15

Airway Epithelia

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1. Introduction

Cystic fibrosis (CF) is a common inherited disorder affecting a variety of epithelial tissues. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) that lead to abnormal secretions, recurrent infection and inflammation, bronchiectasis, and premature death. Because airways disease is the major cause of morbidity and mortality in cystic fibrosis, gene therapy efforts have focused on luminal delivery of vector to the airways of CF patients. Retroviruses are attractive as a gene transfer vector system since integration of the wild-type CFTR cDNA into the host genome may lead to long-term expression and perhaps, a cure. However, simple retroviruses are limited as vectors for airway gene transfer by the low rates of epithelial cell proliferation in human airways (~0.1–0.2%) combined with the traditionally low titers. Advances in vector design and production have improved titers, and the development of human and animal lentiviruses may help overcome the requirement for cell proliferation. These developments have raised hopes for retroviral approaches for treatment of CF lung disease.

The best-known lentiviruses are the human immunodeficiency viruses types 1 and 2 (HIV-1 and 2) and simian immunodeficiency viruses (SIV). HIV, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV) have each been developed as gene transfer vectors for cystic fibrosis. Lentiviral vectors have been shown to transduce a variety of nondividing cell types in vitro and in vivo ***(1–9)***. HIV, EIAV, and FIV vectors have been shown to efficiently transduce aphidicolin-treated (growth-arrested) airway epithelial cells in vitro ***(6–8,10)***.

From: *Methods in Molecular Biology, vol. 229: Lentivirus Gene Engineering Protocols*
Edited by: M. Federico © Humana Press Inc., Totowa, NJ

The envelope proteins of lentiviral vectors bind to cell surface receptors to facilitate entry with subsequent reverse transcription, nuclear import, and integration into the host chromosome culminating in gene expression. To evaluate the polarity of lentiviral transduction, vescicular stomatitis virus glycoprotein-G (VSV-G) pseudotyped vectors have been applied to either the apical or the basolateral surfaces of polarized well-differentiated (WD) human airway epithelial (HAE) cells. Thirty-fold greater transduction efficiency was observed in vitro when HIV VSV-G vectors were applied to the basolateral surface as compared to apical application of vector ***(11)***. Goldman and colleagues demonstrated that luminally applied HIV (VSV-G) vectors failed to transduce WD primary HAE cells in bronchial xenografts ***(11)***. Similar data have evolved for EIAV pseudotyped with VSV-G ***(11a)***, and for amphotropic- and VSV-G-enveloped FIV vectors ***(7,10)***. These data suggest that, while lentiviral vectors can transduce nondividing cells, the receptors for uptake and entry of amphotropic and VSV-G pseudotyped MLV and lentiviral vectors are predominantly localized to the basolateral membrane of polarized epithelial cells.

Two strategies have been proposed to overcome the lack of apical membrane receptor expression: (1) host modification with injury models and agents that permeabilize tight junctions to increase vector access to basolateral membrane receptors and basal cells ***(11)*** and (2) targeting the apical membrane of polarized airway epithelia by pseudotyping lentiviruses with envelope proteins from other viruses that bind and enter across the apical membrane.

Injury models and transient permeabilization of intercellular junctions are methods by which host cells have been modified to increase access to the basolateral receptors. Johnson et al. exposed mice to sulfur dioxide (SO_2) inhalation to increase vector delivery to the basolateral surface and/or basal cells of murine tracheas. Induction of airway cell proliferation peaked at 24 h postinhalation with no significant increase in proliferation occurring in the first 12 h after inhalation ***(12)***. SO_2 also caused a dose-related denuding of the surface epithelium leaving the basal cell layer intact. In regions less severely injured by SO_2, permeability was increased through the tight junctions ***(12)***. Using this model, mice were exposed to SO_2 for 3 h at 500 ppm, and a HIV (VSV-G) vector was delivered onto the nasal or tracheal epithelia of rodents ***(8)***. Efficient reporter gene transfer was observed in nasal and tracheal airway epithelia, whereas no gene transfer was detected in the nasal or tracheal airway epithelia of sham (air)-exposed controls ***(8,12)***. Gene transfer to murine trachea was also more efficient following SO_2 exposure when vector was administered on the same day of exposure (~7% of cells transduced) and when cell proliferation was not increased, as compared to the day after SO_2 inhalation (2% transduction of cells) at which time peak airway cell proliferation occurs.

This finding is consistent with preferential transduction of nondividing cells ***(8)***. These data demonstrate that HIV vectors can efficiently transduce airway epithelia in vivo when access to receptors on the basolateral membrane is increased by injury.

