; Rosenfeld et al., 1992), studies have suggested that these vectors may be less tropic for fully differentiated human airways (Grubb et al., 1994) and higher viral dosages may be required to achieve therapeutic levels of infection (Engelhardt et al., 1993a). Recent advances in the biology of vector-epithelial cell interactions have suggested that the abundance of apical surface integrins (V5; (Goldman and Wilson, 1995; Pickles et al., 1996)) and/or fiber

receptors (Zabner et al., 1997) may in part influence the efficiency of recombinant adenoviral gene transfer to human airways. Several studies have demonstrated much higher levels of adenoviral transduction following injury to human and mouse nasal airways, which may alter the expression patterns of these integrins (Pickles et al., 1996; Pilewski et al., 1997). A second limitation of current recombinant adenoviral vectors is limited persistence in immune-competent animal models due to vector-associated cellular and humoral immune responses (Engelhardt et al., 1994; Yang et al., 1994). Such immunologic responses are the result of residual viral gene expression and proteins associated with viral-particle inoculum. Additionally, transgenes such as β -galactosidase have also been noted to evoke cellular immune responses, which may limit transgene expression. Despite the relatively inefficient infection of fully differentiated human proximal airways with recombinant adenovirus, the ability to use high viral titers for infection may circumvent this limitation if associated toxicity and inflammation can be abrogated by increasing the safety of vector design. Numerous laboratories have focused efforts on altering the vector design of recombinant adenovirus by deleting or mutating viral genes responsible for cellular-associated immunity, in an effort to increase the achievable dose of vector administration in the absence of toxicity (Engelhardt et al., 1994; Yang et al., 1994). Alternative strategies are aimed at altering the host immune responses to allow for higher levels of vector delivery with more prolonged persistence.

Recombinant Adeno-Associated Virus—Adeno-associated virus (AAV; *UNIT 12.9*) is a single-stranded DNA parvovirus and represents an alternative vector for gene delivery to the airway (Flotte, 2005; Flotte and Carter, 1995). Attractive features of this particular parvovirus include its ability to infect nondividing cells and integrate into the host genome. In contrast to recombinant adenovirus, which persists as an episome, rAAV can persist as either an episome or as an integrated provirus. Wild-type AAV also has the ability to integrate specifically within a defined site on chromosome 19 (Samulski, 1993). Although recombinant AAV vectors lose their ability for site-specific integration at this locus, research in this area may ultimately enhance the targeting of AAV to specific sites in the cellular genome. Some researchers have had success in using this rAAV serotype 2 (rAAV2) for persistent gene delivery to the airways of rabbits and nonhuman primates (Flotte, 2005; Flotte and Carter, 1995). However, it is generally accepted that the level of gene expression with rAAV2 in the lung is much less efficient than in other organs such as muscle (Fisher et al., 1997). It was found that intracellular endosomal processing of apically-entering rAAV2 virions is a rate limiting step and is inefficient in airway cells, leading to lack of nuclear targeting and transduction (Duan et al., 1998b; Duan et al., 2000). This defect can be overcome by the addition of proteasome inhibitors during infection, which enhance rAAV2 transduction by several orders of magnitude (Duan et al., 2000). Furthermore, other serotypes of rAAV have proven more efficient in apical transduction of airway epithelial cells, such as serotype 1, 6, and 9 (Halbert et al., 2001; Limberis et al., 2009; Yan et al., 2012; Yan et al., 2006; Zincarelli et al., 2008). In addition, mutagenesis, generation of hybrid vectors, and directed evolution of viral capsid sequences has also led to an increased repertoire of potential rAAVs that may be useful for airway gene transfer (Asokan et al., 2012; Wu et al., 2006). A combination of these approaches may serve to identify the capsid sequences most suitable for airway gene transfer by rAAV. Also, further investigation into the cellular factors that promote high-level rAAV gene expression in the airway may ultimately enhance the use of this vector for lung gene transfer by modulating target cells prior to infection.

Recombinant Retrovirus and Lentivirus—Retroviruses and lentiviruses (*UNIT 12.5, 12.10*), which fall into the classification of RNA viruses, have attractive advantages as vectors due to their efficient integration. However, the application of retroviral vectors in the

airway is limited by the need for cell division to achieve transduction and the low titers achievable with this vector. Despite these limitations, several groups have focused efforts on modifying the cell cycle with growth factors as a mechanism for increasing infection in the airways (Wang et al., 1999). Alternatively, these vectors may prove useful in approaches of in utero gene therapy for CF, where the target cell population is much reduced and cells are normally actively dividing. The principles of in utero applications of these vectors have been tested in sheep (Pitt et al., 1995) and xenograft models (Duan et al., 1998a; Engelhardt et al., 1992). Furthermore, retroviruses have been useful in addressing aspects of progenitor-progeny relationships within the human airway using the human bronchial xenograft model described in this unit (Engelhardt et al., 1995). By contrast, lentiviral vectors do not require cellular division for transduction. The development of pseudotyped lentiviral vectors capable of infected the airway in various species are increasing and may also prove attractive as a gene therapy agent (Gill et al., 2010; Liu et al., 2010; Mishra et al., 2011; Sinn et al., 2012).

