

# Species-Specific Differences in Mouse and Human Airway Epithelial Biology of Recombinant Adeno-Associated Virus Transduction

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Differences in airway epithelial biology between mice and humans have presented challenges to evaluating gene therapies for cystic fibrosis (CF) using murine models. In this context, recombinant adeno-associated virus (rAAV) type 2 and rAAV5 vectors have very different transduction efficiencies in human air-liquid interface (ALI) airway epithelia (rAAV2  $\cong$  rAAV5) as compared with mouse lung (rAAV5  $\gg$  rAAV2). It is unclear if these differences are due to species-specific airway biology or limitations of ALI cultures to reproduce *in vivo* airway biology. To this end, we compared rAAV2 and rAAV5 transduction biology in mouse and human ALI cultures, and investigated the utility of murine  $\Delta$ F508 cystic fibrosis transmembrane conductance regulator (CFTR) ALI epithelia to study CFTR complementation. Our results demonstrate that mouse ALI epithelia retain *in vivo* preferences for rAAV serotype transduction from the apical membrane (rAAV5  $\gg$  rAAV2) not seen in human epithelia (rAAV2  $\cong$  rAAV5). Viral binding of rAAV2 and rAAV5 to the apical surface of mouse ALI airway epithelia was not significantly different, and proteasome-modulating agents significantly enhanced rAAV2 transduction to a level equivalent to that of rAAV5 in the presence of these agents, suggesting that the ubiquitin/proteasome pathway represents a more significant intracellular block for rAAV2 transduction of mouse airway epithelia. Interestingly, cAMP-inducible chloride currents were enhanced in  $\Delta$ F508CFTR mouse ALI cultures, making this model incompatible with CFTR complementation studies. These studies emphasize species-specific differences in airway biology between mice and humans that significantly influence the use of mice as surrogate models for rAAV transduction and gene therapy for CF.

**Keywords:** recombinant adeno-associated virus; airway model; serotype; tropism

Recombinant adeno-associated virus (rAAV) has attracted considerable interest as a vector for gene therapy due to its ability to transduce multiple tissue types and its low immunogenicity. To date, at least nine different serotypes of rAAV vectors (types 1–9) have been used to express recombinant genes (1–9). However, rAAV serotypes 2 and 5 are the most extensively studied vectors for gene transfer. Type 2 and 5 capsid serotypes have been demonstrated to have different tropisms for transducing many organs and/or specific cell types in the brain, eye, liver, muscle, central nervous system, and lung (1, 7–13). Most studies

have used mouse models to examine the efficiency of gene delivery. A second interesting aspect of serotype-specific transduction occurs in polarized epithelium. In this context, polarized human airway epithelial cells demonstrate significant preference for transduction from the basolateral membrane by rAAV2 (10).

It is generally accepted that rAAV2 is inherently ineffective at transducing the apical membrane of human and mouse airway epithelia (9, 10, 12, 14). In human airway epithelia, this ineffectiveness appears to be due to a postentry block in transduction specific for the apical compartment that limits nuclear uptake of virus (11, 15, 16). However, such intracellular blocks in rAAV2 transduction have not been studied in mouse airway epithelia. In contrast, rAAV5 has been shown to be considerably more effective at transducing mouse lung than has rAAV2 (9, 12). However, in terms of polarized human airway epithelia, there is disagreement as to whether rAAV5 has a greater (12) or similar capacity (11, 17, 18) to transduce the apical membrane of human polarized airway epithelia.

Previous studies have indicated that air-liquid interface (ALI) cultures of polarized airway epithelia from different species are capable of reproducing many of the morphologic and electrophysiologic characteristics of the native airway epithelia from which they are derived (19–23). However, it is currently unclear if such ALI models of polarized airway epithelia retain gene transfer characteristics of native airways *in vivo*. This is of particular importance when drawing conclusions about the validity of this model for predicting the therapeutic efficacy of rAAV vectors for cystic fibrosis (CF) clinical trials.

In the current study, we sought to investigate whether differences in rAAV2 and rAAV5 transduction seen in mouse lungs *in vivo* (rAAV5  $\gg$  rAAV2), when compared with transduction of polarized human airway epithelia (rAAV5  $\cong$  rAAV2), were due to species (human and mouse) or model system (*in vitro* ALI culture and *in vivo* lung) discrepancies. With the hypothesis that the airway ALI culture model may not clearly reproduce *in vivo* biology of the airway, we sought to test whether mouse airway ALI cultures retained the serotype specificity of infection seen in mouse lung. Furthermore, we sought to compare the electrophysiologic characteristics of both mouse and human non-CF- and  $\Delta$ F508 CF-polarized airway epithelia *in vitro* to determine whether  $\Delta$ F508 cystic fibrosis mouse airway epithelia could be used as a model for studying CF transmembrane conductance regulator (CFTR) complementation.