Transient permeabilization of tight junctions is another method by which to increase vector delivery the basolateral membrane and basal cells. Nonprimate lentiviral vectors have been coadministered with calcium chelators to airways to increase vector access to basolateral receptors, presumably by modulating paracellular permeability ***(7,10)***. Wang et al. applied FIV-CFTR vectors in combination with hypotonic solution and ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid (EGTA) to the apical membrane of polarized WD CF airway epithelial cells ***(7,10)***. Restoration of chloride secretory function was achieved following infection with FIV-CFTR in primary CF airway epithelial cells that was similar to levels of correction achieved with an adenoviral (Ad)CFTR vector. The correction of chloride secretory function mediated by FIV-CFTR persisted for up to six months in culture, whereas the AdCFTR-mediated Cl- correction had resolved by 21 d after transduction. Subsequently, these investigators demonstrated relatively efficient transduction of ciliated and nonciliated cells within rabbit airways in vivo comprising approx 5% of airway cells infected with vector at d 5 and decreasing to approx 2.5% of cells at 6 wk ***(7)***. The mechanism of attenuation of transgene expression was not evaluated. Nevertheless, FIV-CFTR vectors offer promise as vectors for gene therapy of cystic fibrosis.

Olsen and colleagues have developed a mammalian lentiviral vector based on EIAV ***(6)***. As compared to HIV-1, EIAV has a much simpler genome. Horses infected with the wild-type EIAV virus typically survive the infection (~95%), and wild-type EIAV does not replicate in human cells. Olsen demonstrated efficient transduction of WD primary HAE cells after apical application of EIAV (VSV-G) β-galactosidase (lacZ) vector in the presence of ethylenediaminetetraacetic acid (EDTA) or EGTA to increase access to basolateral receptors by permeabilizing the tight junction. Persistence of transgene expression in vitro lasted for up to 24 d posttransduction, the longest period tested. In preliminary studies, EIAV vectors with internal promoters have been shown to express reporter genes in murine tracheal airway epithelia following SO_2 injury to increase delivery to basal cells and the basolateral membrane ***(13)***. The efficiency of transduction was dose-related with minimal transduction at vector titers of 10^8 infectious units (i.u.)/mL, whereas vectors with approx 10^{10} i.u./mL yielded transduction efficiencies that were significantly greater than those observed at lower EIAV lentiviral titers ***(13)***.

Targeting lentiviral vectors to receptors on the apical membrane is an alternative strategy to increase airway gene transfer. The usual paradigm for

retroviral targeting is the generation of pseudotypes from envelope proteins of other viruses that target specific cell types. Toward this goal, investigators have screened enveloped viruses for their ability to infect WD HAE cells from the apical surface. Although some efforts have focused on common respiratory viruses, others have also included nonrespiratory viruses. Of the respiratory viruses considered as candidates for pseudotyping, respiratory syncytial virus (RSV), human corona virus, and influenza virus have received the most attention. Wild-type and replication-competent recombinant RSV have been shown to infect or enter cells across the apical membrane of WD HAE cells ***(14,15)***. Human corona virus 229E (HcoV229E) has also been shown to bind and enter across the apical membrane of WD HAE cells ***(16)***. However, no successful lentiviral pseudotypes derived from the Env proteins of these respiratory viruses have been reported.

Influenza A virus subtype H2N2/Japan/305/57 has been shown to preferentially infect the apical, rather than the basolateral, membrane of polarized WD HAE cells, whereas apical infection of HAE cells with subtypes H1N1 and H3N2 was inefficient ***(17)***. The binding of the H2N2 subtype appeared to be specific for sialic acid α2,3-gal residues, suggesting that these sialic acid residues may serve as an apical membrane receptor for targeting of lentiviral pseudotypes. Recently, Morse et al. have shown in preliminary studies that coexpression of influenza M2 and neuraminic acid (NA) proteins in producer cells enhances titers of influenza hemagglutinin pseudotyped EIAV vectors ***(18)***. Moreover, these HA/M2/NA chimeric pseudotyped vectors transduced polarized WD HAE cells following luminal application.

The Env proteins of other viruses have also been considered. In a preliminary study, FIV-based vectors were pseudotyped with amphotropic, xenotropic, VSV-G, RD-114, 10A1, ecotropic, gibbon ape leukemia virus (GALV), Marburg, and Ebola envelope glycoproteins by transient transfection techniques. Only FIV vectors pseudotyped with the Marburg virus envelope glycoprotein efficiently transduced WD HAE cells following luminal application ***(19)***.

Kobinger and colleagues further explored the potential of this family of viruses, known as the Filoviridae, to target the apical membrane of airway epithelia ***(20)***. Filoviridae are enveloped, nonsegmented negative sense RNA viruses, which include Ebola virus and Marburg virus among its members. To identify viral envelopes capable of mediating apical transduction of polarized air-liquid interface cultures, HIV vectors were pseudotyped with murine leukemia virus (MLV), influenza-hemagglutinin, RSV F and G proteins, Mokola, Ebola Reston (Ebo-R), or Ebola Zaire (EboZ) envelopes. Concentrated stocks of EboZ, but not EboR or other pseudotypes, efficiently transduced polarized HAE cells following apical application with gene transfer in up to 70% of cells as compared to up to 40% of cells following basolateral

transduction. EboZ-pseudotyped HIV vectors also transduced WD HAE cells in xenografts and in freshly excised human tracheal explants. Thus, lentiviral vectors may overcome the limitations of low rates of cellular proliferation, and pseudotyping lentiviral vectors with specific envelope proteins may overcome barriers to titer and the lack of apical membrane receptors on polarized WD HAE cells.