Liposomes—Liposome-mediated gene transfer to the airway has considerable advantages due to the low level of toxicity. However, limitations include transient low-level expression in differentiated airway epithelia (Griesenbach et al., 2010; Matsui et al., 1997; McLachlan et al., 2011). Despite this apparent limitation, several laboratories have had considerable success with the use of cationic liposome-mediated gene transfer in several animal models including mouse and rat lung (Alton et al., 1993; Hyde et al., 1993; Rosenecker et al., 2006; Tagalakakis et al., 2008) and human tracheal xenografts (Zhang et al., 1998).

Critical Parameters and Troubleshooting

The most critical parameter in generating well-differentiated models of the human airway is the quality of primary airway epithelial cells. Freshly isolated lung transplant tissue is better than post-mortem tissue. The best polarized airway epithelia are obtained when unpassaged, freshly isolated airway epithelial cells are used. High seeding densities are also critical to the success of polarized airway epithelial cell models. With good viable airway samples, polarized epithelia can also be generated from passage-1 primary cells with some success. Exposure of the apical surface of polarized epithelial cultures to air is also a necessary feature for promoting ciliogenesis. Human tracheal xenografts can be reproducibly generated from primary cells that have been expanded in culture by serial propagation one time. Twice-passaged primary cells can also be used for xenografts; however, the success will be dependent on the quality of airway samples obtained. Newborn ferret tracheal xenografts, on the other hand, must be generated from freshly isolated intact tracheas and cannot be cultured before engraftment. The generation of CF airway models such as xenografts and polarized epithelia requires the early use of very high levels of antibiotics and antifungal agents to prevent infection. Since these agents (especially antifungal agents) are toxic to primary cells, the exposure should be limited to 1 to 3 days post seeding. Prolonged culturing in the presence of antibiotics at levels outlined for medium C (see Reagents and Solutions) can be used without deleterious results on viability. Infections in CF samples can also be avoided by extensive washing of airway tissue and cells just following seeding.

Another parameter critical to maintaining primary cultures capable of serial passage and reconstituting differentiated airways in xenografts is minimizing exposure to fetal bovine serum (FBS). Although serum is needed in the initial preparation of cells from tissue to promote adherence to tissue culture plastic, subsequent exposure to serum following trypsinization will lead to differentiation of airway cells and reduced capacity for growth in serial passaging. Therefore methods have been developed that utilize trypsin inhibitor rather than serum to inactivate trypsin during passaging of cells. Healthy proliferating airway

progenitor cells have a morphologic appearance of small uniform cells with a high nuclear to cytoplasmic ratio. Differentiation leads to larger, more irregularly shaped flattened cells, and is a sign that cultures will no longer have the capacity for good regeneration in the bronchial xenograft model.

Parameters that affect the efficiency of gene transfer to airway models are quite diverse and dependent on the vector used for study. Retroviruses require proliferation for productive transduction and hence are most suitable for primary proliferating cultures of airway cells grown on plastic. In contrast, recombinant adenoviruses do not require proliferation for infection and expression of transgenes; however, fully differentiated airway epithelia are more resistant to apical infection by this vector. Toxicity of recombinant adenoviral vectors should also be closely monitored when evaluating functional endpoints. Because, at high titers of infection, traditional E1-deleted adenoviral vectors can have cryptic expression of viral genes, irrelevant transgene vector controls such as β -galactosidase or alkaline phosphatase should always be used when interpreting functional effects. In the context of CF research, these negative controls are absolutely necessary when interpreting functional electrophysiologic endpoints which can be effected by the integrity of the epithelial tight junctions. Like adenovirus, rAAV has the capacity to transduce nondividing cells, albeit at lower levels than actively proliferating cells. The major limitation, currently, to rAAV infection in airway cells is obtaining adequate titers for infection. This vector has substantially less toxicity than adenovirus and hence is attractive if technical limitations of production can be overcome. Of importance in the use of rAAV is the extent of contaminating helper adenovirus if this virus is used in the amplification method; adenovirus can significantly increase the level of transduction with rAAV. Typically, the extent of adenoviral contamination can be easily assessed by growing rAAV with recombinant adenovirus harboring an alternative transgene indicator. Hence, adenoviral contamination can be quantitated by direct histochemical staining of rAAV stocks. The purity of rAAV stocks is also very important to maintaining non-toxicity when applied to primary airway cells. Contaminating proteins such as hexon and fiber, which are generated during AAV propagation, are extremely toxic to primary airway cells. Hence, at a minimum, three rounds of serial CsCl density centrifugation should be performed when generating rAAV stock.