Findings from these studies demonstrate that both cAMP-mediated chloride currents and the serotype preferences for apical infection with rAAV5 vectors were significantly different between mouse and human ALI airway epithelia. Our results demonstrate that mouse airway ALI cultures are transduced  $\sim$  50- to 100-fold more efficiently with rAAV5 after apical infection than with rAAV2, which was similar to transduction efficiencies seen *in vivo* in mouse lung and trachea. Despite these differences, mouse and human polarized airway epithelia retained both significant polarity of infection with rAAV2 vectors (basal  $\gg$  apical) and responsiveness to proteasome modulating

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agents capable of enhancing transduction. Such findings suggest that ALI cultures of airway epithelia reproduce biologic features of rAAV gene delivery seen *in vivo*, and hence human ALI cultures may be reliable surrogate models for evaluating rAAV gene delivery to the human airway. However, species-specific differences in the regulation of chloride transport makes *in vitro* mouse airway epithelia a poor model for evaluating functional correction of CFTR.

## MATERIALS AND METHODS

### Mouse Tracheal Epithelial Cell Isolation

Four- to eight-week-old male and female wild-type and  $\Delta F508CFTR$  mutated ( $CFTR^{m1Kth}$ ) mice (33) on a C57Bl/6  $\times$  Balb/C F1 background were used to generate ALI tracheal cultures. All animal experiments were monitored regularly and maintained in accordance with the principles and procedures outlined in the National Institutes of Health guidelines for the care and use of experimental animals. Mouse tracheal epithelial cells were isolated following previously described methods, with modifications (21, 22). Mouse tracheas (from the larynx to the bronchial main branches) were dissected and washed in ice-cold 1% Pen/Strep containing 1.0  $\mu\text{g/ml}$  fungizone in Ham's F12 medium (all from Invitrogen, Carlsbad, CA). Tracheas were then opened longitudinally and incubated in the same medium containing 1.5 mg/ml pronase (Roche Molecular Biochemicals, Indianapolis, IN) for 18–24 h at 4°C, with occasional rocking. FBS was then added to the digestion tube at a final concentration of 10%, and tracheal epithelial cells were dissociated by inverting the tube 10–20 times. The tracheas were washed twice in this manner, and the cell suspensions were pooled and collected by centrifugation at  $500 \times g$  for 10 min at 4°C. Cells were then resuspended in Ham's F-12 medium containing 1% Pen/Strep, 0.5 mg/ml crude pancreatic DNase I (Sigma-Aldrich, St. Louis, MO), and 10 mg/ml BSA, and were incubated on ice for 5 min. DNase I was removed from the cells by centrifugation and washing with Ham's F-12 medium with 10% FBS before plating on Primaria tissue culture plates (Becton-Dickinson Labware, Franklin Lakes, NJ) for 2 h in 5%  $\text{CO}_2$  at 37°C to adhere fibroblasts. Nonadherent cells were collected by centrifugation and resuspended in 5% FBS/bronchial epithelial medium (BEGM; Cambrex, East Rutherford, NJ). Total cell numbers were then counted using a hemocytometer (Hausser Scientific, Horsham, PA). BEGM medium was made by adding reagents from one BEGM SingleQuot kit (given a final concentration of 10  $\mu\text{g/ml}$  insulin, 1.0  $\mu\text{g/ml}$  cholera toxin, 40  $\mu\text{g/ml}$  bovine pituitary extract, 1.0  $\mu\text{g/ml}$  human endothelial growth factor (hEGF), 1.0  $\mu\text{g/ml}$  epinephrine, 20  $\mu\text{g/ml}$  transferrin, 0.0001  $\mu\text{g/ml}$  retinoic acid) to 500 ml of 50% DMEM/50% Ham's F12 medium supplemented with 1% Pen/Strep, 0.25  $\mu\text{g/ml}$  fungizone, 15 mM HEPES, and 3.6 mM  $\text{Na}_2\text{CO}_3$ .