Previously, we have published methods for retroviral infection of primary HAE cells on plastic and for functional characterization of CFTR function in vitro ***(21)***. In this chapter, we focus on methods for lentiviral transduction of polarized, well-differentiated primary human airway epithelia in vitro. An EIAV vector pseudotyped with VSV-G will serve as the prototypical vector for which the methods have been written. However, the methods should also be applicable to HIV, VSV-G, and other pseudotyped lentiviral vectors. Enhancement of luminal gene transfer will be described for host modification approaches shown to be useful for at least one of the currently employed VSV-G pseudotyped lentiviral vectors studied in airways.

2. Materials

2.1. EIAV Lentiviral Vector Production

2.1.1. Culture of 293T Cells and BiG-45 EIAV Packaging Cells

1. Dulbecco's modified Eagle medium, high glucose (DMEM-H), with 4500 mg/L glucose and L-glutamine (Invitrogen, Carlsbad, CA).
2. 293T cells, cultivated in DMEM-H supplemented with 10% fetal bovine serum (FBS) (Invitrogen).
3. BiG-45 cultivated in DMEM-H supplemented with 10% Tet System Approved FBS (Clontech, Palo Alto, CA) (*see* **Note 1**).
4. Penicillin (50 U/mL) and streptomycin (50 μg/mL) as antibiotics.

2.1.2. Production of Lentiviral Vectors

We generally use $CaPO_4$-mediated transfection to generate virus by transient transfection. We make our own reagents, although we have on occasion used the Calcium Phosphate Transfection System from Life Technologies (Carlsbad, CA) with good results.

2.1.2.1. Calcium Phosphate Transfection Reagents

1. 0.5 *M* HEPES (pH 7.1): Dissolve 12 g HEPES (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid [Roche Applied Science, Indianapolis, IN] in H_2O to a final volume of 100 mL. Adjust pH to 7.1 ± 0.05 with 3 *N* NaOH. Sterilize by filtration using a 0.2-μm filter (*see* **Note 2**).
2. 2 *M* NaCl: Dissolve 11.7 g NaCl in H_2O to a final volume of 0.1 L. Sterilize by filtration using a 0.2-μm filter.

3. 2 *M* $CaCl_2$: Dissolve 29.4 g $CaCl_2$•$2H_2O$ in H_2O to a final volume of 0.1 L. Sterilize by filtration using a 0.2-μm filter.
4. 150 m*M* phosphate buffer (pH 7.0): Dissolve 4.02 g Na_2HPO_4•$7H_2O$ in H_2O to a final volume of 100 mL. Dissolve 2.08 g NaH_2PO_4•H_2O in H2O to a final volume of 100 mL. Adjust 42.3 mL NaH_2PO_4 solution to pH 7.0 with 57.7 mL of the Na_2HPO_4 solution. Sterilize by filtration using a 0.2-μm filter.
5. 2X HEPES-buffered saline (HBS) solution: Mix 1.53 mL sterile H_2O, 200 μL 0.5 *M* HEPES (pH 7.1), 250 μL 2 *M* NaCl, and 20 μL 150 m*M* phosphate buffer (pH 7.0). Make up fresh 2X HBS solution on the day of transfection.

2.1.2.2. Induction Reagents

1. 500 m*M* sodium butyrate (50X stock): Dissolve 0.55 g (Sigma, St. Louis, MO) in H_2O to a final volume of 10 mL. Sterilize by filtration using a 0.2-μm filter. Store at –20°C.
2. 1.5 mg/mL doxycycline (1000X stock in 95% ethanol, obtained from Sigma). Store in dark (light sensitive) at –20°C.

2.1.2.3. Concentration of Virus by Low Speed Centrifugation

1. Formulation buffer: 19.75 m*M* Tris-HCl (pH 7.0), 40 mg/mL lactose, 37.5 m*M* NaCl, 5 μg/mL protamine sulfate. Sterilize by filtration using a 0.2-μm filter. Store at 4°C.
2. Centrifuge tubes and adapters for the Beckman SW 28 rotor: 30-mL Konical tubes (Beckman Coulter, Fullerton, CA) and adapters.