Expression of transgenes in the airway can also be affected by the promoters used within viral cassettes. This is most notable for retroviruses and AAV. CMV enhancer/ β -actin promoter retroviral vectors demonstrate stable transgene expression at high levels in all cell types in human tracheal xenografts. In contrast, LTR-driven retroviral reporters have some level of cell type-specific expression in xenograft epithelia (lower in ciliated cells than other cell types). Although there is substantially less information on promoter effects with rAAV vectors in the airway, promoters such as CMV have been suggested to be dramatically less efficient at expressing transgenes in the liver than RSV or LTR promoters. Similar concerns may be highly relevant in models of rAAV gene transfer to the airway. The K18 promoter has also been used to deliver more specific airway expression.

Delivery of viral vectors is most variable for in vivo gene transfer to lungs of intact animals. Such variability can be caused by the position of animals at the time of vector delivery, respiration during administration of vector, and the amount of vehicle delivered to the lung. For delivery to alveolar regions, larger volumes will increase gene transfer. In contrast, delivery to more proximal airways can be enhanced by lower volumes of vector or inhalation protocols. Efforts should be taken to evaluate the largest sampling of lung tissue when studying functional effects and/or transgene expression. Often the use of serial sections can be used to localize areas of transgene expression in one section, while a second serial section can be used to evaluate a second functional endpoint.

Anticipated Results

Critical to the use of airway models for studies of gene therapy is the extent of gene transfer needed to obtain a functional effect. Functional effects can be measured by complementation of primary defects caused by a genetic defects (i.e., chloride secretion in the case of CF) or in terms of ectopic overexpression of an endogenous gene of interest. In setting up these model systems it is advantageous to start with a known, easily detectable reporter gene to determine the baseline efficiencies of the vector type and cassette used for expression. Each of the airway model systems has different requirements that influence the choice of optimal vector types. For proliferating airway cells, retroviral vectors provide the most stable transduction (up to 50%) since these vectors integrate their transgenes. However, recombinant adenovirus can give complete transduction with appropriate MOIs. Toxicity of vectors may also play a role in choosing which is most appropriate (adenovirus is more toxic than retroviral infection). Complete transduction can be achieved by serial infections; however, increased toxicity is also seen. Furthermore, when immune-competent animal models are used, immune responses to viral gene products will limit the duration of transgene expression; typically, transgene expression from recombinant E1-deleted adenoviruses will be lost by 14 days post infection due to cellular immune responses. However, cellular immune responses to reporter genes can also affect the duration of expression. This is not the case in human tracheal xenografts, for which the *nu/nu* hosts are deficient in T cells. Transgene expression from recombinant E1-deleted adenovirus is stable for at least 8 weeks in human tracheal xenografts. Hence, toxicity of initial infections can be diminished by a waiting period following infection, and prior to functional assessment of transgene-related effects.

Time Considerations

Primary cultures of airway epithelia generally take ~1 week to establish. Polarized airway epithelial monolayers take somewhat longer, and require at least 3 weeks to differentiate and form a ciliated epithelium. These polarized epithelia are stable for several months without diminution in bioelectric resistance. Bronchial xenograft models are by far the most time-consuming and technically difficult models described in this unit. Each xenograft takes ~5 hr of processing time, which includes primary cell culturing, generation of xenograft cassettes, surgery, and xenograft maintenance to 4 weeks of differentiation. A typical experiment of 12 xenografts is about the largest one should undertake at any one time. From a biologic standpoint, tracheal xenografts typically take ~4 to 5 weeks to become fully differentiated following seeding of primary airway epithelial cells.

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Figure 13.9.1.

In vitro cultures of polarized airway epithelial cells. The monolayer of primary airway epithelial cells are grown on collagen-coated Millipore cell culture inserts (the total growth area is 0.6 cm^2) placed in wells of a 24-well tissue culture plate. The support filter is made from mixed esters of cellulose nitrate and acetate and subsequently coated with liquid collagen. During culturing the basolateral sides of the human airway epithelia are exposed to Ussing chamber culture medium ($\sim 500 \mu\text{l}$ is added to the outside of each insert). The apical surfaces are exposed to the air. Scanning electron micrographs kindly provided by Zabner et al., et al., 1996; Bar: right panels, $5 \mu\text{m}$; left panels, $37.5 \mu\text{m}$. Reproduced with permission from American Society for Microbiology.