### *In Vitro* Culture of Polarized Mouse and Human Airway Epithelia

Human polarized airway epithelia from non-CF and  $\Delta F508/\Delta F508$  CF tracheobronchial tissue were generated as previously described (18, 19). *In vitro* cultures of polarized mouse tracheal epithelial cells were generated as previously described, with minor modifications (21, 22). Briefly, polycarbonate/polyester porous (0.4  $\mu\text{m}$  pores) transwell membranes (PCF Millicell inserts; Millipore, Bedford, MA) were precoated with filter-sterilized 60  $\mu\text{g/ml}$  type IV human placental collagen (Sigma). The 0.6  $\text{cm}^2$  Millicell insert membranes were seeded with  $1 \times 10^5$  mouse tracheal cells (cells from approximately 1 mouse trachea) and incubated with 5% FBS/BEGM medium in the apical and basolateral compartment of a 24-well plate at 37°C/6%  $\text{CO}_2$  for  $\sim$  18–24 h. The membranes were then washed with prewarmed PBS to remove unattached cells and refed with 5% FBS/BEGM medium. The medium was changed every 2 d until the transmembrane resistance was greater than  $\sim$  1,000  $\Omega/\text{cm}^2$  (usually 2–5 d after seeding). The medium was then removed from the apical compartment, and fresh BEGM medium (without FBS) was fed to the basolateral side of the chamber to establish an ALI. Cultures were refed with fresh BEGM medium every 2–3 d. Differentiation of airway epithelia was achieved by  $\sim$  10–15 d after ALI.

### Electron Microscopy

Millicell membranes were fixed with 2.5% glutaraldehyde, stained with 1.25% osmium tetroxide in PBS, dehydrated, and sputter-coated before visualization on an S-450 microscope (Hitachi, Tokyo, Japan) for scanning electron microscopy (EM). For transmission EM, membranes were fixed as for scanning EM, followed by infiltration with Spurr resin after dehydration. Eighty nanometere serial sections were then viewed on an H-7000 Electron Microscope (Hitachi, Tokyo, Japan).

### Immunofluorescent Staining

Two-week-old differentiated murine tracheal epithelial cultures were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, washed in PBS for 5 min three times, and then permeabilized with 0.3% Triton X-100 for 20 min at room temperature. To block nonspecific antibody binding, we preincubated intact epithelia with 5% normal serum (from the same species to generate the secondary antibody) in PBS for 1–2 h at room temperature. Primary antibodies to zona occludens protein-1 (ZO-1) (1:100 dilution; Zymed Lab Inc, San Francisco, CA) or Aquaporin 4 (1:100 dilution; Chemicon International, Temecula, CA) were incubated with filter membranes overnight at 4°C in the presence of 1.5% serum/PBS. Primary antibody binding was detected using the appropriate FITC-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Membranes were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA). For immunostaining analysis on sections, tracheal tissue or membranes (3-wk culture) were embedded with Tissue-Tek OCT compound (Sakura, Torrance, CA), and immunostaining was conducted on 6  $\mu\text{m}$  sections with rabbit anti-human keratin 14 (1:200 dilution; NeoMarkers, Fremont, CA) or mouse anti-tubulin IV (1:200 dilution; BioGenex, San Ramon, CA), rat anti-heparan sulfate proteoglycan monoclonal antibody (1:100 dilution; Chemicon International, Temecula, CA), and FITC-labeled *Maackia amurensis* lectin (1:200 dilution; Vector Laboratories), followed by incubation with the appropriate fluorescence-labeled secondary antibody, where applicable. Section specimens were mounted in Vectashield mounting medium with 4',6'-diamidino-2-phenylindole to demarcate the nucleus (H-1200; Vector Laboratories).

### Electrophysiologic Characterization

Transepithelial short circuit currents ( $I_{sc}$ ) were measured on polarized airway epithelial cultures using an epithelial voltage clamp (Model EC-825) and a self-contained Ussing chamber system (both from Warner Instruments Inc., Hamden, CT), as previously described (52). Throughout the experiment, the chamber was kept at 37°C and the chamber solution was aerated. The basolateral side of the chamber was filled with buffered Ringer's solution containing 135 mM NaCl, 1.2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 2.4 mM  $\text{KH}_2\text{PO}_4$ , 0.2 mM  $\text{K}_2\text{HPO}_4$ , and 5 mM Hepes, pH 7.4. The apical side of the chamber was filled with a low-chloride Ringer's solution containing 135 mM Na-gluconate, 1.2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 2.4 mM  $\text{KH}_2\text{PO}_4$ , 0.2 mM  $\text{K}_2\text{HPO}_4$ , and 5 mM Hepes, pH 7.4. The following chemicals (all from Sigma) were sequentially added into the apical chamber: (1) amiloride (100  $\mu\text{M}$ ) for inhibition of epithelial sodium conductance; (2) 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS) (100  $\mu\text{M}$ ) to inhibit non-CFTR chloride channels; and (3) cAMP agonists forskolin (10  $\mu\text{M}$ )/3-isobutyl-1-methylxanthine (IBMX) (100  $\mu\text{M}$ ) to activate CFTR chloride channels. Finally, bumetanide (100  $\mu\text{M}$ ) was added to the basolateral chamber to block transepithelial  $\text{Cl}^-$  secretion. The transepithelial voltage was clamped at zero, and the resulting  $I_{sc}$  was recorded by Quick DataAcq DT9800 series real-time data acquisition universal serial bus board (Data Translation Inc., Marlboro, MA) and analyzed with Excel files (Microsoft Corporation, Redmond, WA).