2.2. Primary Culture of Human Airway Epithelial Cells

1. Phosphate-buffered saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 2.16 g Na_2HPO_4•$7H_2O$, and 0.2 g KH_2PO_4 in 800 mL distilled deionized H_2O. Adjust to pH 7.2 and bring to 1 liter final volume.
2. 1.0 mg/mL protease Type XIV (Sigma) plus 0.1 mg/mL deoxyribonuclease I (Sigma) in Jolik's minimum essential medium (JMEM) (0.2-μm filter sterilized).
3. Trypan blue (0.4%) in PBS (Sigma).
4. Purified collagen (Vitrogen-100, Cohesion, Palo Alto, CA).
5. Collagen-coated plastic tissue culture dishes (*see* **Note 3**).
6. Trace Elements
   Make stock solutions of all trace elements in tissue culture grade H_2O as specified below.

Selenium ($NaSeO_3$)	52.0 mg in 100 mL	3.0 m*M*
Manganese ($MnCl_2$•$4H_2O$)	1.26 mg in 100 mL	0.1 m*M*
Silicone (Na_2SiO_3•$9H_2O$)	1.42 g in 100 mL	50 m*M*
Molybdenum (NH_4)$6Mo_7O_{24}$•$4H_2O$)	12.4 mg in 100 mL	100 μ*M*
Vanadium (NH_4VO_3)	5.9 mg in 100 mL	0.5 m*M*
Nickel ($NiSO_4$•$6H_2O$)	1.3 mg in 100 mL	0.1 m*M*
Tin ($SnCl_2$•$2H_2O$)	1.1 mg in 100 mL	50 μ*M*

Add 1 mL of each stock and 1 ml of 36% HCl to 992 mL distilled deionized H_2O and filter-sterilize.

7. Divalent Cation stock
   Make stock solution as specified below:

Ferrous Sulfate ($FeSO_4$•$7H_2O$)	0.042 g (1.5×10^{-4} *M*)
Magnesium Chloride ($MgCl_2$•$6H_2O$)	12.20 g (6.0×10^{-2} *M*)
Calcium Chloride ($CaCl_2$•$2H_2O$)	0.411 g (2.8×10^{-3} *M*)
Hydrochloric Acid (concentrated)	0.5 mL

   Bring volume to 1 liter with tissue culture grade H_2O, and filter-sterilize.
8. Bovine pituitary extract (BPE) (*see* **Note 4**).
9. Transwell-col tissue culture substrates (0.4-μ*M* pore size, Corning Costar, Acton, MA).
10. JMEM calcium-free and magnesium-free (Life Technologies).
11. DMEM-H.
12. Growth medium for primary airway epithelial cells on plastic (*see* **Note 5**): LHC Basal media (Biofluids, Rockville, MD) supplemented with hormones and growth factors **(Table 1)**. Penicillin (50 U/mL), streptomycin (50 μg/mL), and gentamicin (50 μg/mL), amphotericin B (250 μg/mL) are added as antibiotics.
13. Air–liquid interface (ALI) medium. Prepare a 1 : 1 mixture of LHC basal medium and DMEM-H. Add growth factors and hormones used for the growth medium, but reduce epidermal growth factor (EGF) concentration reduced to 0.5 ng/ml and omit amphotericin B and gentamicin. Filter-sterilize.

2.3. Reagents for Gene Transfer to Undifferentiated and Polarized Well-Differentiated HAE Cells

1. Polybrene (Sigma) stock of 4 mg/mL and sterilized by passing through a 0.2-μm syringe filter.
2. EGTA, 0.1 *M* stock. Dissolve 9.51 g in 200 mL PBS. Adjust pH to 7.4. Bring to 250 mL final volume and sterilize through a 0.2-μm filter.

3. Methods

3.1. Production of Lentiviral Vectors Based on EIAV

EIAV vectors can be efficiently produced by three-plasmid transfection of 293T cells (similar to other vector production systems discussed in this volume) or from recently developed EIAV packaging cell lines. We use the BiG-45 packaging cell line, which is a 293-based cell line that has been modified to express the gag-pol and rev proteins from EIAV and the VSV-G envelope glycoprotein. These cells are useful for producing EIAV lentiviral vectors by a single plasmid transfection of the gene transfer vector (method follows) or alternately can be stably modified with the gene transfer vector to create a full-fledged vector producing cell line. These cells were derived from a subclone (293.101) of the human embryonic kidney cell line, 293, in two steps. First, stable transfection of 293.101 cells with the helper plasmid pEV53B resulted in