Figure 13.9.2.

Human tracheal xenograft model. Subcutaneous implants of xenograft human airways are generated from primary human airway cells transplanted onto denuded rat tracheas. Primary cells can be genetically manipulated ex vivo with vectors such as recombinant retrovirus and adeno-associated virus (see Basic Protocol 2). Alternatively, xenografts can be generated from genetically unaltered primary cells and gene-transfer studies performed in vivo following reconstitution of grafts. Typically, a fully differentiated mucociliary epithelium is obtained by 4 weeks post-transplantation. Identifiers on micrograph: B, basal cell; Ci, ciliated cell; I, intermediate cell; G, goblet cell. Bar = 20 μm).

Figure 13.9.3.

Xenograft cassettes are generated from a series of defined types of tubing as defined in (A). Tube a, 2.5-cm Silastic tubing; tube b, 1.9-cm Silastic tubing; tube c, 4.5-cm Silastic tubing; tube d, 3.2-cm Teflon tubing; tube e, adapter. Tubing is connected to denuded rat tracheas by a series of suture ligations as shown in (B).

Figure 13.9.4.

Transplantation of tracheal xenografts in *nu/nu* mice. (A) The mouse on the left represents a schematic view of subcutaneous transplantation of the xenograft cassette. (B) Four incisions are made as marked by arrows and the xenograft cassette guided subcutaneously using forceps, so that one port exits through the back of the neck and the other port through the main incision. Surgical staples are used to close incisions as marked by open arrowheads. (C) The right diagram shows the resultant xenografted mouse after 1 week post transplantation, when staples are removed. The staples marked by closed arrowheads in panel C are used to maintain the position of the cassette and prevent subcutaneous migration (typically it may be necessary to leave these staples in for 2 to 3 weeks).

Table 13.9.1**Choice of Airway Model System**

Airway model system	Advantages	Disadvantages
Proliferating cultures of human airway cells (Basic Protocols 1 and 2)	Ease of use; rate of gene transfer is typically higher than in differentiated models of the airway	Cells are undifferentiated
Polarized human airway epithelial monolayers (Basic Protocols 3 and 4)	Good differentiation; experimentally very flexible for functional measurements (e.g., bioelectric properties)	Requires freshly isolated airway cells; differentiated monolayers may not contain all cell types found in vivo
Human tracheal xenografts (Basic Protocols 5 and 6)	Excellent differentiation which appears to contain the native distribution of all cell types in the human airway; partially reconstitutes submucosal glands; partial immune response present	Technically difficult; expensive; immune incompetent with respect to T cells
Ferret newborn Tracheal xenografts	Excellent differentiation which appears to contain the native distribution of all cell types in the human airway; fully reconstitutes submucosal glands; partial immune response present	Technically difficult; expensive; immune incompetent with respect to T cells
Intact lung of animal models (Basic Protocol 7)	Can assess aspects of intact lung function and gene delivery	Not human; airway cell types may differ from that of human

Table 13.9.2Choice of Vector System^a

Airway model system	Efficiency of gene transfer
Proliferating cultures (Basic Protocols 1 and 2)	Adenovirus > retrovirus > adeno-associated virus
Polarized monolayers (Basic Protocols 3 and 4)	Adenovirus > adeno-associated virus ^b > retrovirus
Tracheal xenografts (ex vivo gene delivery; Basic Protocols 5 and 6)	Retrovirus > adeno-associated virus > adenovirus
Tracheal xenografts (in vivo gene delivery; Basic Protocols 5 and 6)	Adenovirus > adeno-associated virus ^b > retrovirus
Lung of animal models (Basic Protocols 7)	Adenovirus > adeno-associated virus ^b > retrovirus

^aOther considerations influencing vector choice and efficiency of gene transfer, such as the potential for integration, stability of transgene expression, the species being used, whether EGTA is being used to expose the basolateral surface of airway epithelia, and the potential for inflammatory responses, are described in the respective units dealing with viral vectors—including adenovirus (*UNIT 12.4*), retroviruses (*UNIT 12.5*), and adeno-associated virus (*UNIT 12.9*).

^bIt is difficult to make generalization for rAAV since there are so many serotypes. But in generally, with the use of proteasome inhibitors, these vectors perform at the indicated efficiency relative to the other vectors.