### Generation of rAAV Luciferase Reporter Vectors and rAAV Infection

AV2/2.CMVLuc and AV2/5.CMVLuc viruses were generated with an AAV2 inverted terminal repeat (ITR) proviral backbone and packaged into serotype 2 (rAAV2/2) or 5 (rAAV2/5) capsids using a triple plasmid transfection method in 293 cells, as previously described (10, 11). Physical titers of rAAV were determined by slot-blot hybridization. ALI cultures were infected with  $2.0 \times 10^3$  particles/cell applied to the apical

or basolateral surface of the epithelia in the presence or absence of 40  $\mu$ M N-acetyl-L-leucyl-L-leucyl-norleucine (LLnL) and 5  $\mu$ M doxorubicin, as previously described (11). The cells were incubated at 37°C for 16 h before loaded virus was removed and the epithelia were returned to fresh medium lacking virus and/or proteasome inhibitors. Luciferase activity was evaluated at 3 and 15 d after infection. *In vivo* mouse lung infections with rAAV were performed in 4- to 6-wk-old C57BL/6 mice by intratracheal instillation of rAAV vectors. A total of  $2 \times 10^{10}$  particles of rAAV vector were diluted in 40  $\mu$ l PBS and intratracheally instilled into each mouse. Luciferase activity was evaluated in the lungs and tracheas at 2 wk and 3 mo after infection, as previously described (11).

### Analysis of rAAV Binding and Viral Genomes after Infection of Polarized Murine Tracheal Epithelial Cultures

Southern blotting for viral genomes was used to evaluate viral binding and genome conversion after infection as previously described (16). To evaluate viral binding, cultures were incubated with virus at 4°C or 37°C for 1 h, followed by extensive washing before Hirt DNA preparation. Similarly, cultures were also incubated with virus overnight at 37°C and harvested at 3 and 15 d after infection for Hirt DNA preparation. Hirt DNA preparation and Southern blots were performed as previously described (16), and blots were evaluated using a transgene-specific luciferase probe.

## RESULTS

### Generation of Human and Mouse Primary Airway Epithelial ALI Cultures

ALI cultures of human CF polarized airway epithelia reproduce defects in CFTR-mediated chloride transport seen in human CF nasal epithelia *in vivo* and are good models for studying CFTR complementation with rAAV CFTR vectors (18, 24, 25). CF mouse models have been less helpful in studying CFTR-mediated gene therapy, since alternative  $\text{Ca}^{2+}$ -activated chloride channels appear to compensate for CFTR deficiency in knockout (KO) mice (26). Nonetheless, nasal epithelia in mice with CF have been used successfully to test CFTR gene delivery, as this epithelium mimics abnormalities seen in CF human airway epithelia (27–29). In the present report, we sought to evaluate *in vitro* polarized airway epithelia from mouse and human tracheobronchial airways for their biologic characteristics in terms of rAAV transduction and CFTR function. Such studies will aid in determining how closely ALI cultures of airway epithelia reproduce *in vivo* biology and their utility for modeling gene therapy of CF airway disease.

We have developed a method for expanding primary mouse tracheal epithelial cells on permeabilized supports using BEGM medium before establishing an ALI. As previously reported using similar methods (21, 22), transepithelial resistance of murine primary tracheal ALI cultures stabilized by 2 wk after the establishment of ALI (data not shown). Morphologic analysis by scanning and transmission EM of mouse ALI epithelia generated using this procedure demonstrated abundant ciliated and nonciliated columnar cells (Figures 1A–1C), as previously observed (21). Immunostaining studies indicated keratin 14–positive cells in the basal layer of the cultures (Figure 1G), and the expression of tubulin IV in the ciliated cells (Figure 1H), as similarly seen in native murine tracheal epithelia (Figures 1D and 1E). ZO-1 immunostaining of epithelial tight junctions (Figure 1F), together with expression of aquaporin 4 in basolateral membranes (Figure 1I) (21), suggested that the ALI cultures were well differentiated.