Table 1
Hormone and Growth Factors for Growth Medium

Additive	Stock Prep	Stock Conc.	Final Conc.	Storage
Insulin (INS)	250 mg/47.5 mL H_2O + 2.5 mL acetic acid	5 mg/mL	5 μg/mL	–20°C
Hydrocortisone (HC)	1) 10 mg/13.8 mL 70% ETOH	0.72 mg/mL		–20°C
	2) Dilute 1:10 in PBS	0.072 mg/mL	0.072 μg/mL	
Epidermal Growth Factor (EGF)	100 μg/4 mL PBS	25 μg/mL	25 ng/mL ALI conc =0.5 ng/mL	–20°C
Triiodothyronine (T_3)	1) 6.5 mg/5 mL 0.2 *M* NaOH	1×10^{-5} *M*	1×10^{-8} *M*	–20°C
	2) Add 5 mL H_2O			–20°C
	3) Dilute 1:100 in H_2O			–20°C
Transferrin (T_f)	500 mg/50 mL PBS	10 mg/mL	10 μg/mL	–20°C
Epinephrine (EPI)	1) 30 mg/5 mL 0.1 *N* HCl	0.6 mg/mL	0.6 μg/mL	–20°C
	2) QS to 50 mL with H_2O			
Phosphoethanolamine (Phosphoeth)	7 mg/100 mL PBS	0.5 m*M*	0.5 μ*M*	–20°C
Ethanolamine (Eth)	3.1 μL/100 mL PBS	0.5 m*M*, 0.031 mg/mL	0.5 μ*M*, 0.031 μg/mL	23°C
Bovine Pituitary Extract (BPE)	BPE stock solution (*see* **Subheading 2.2.**)	100X	1X	–20°C
Bovine Serum Albumin (BSA)	150 mg/mL in F12(1X)	150 mg/mL in F12(1X)	0.5 mg/mL	–20°C
Calcium Chloride ($CaCl_2$)	8.88 g/L in ddH_2O	0.08 *M*	0.08 m*M*	23°C
Trace Elements	Trace Elements stock solution (*see* **Subheading 2.2.**)	100X	1X	4°C
Divalent Cation Stock	*see* **Subheading 2.2.**	100X	1X	23°C
Zinc Sulfate Heptahydrate ($ZnSO_4 \cdot 7H_2O$)	0.863 g/L in ddH_2O Filter-sterilize.	3×10^{-3} *M*	3×10^{-6} *M*	–20°C
Retinoic Acid (RA)	1) 12.5 mg/40 mL ethanol for 1×10^{-3} *M* stock.	5×10^{-5} *M*	5×10^{-8} *M*	–20°C
	2) Add 3 mL 1×10^{-3} stock to 50 mL 12 mg/mL endotoxin-free BSA in PBS.			
	3) Bring to final vol. 60 mL w/PBS.			

B-241 cells. B-241 cells were then modified to express VSV-G under regulated expression by the tetracycline repressor. Cells are routinely maintained on poly-L-lysine-coated dishes. Cells are passaged 1:4 every fourth day. Do not allow the cells to overgrow. New cultures of BiG-45 cells are thawed every 1–2 mo.

3.1.1. Preparation of VSV-G Pseudotyped EIAV Vectors Following Transient Transfection of 293T Cells or BiG-45 Cells

The following recipe is for 10-cm plates. The procedure can be modified by changing volumes/quantities proportionally for scale-up or scale-down. Note that the expression of EIAV viral proteins in BiG-45 cells is inducible by both doxycycline and sodium butyrate. Sodium butyrate treatment also results in increased vector yield from 293T cells. Omission of sodium butyrate will result in reduced vector titers.

1. Seed cells to obtain approx 80% confluence the next day. For 293T cells, the plating density is about 5×10^6 cells/100-mm polystyrene tissue culture dish. BiG-45 cells are plated at approx $1.0–1.2 \times 10^7$ cells/100-mm tissue culture dish. DNA transfer will be most efficient if the cells are just subconfluent at the time of transfection. Incubate overnight at 37°C in a humidified incubator with 5% CO_2 (*see* **Note 6**).
2. On the day of transfection, make up the 2X HBS solution.
3. For a three-plasmid transfection of 293T cells: In polystyrene tubes, mix 12 μg each of the gag-pol expression vector (pEV53B) and the gene transfer vector and 6 μg of the envelope expression vector (pCI-VSV-G) with H_2O to give a final volume of 262.5 μL. For a single plasmid transfection of BiG-45 cells, mix 12 μg of the gene transfer vector DNA with H_2O to give a final volume of 262.5 μL. To the DNA solution, add 37.5 μL 2 *M* $CaCl_2$ (final concentration of 250 m*M* $CaCl_2$).
4. For each transfection, aliquot 300 μL 2X HBS solution into a polystyrene tube. Add the 300 μL DNA/$CaCl_2$ mixture dropwise, then bubble air through the solution 5–10 times with a 1 mL pipet tip. Alternately, bubble air through a 1 mL pipet during addition.
5. Incubate the mixture 20–30 min at room temperature.
6. Remove medium from cells and replace with 6 mL fresh medium. Then add the 600 μL sample to the cells, dropwise. Swirl the plate gently to mix. Incubate overnight at 37°C in a humidified incubator with 5% CO_2.
7. Next day, remove the medium. Add 6 mL of fresh growth medium per plate containing 10 m*M* sodium butyrate per plate. For induction of VSV-G expression in BiG-45 cells, include 1.5 μg/mL doxycycline. Incubate 24 h at 37°C in a humidified incubator with 5% CO_2.
8. Next day: Swirl the plates gently, then remove cell supernatant containing virus. Filter virus through a syringe filter containing 0.22-μm cellulose acetate or

polyethersulfone (PES) membranes. Store virus in aliquots at –70°C. For large batches (100 mL to 750 mL), remove large cell debris by centrifugation (100*g*, 10 min) prior to filtration through a Corning 0.22-μm PES membrane filter system (Fisher Scientific, Pittsburgh, PA, 250 mL or 500 mL).