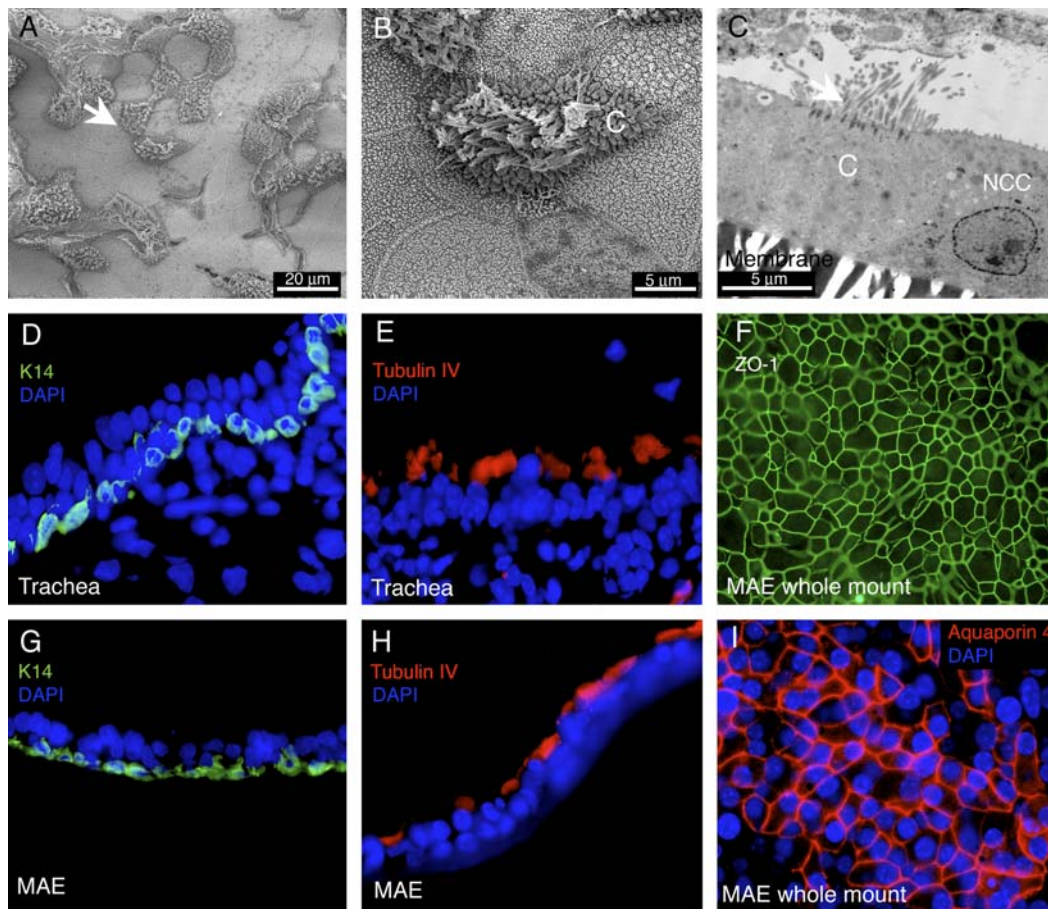
### Primary Tracheal Epithelial ALI Cultures Generated from $\Delta$ F508CFTR Mice Retain cAMP-Inducible Transepithelial $\text{Cl}^{-}$ Secretion

Previous studies in polarized airway epithelia generated from mouse tracheas have demonstrated that these cultures maintain

both amiloride-sensitive sodium channels and cAMP-stimulated chloride channels consistent with CFTR expression (22, 30). The electrophysiologic characteristics of polarized mouse tracheal epithelia grown at an ALI are also similar to those seen in whole-mount mouse tracheas (31). It was originally suggested in the first report on CFTR KO mice that *in vitro* polarized nasal and tracheal epithelial ALI cultures had defective cAMP-inducible chloride currents (32). However, later studies, using whole-mount excised CF and non-CF mouse tracheas, have suggested that alternative  $\text{Ca}^{2+}$ -activated channels are activated by cAMP pathways in CF epithelia, giving rise to “CFTR-like” channel activity (31). This finding is thought to explain why mice with CF do not get the overt lung disease phenotype seen in humans (26).