9. If desired, a second harvest of vector can be collected. In this case, add 6 mL of fresh growth medium without sodium butyrate to each culture dish (include 1.5 μg/mL doxycycline if using BiG-45 cells). Incubate 24 h at 37°C in a humidified incubator with 5% CO_2 and harvest virus as described in **step 8**.

3.1.2. Concentration of EIAV Vectors by Low Speed Centrifugation

The advantage of concentrating lentiviruses by low speed centrifugation is that it provides a more gentle alternative than ultracentrifugation which can result in the loss of certain labile envelope glycoproteins ***(22)***. We have used this approach for successfully concentrating EIAV vectors pseudotyped with the VSV-G, murine amphotropic, Jaagsiekte sheep retrovirus, and influenza hemagglutinin (HA) envelopes. The following protocol has been used with the Beckman SW 28 rotor using the conically shaped Beckman brand Konical tubes (30 mL capacity) to pellet virus, but should be adaptable for use with other rotors and tubes. Using this protocol, it is possible to process up to 180 mL of virus-containing medium. For larger volumes (250 mL to 3 L) the Sorvall (Newton, CT) H6000A rotor has been used successfully ***(22)***.

1. Prerinse polyallomer tubes with approx 20 mL aliquots of acetone (three times), 95% ethanol (twice), and tissue culture grade H_2O (three times). Allow tubes to drain in tissue culture hood.
2. Fill tubes with filtered virus from the producer cells. Place tubes into rotor buckets. (If the Beckman Konical tubes are used, be sure to use special adapters to prevent tube collapse.)
3. Pellet the virus using a swinging bucket rotor (SW 28) at 5000*g* for 20 h at 4°C.
4. Decant supernatant and remove excess liquid from sides of tube above pellet with UV-sterilized Kimwipes or other lintless laboratory wipers.
5. Suspend virus in a small volume of formulation buffer and use for immediate gene transfer or store in aliquots at –70°C.

3.2. Primary Culture

3.2.1. Tissue Procurement

1. Obtain excess excised nasal or bronchial tissues from fresh surgical specimens (e.g., nasal polyps or lobes of the lung; *see* **Note 7**).
2. Remove and discard loose connective tissue by blunt and sharp dissection.
3. Incubate in excess volume (1:10 tissue:medium) of chilled (4°C) JMEM containing penicillin (50 U/mL), streptomycin (50 mg/mL), and gentamicin (50 μg/mL) for 2–24 h.

3.2.2. Isolation of HAE Cells

1. Rinse tissue with fresh JMEM plus antibiotics and incubate in 1:10 v/v (tissue:medium) 0.1% protease Type XIV plus 1.0 μg/mL deoxyribonuclease I in JMEM at 4°C for 16–48 h on a rocker shaker (*see* **Note 8**).
2. Transfer the tissue to a sterile 10-cm culture dish and gently scrape cells from the mucosal side of the tissue with the convex edge of a scalpel blade. Then rinse the cells away from the tissue with the protease solution.
3. Add FBS to cells suspensions from **steps 1** and **2** to 10% (v/v) to neutralize the protease.
4. Remove tissue pieces, pellet cell suspension (500*g* for 5 min at 4°C).
5. Wash cells with JMEM containing 10% FBS and pellet at 500*g* for 5 min.
6. Stain aliquot of cells with an equal volume of trypan blue solution and count viable and dead cells in a hemacytometer.
7. Resuspend cells in growth medium to desired volume.

3.2.3. Primary Culture of Undifferentiated Cells on Plastic

1. Seed isolated cells onto collagen-coated 10-cm plastic dishes at a density of 1×10^6 cells/dish in growth medium.
2. Feed cells 24 h after seeding with fresh medium and every other day thereafter.
3. On culture days 5 and 6, wash cells with PBS, incubate cells in 0.1% trypsin/1.0 m*M* EDTA at 37°C until they dissociate (~5 to 10 min), then add 20% by volume soybean trypsin inhibitor (1 mg/mL in serum-free DMEM). Harvest cells and pellet at 500*g* for 5 min.
4. Wash cells with 5 mL fresh medium, repellet, and seed cells at 2.5×10^5 cells/well of a 6-well plate in growth medium to create passage 1 (p1) primary cells.
5. Feed cells after 24 h and every 2–3 d until used for studies.