In the present study, we sought to revisit whether ALI cultures of mouse CF and non-CF tracheal epithelia retain the electrophysiologic characteristics seen in human CF proximal airway epithelia, and whether such CF mouse ALI models may be useful for studying CFTR gene therapy. In human primary airway epithelial ALI cultures, cAMP agonists (IBMX/forskolin) stimulated CFTR-mediated  $\text{Cl}^{-}$  secretions in non-CF (Figure 2A), but not in  $\Delta$ F508CFTR CF, cultures (Figure 2B). Although there was no difference in the baseline of *I*<sub>sc</sub> between non-CF human ( $41.66 \pm 10.43 \mu\text{A}/\text{cm}^2$ ; values are  $\pm$  SEM) and  $\Delta$ F508CFTR CF human ( $37.5 \pm 3.04 \mu\text{A}/\text{cm}^2$ ) ALI epithelia, the responses to amiloride ( $27.6 \pm 5.85 \mu\text{A}/\text{cm}^2$  for non-CF;  $21.5 \pm 1.68 \mu\text{A}/\text{cm}^2$  for  $\Delta$ F508CFTR CF) and DIDS ( $2.8 \pm 0.41 \mu\text{A}/\text{cm}^2$  for non-CF;  $0.61 \pm 0.14 \mu\text{A}/\text{cm}^2$  for  $\Delta$ F508CFTR CF) were slightly reduced in  $\Delta$ F508CFTR specimens (Figure 2C). As anticipated from previous studies, the IBMX/forskolin-stimulated CFTR-mediated  $\text{Cl}^{-}$  current was absent in human  $\Delta$ F508CFTR ( $-0.16 \pm 0.07 \mu\text{A}/\text{cm}^2$ ) as compared with non-CF ( $6.6 \pm 0.78 \mu\text{A}/\text{cm}^2$ ) human airway epithelial cultures.

To investigate the transepithelial ion transport properties of CF mouse tracheal epithelia, ALI cultures from  $\Delta$ F508CFTR (CFTR<sup>*m1Kth*</sup>) mice (33) on an F1 BL6/Balb/C background and their non-CF littermates were used to generate tracheal epithelial ALI cultures. These mice were generated by breeding F1 offspring derived from a cross between inbred  $\Delta$ F508CFTR C57/BL6 mice (> 15 generations) and Balb/C mice. Offspring from this cross have less severe intestinal defects and increased survival after weaning. In contrast to differences seen between human non-CF and CF airway epithelia, we observed no qualitative differences in the *I*<sub>sc</sub> profiles of mouse CF and non-CF epithelia in response to amiloride, DIDS, IBMX/forskolin, or bumetanide (Figures 2D–2F). However, quantitative differences in the extent of IBMX/forskolin-stimulated  $\text{Cl}^{-}$  current were greater in murine  $\Delta$ F508CFTR tracheal epithelia ( $24.52 \pm 1.80 \mu\text{A}/\text{cm}^2$ ) than in non-CF tracheal epithelia ( $11.72 \pm 0.76 \mu\text{A}/\text{cm}^2$ ). Similarly, bumetanide-mediated inhibition of IBMX/forskolin-stimulated  $\text{Cl}^{-}$  current was greater in  $\Delta$ F508CFTR tracheal epithelia ( $8.60 \pm 0.94 \mu\text{A}/\text{cm}^2$  for non-CF;  $18.52 \pm 1.97 \mu\text{A}/\text{cm}^2$  for  $\Delta$ F508CFTR) (Figure 2F), demonstrating that transepithelial  $\text{Cl}^{-}$  secretion was responsible for changes in IBMX/forskolin-stimulated  $\text{Cl}^{-}$  currents. *I*<sub>sc</sub> profiles between primary murine tracheal ALI cultures generated from normal C57/BL6 and Balb/C mice were also similar (data not shown). Our results evaluating the bioelectric properties of non-CF murine tracheal epithelia cultures are consistent with the previous studies evaluating murine tracheal ALI culture models (22, 34). They also agree with studies on freshly excised CF and non-CF mouse tracheas (31, 35), but disagree with ALI studies in the original report describing the CF mouse model (32). In summary, our data demonstrate that CF murine ALI cultures of tracheal epithelia are not suitable models for studying CFTR complementation.