3.2.4. Primary Culture of Polarized WD Airway Epithelial Cells

1. Seed p0 or p1 cells at a density of 2×10^5 cells/12 mm (0.4-μm pore size) Transwell-Col insert in ALI medium (*see* **Note 9**).
2. When cultures reach confluence (~5–7 d), aspirate medium gently from the apical surface creating an air-liquid interface.
3. Maintain in culture by feeding every 1–2 d for an additional 2–3 wk.
4. Confirm polarization by measurement of transepithelial resistance (R_t) beginning on days 18 through 21 in culture with an ohmmeter (EVOM, World Precision Instruments, Sarasota, FL) (*see* **Note 10**).
5. Confirm the presence of WD phenotype by the presence of cilia on greater than 10–20% of the cells upon visualization by phase contrast microscopy (typically 3–4 wk in culture).

3.3. Gene Transfer to Undifferentiated Primary HAE Cells Grown on Plastic

Primary HAE cells are a good host for lentiviral vectors containing either the amphotropic envelope or pseudotyped with the VSV-G envelope glycoprotein

with gene transfer efficiencies as high as 50–100% of primary cells transduced after a single infection.

1. Plate cells in plastic tissue culture dishes as described in **Subheading 3.2.3.**
2. At 24 h after plating, infect cells by replacing medium with 0.5–1 mL lentiviral vector. Add polybrene to 8 μg/mL and return cells to incubator.
3. After 2 h, remove virus and replace with growth medium. Analyze cells for expression 48–72 h after infection.

3.4. Gene Transfer to Polarized WD Airway Cells

Polarized WD primary HAE cells are resistant to luminal transduction by VSV-G pseudotyped, amphotropic enveloped, and GALV pseudotyped vectors. Methods that increase access of vectors to the basolateral surface where receptors appear to be expressed at higher density, appear to increase gene transfer efficiency following luminal application of vector. Several techniques have been developed to enhance gene transfer. These include basolateral infection, injury with pipet tip, and modulating paracellular permeability.

3.4.1. Basolateral Infection

1. Seed and grow primary human cells on transwell-col (12 mm) inserts until they develop cilia and R_t as described in **Subheading 3.2.**
2. Remove transwells from tissue plate and invert in 10-cm dish.
3. Gently aspirate medium from basolateral surface and apply 100 μL vector containing 8 μg polybrene to the basolateral surface of inverted transwell.
4. Incubate for 2 h at 37°C, then return transwells to 12-well plate in the upright position.
5. Harvest cells and test for gene expression 72 h later.

3.4.2. In Vitro Pipet Injury Model

This model permits the investigator to assess the ability to infect from the apical or basolateral surface in the same culture. A pipet is used to scratch the epithelial surface such that transduction by vectors that preferentially transduce basolateral surfaces occurs along the line of injury that exposes the basolateral surface, but not in the uninjured areas of the epithelium.

1. Score the luminal surface of a polarized well differentiated HAE cells with a sterile Pasteur pipet across the diameter of the insert.
2. Immediately apply 100–200 μL vector to the lumen of the culture in the presence of 8 μg/mL polybrene and incubate at 37°C for 2 h.
3. Aspirate medium from surface and evaluate for gene expression by histochemistry, immunolabeling, or fluorescence microscopy 72 h later. Gene expression will occur along the line of injury if the vector efficiently transduces basolaterally localized receptors.

3.4.3. Modulating Paracellular Permeability

Calcium chelating agents and medium chain fatty acids have been shown to enhance gene transfer to polarized airway epithelia mediated by Ad, Adenovirus-associated vectors (AAV), and lentiviral vectors. Of these the chelating agents EGTA and EDTA have been most effective with lentiviral vectors. EGTA has been routinely used with both pretreatment and coadministration of vector. We describe strategies for EGTA- or EDTA-mediated enhancement of lentiviral vector gene transfer.

3.4.3.1. Pretreatment Strategies

1. Measure baseline R_t, then add EGTA (10 m*M*) in Ca^{2+}-free HEPES-buffered Ringer solution to the luminal surface of polarized WD cells with Ca^{2+}-containing HEPES-buffered Ringer solution on the basolateral surface (*see* **Note 11**).
2. Measure R_t every 5–10 min until R_t falls by 90% or more of baseline resistance. Residual resistance should be approx 50–80 Ohms-cm^2 after correction for the blank. Generally takes more than 45 min (*see* **Note 12**).
3. Wash cells with medium bilaterally and feed basolateral surface with 1 mL ALI medium.
4. Apply vector containing 8 μg/mL polybrene in medium to lumen of culture and incubate 2 h at 37°C in tissue culture incubator.
5. Aspirate medium, wash cells twice, and maintain in culture for 72 h or until desired for gene expression analysis.