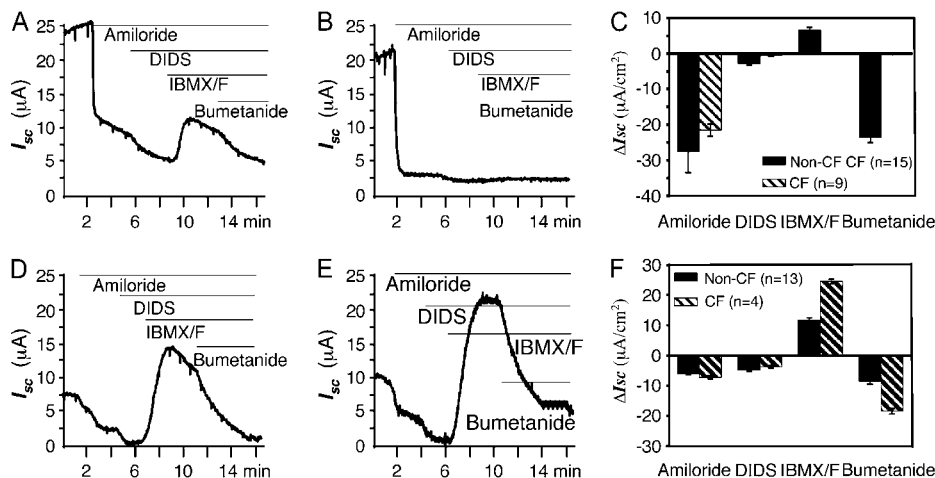


**Figure 1.** Differentiation and polarization of cultured murine tracheal epithelia. Murine tracheal epithelia grown at an air-liquid interface (ALI) were evaluated by (A, B) scanning electron microscopy and (C) transmission electron microscopy at 2–3 wk of culture. Cilia are marked by arrows. (D–I) Keratin 14 and tubulin IV immunostaining of native mouse tracheal section (D, E) are compared with the staining pattern in section of murine ALI airway epithelia (MAE) (G, H) and whole-mount staining of MAE for ZO-1 and aquaporin 4 (F, I). C, ciliated cell; NCC, nonciliated cell.

### Species-Specific Differences in rAAV5 and rAAV2 Transduction of Murine and Human Airway Epithelia

Although CF mouse models are not compatible with studying electrophysiologic complementation of CFTR in their tracheo-bronchial airways, the mouse remains one of the most widely used species for evaluating suitable vectors for gene therapy of CF and other lung diseases. Studies evaluating rAAV-mediated

gene delivery to mouse lung have demonstrated that rAAV5 is far more efficient than rAAV2 (50- to 100-fold) (9). In contrast, studies in polarized human airway epithelia demonstrate that both rAAV2 and rAAV5 vectors have similar levels of apical transduction in polarized human airway epithelia (11), and similar abilities to correct CFTR-mediated  $\text{Cl}^-$  currents in CF epithelia (18). We sought to resolve whether this discrepancy between



**Figure 2.** Short-circuit current ( $I_{sc}$ ) profiles of cystic fibrosis (CF) and non-CF primary cultures of human and murine proximal airway epithelium.  $I_{sc}$  tracings from human and mouse airway epithelial cultures were assessed under secretory conditions as described in MATERIALS AND METHODS after sequential addition of amiloride, 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS), cAMP agonists (3-isobutyl-1-methylxanthine [IBMX]/forskolin), and bumetanide. (A and B) Representative recordings of  $I_{sc}$  from human ALI cultures of non-CF (A) and  $\Delta F508$  cystic fibrosis transmembrane conductance regulator (CFTR) (B) airway epithelia. (D and E) Representative recordings of  $I_{sc}$  from mouse ALI cultures of non-CF and  $\Delta F508$ CFTR airway epithelia. (C and F) Delta responses in  $I_{sc}$  ( $\Delta I_{sc}$ ) after the addition of amiloride, DIDS, IBMX/F, and bumetanide. Values are the mean  $\pm$  SEM for  $n$  number of independent epithelia measured.

cAMP agonists, and bumetanide to human (C) and mouse (F) airway epithelial cultures.  $\Delta I_{sc}$  was calculated as the peak current after stimulation minus the current immediately before chemical addition. Values are the mean  $\pm$  SEM for  $n$  number of independent epithelia measured.

rAAV5 transduction of mouse and human airway epithelium was the result of inadequacies of the ALI culture model to reproduce normal airway cell biology. To this end, we evaluated the biologic features of rAAV5 and rAAV2 transduction in mouse ALI tracheal epithelial cultures and compared those properties to *in vivo* transduction in mouse trachea and lung.

Results from this analysis demonstrated that rAAV2/5 (i.e., AAV2 proviral genome pseudotyped with a AAV5 capsid) transduced the apical membrane of polarized mouse airway epithelia  $\sim 50$ -fold more efficiently than rAAV2/2 vectors (Figure 3A). A similar tropism preference (rAAV2/5  $\gg$  rAAV2/2) was seen *in vivo* in mouse trachea and lung epithelia (Figures 3E and 3F). In contrast, rAAV2/5 and rAAV2/2 demonstrated near equivalent levels of transduction from the apical membrane of human airway epithelia (Figure 3C). These results demonstrate that mouse polarized airway epithelial models maintain properties of *in vivo* airways with respect to rAAV2 and rAAV5 capsid-mediated transduction, and, as such, may be good predictors of tropism preferences for rAAV in the human airway.