3.4.3.2. Coadministration with Vector

1. Dilute concentrated EGTA or EDTA stock (0.1–0.5 m*M*) admixed with vector to final concentration 10 m*M*.
2. Wash the lumen of WD HAE cells with PBS or medium, then apply vector containing 8 μg/mL polybrene to apical surface.
3. Incubate at 37°C for 2 h, then remove vector and wash cells twice with medium, then feed cells with fresh medium.
4. Assay for gene expression at 72 h or when desired.

4. Notes

1. BiG-45 cells are maintained on tissue culture plates coated with poly-L-lysine (PLL) to improve adherence of cells to the plate. PLL coating solution is made up by adding 16 mL of tissue culture grade poly-L-lysine (Sigma, St. Louis, MO) to 500 mL tissue culture grade H_2O. To coat 10-cm tissue culture plates, 6 mL of PLL coating solution is added to the plate and incubated for 2 h at room temperature. The PLL solution is removed, and the remaining PLL is allowed to collect near one edge by raising one side of the plate. After removing the solution with a Pasteur pipet, the plates are allowed to dry completely in a tissue culture hood for several hours.

2. It is prudent to make up several HEPES stocks from pH 7.05 to pH 7.2, and test them functionally.
3. Collagen-coated plastic tissue culture dishes are prepared as follows. Dilute sterile concentrated purified collagen 1:75 in sterile H_2O. Apply 0.5 mL/well to 12-well culture plate, 1.0 mL/well to 6-well culture plate, or 4 mL/well to 10-cm plate. Incubate from 2 h to overnight at 37°C. Remove collagen by aspiration and air dry in tissue culture hood. UV sterilize under tissue culture hood for 10–15 min. Seal plates with parafilm and store at 4°C for up to 6 wk.
4. We prepare our own BPE stocks. However, BPE can also be purchased commercially (Clonetics, San Diego, CA). Thaw mature unprocessed bovine pituitaries (Pel Freeze, Rogers, AZ) at 4°C. Drain blood and rinse pituitaries with chilled PBS. Add 200 g of tissue to 400 mL cold PBS and mince in Hamilton-Beach (Washington, NC) Blendmaster-7-speed blender for 10 min. Transfer blended solution into 500 mL Beckman centrifuge canisters (Beckman JA-10 Rotor) and centrifuge at 5000*g* for 10 min at 4°C. Combine supernatants and aliquot. Before use, respin aliquot at 5000*g* for 10 min at 4°C and pass supernatant through a 0.4-μm filter.
5. We prepare our own growth medium ***(23,24)***, but a similar commercial product is available (BEGM, Clonetics, San Diego, CA). For culture of airway epithelial cells from the lungs or cystic fibrosis subjects, additional antibiotics are required ***(24)***.
6. For 293T cells, as with BiG-45 cells, higher vector yields are obtained if poly-L-lysine-coated plates are used for transfection. We attribute this to better adherence of the cells to the plate during medium changing.
7. The use of human tissues generally requires approval from your local institutional review board (IRB) for the protection of human subjects. Although some uses may be exempt from review, we recommend that all investigators submit a protocol for review. An exemption, if appropriate, will be granted by the IRB.
8. Epithelial cell yield is greater after 48 h of protease digestion than after 24 h, without significant loss of viability. By 72 h of protease digestion, cell viability has decreased significantly.
9. Other commercially available permeable substrates can be used for culture of polarized HAE cells including Millipore filters (Bedford, MA) and Transwell-Clear (Corning Costar).
10. The EVOM electrodes should be sterilized before use by soaking in 70% ethanol and equilibrated in ALI medium prior to use. All measurements should be made in a tissue culture hood at room temperature. Measure the resistance across a blank T-Col and record. Subtract the blank resistance from measured resistance across HAE cells on a T-cols and multiply by the surface area to generate the actual R_t. When the culture has fully differentiated into an epithelial sheet with tight junctions, polarized HAE cells will have a R_t of 200 to 1200 Ohms-cm^2. Typically p0 and p1 cells have a higher resistance than p2 cells.
11. Some investigators apply a hypotonic Ca^{2+}-free solution (10 m*M* HEPES, pH 7.4) containing EGTA or EDTA to the luminal bath.

12. Bilateral application of EGTA or EDTA leads to a rapid fall in resistance (within minutes). However, epithelium may lift off from the substrate if chelators are left on the basolateral surface too long.

Acknowledgments

The authors thank Miriam Kelly Vanhook (Johnson Laboratory) and W. Randall Lampe of the Cystic Fibrosis Pulmonary Research and Treatment Center's Tissue Culture Core at the University of North Carolina for their assistance in the preparation of this manuscript. This work was supported by grant HL 58342 (LGJ) from the National Heart Lung and Blood Institute, National Institutes of Health.

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