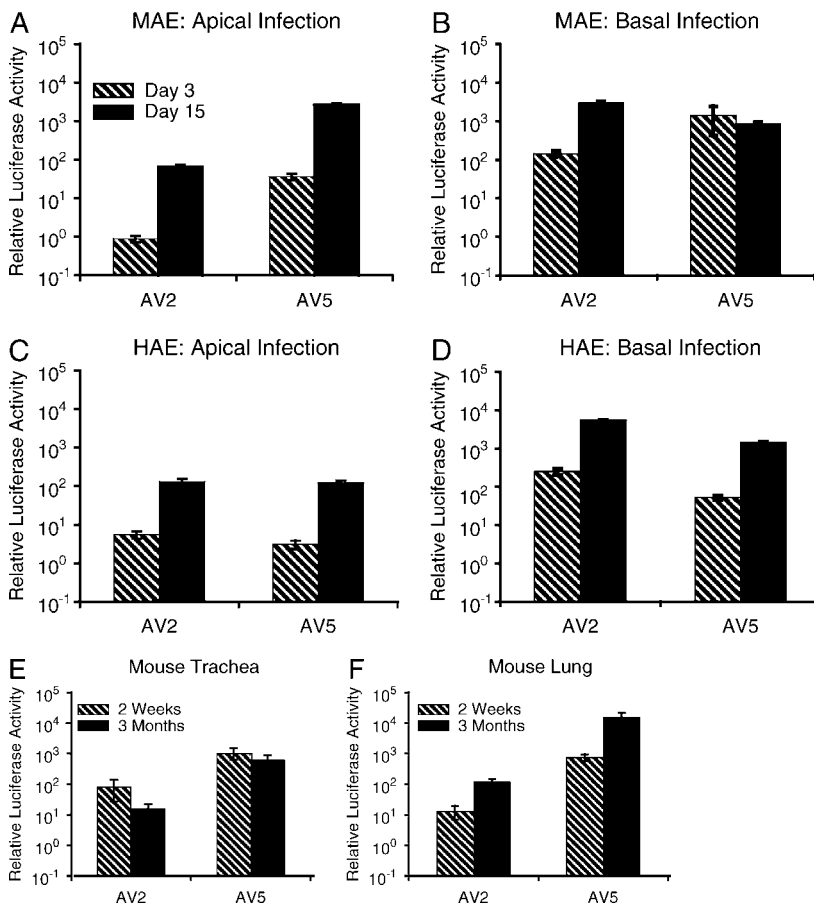
#### Murine and Human Airway Epithelia Demonstrate Conservation in the Polarity of rAAV2, but not rAAV5, Transduction from Apical and Basolateral Membranes

More efficient transduction from the basolateral, as compared with the apical, membrane with rAAV and other viral vectors is a recurrent theme in viral transduction of polarized human airway epithelia (10, 14, 16, 36, 37). We sought to compare the polarity of transduction between rAAV2 and rAAV5 serotypes in mouse ALI cultures to determine whether this aspect of rAAV biology is similar to that in human airway epithelia. Indeed, our

results demonstrate that a preference for transduction from the basolateral membrane of ALI cultures was conserved in both mouse and human ALI cultures (Figures 3A–3D). Both rAAV2 and rAAV5 serotypes were more effective at transducing the basolateral membrane of mouse and human ALI cultures (50- to 200-fold) at 3-d time points. In all but one case, the kinetics of transduction from the apical and basolateral membranes were similar for the two serotypes in both mouse and human airways (increasing from Day 3 to Day 15) (Figures 3A–3D). However, one exception was that rAAV5 virus more rapidly transduced the basolateral membrane of mouse ALI cultures (Figure 3B); rAAV5 demonstrates slower kinetics of transduction from the apical membrane, but ultimately has an equivalent capacity to transduce both basolateral and apical membranes over time. These findings suggest that, although serotype differences exist for rAAV5 transduction in mouse and human airway epithelia, there are also conserved biologic features of cell polarity that limit rAAV2 transduction from the apical membrane in both species.

#### Proteasome Modulating Agents Enhance rAAV5 and rAAV2 Transduction in Both Mouse and Human Primary Tracheal Epithelial ALI Cultures

Postentry blocks mediated by the ubiquitin/proteasome system appear to play an important role in limiting rAAV2 and rAAV5 transduction of human airway epithelia (11, 16). Several laboratories have also demonstrated that intracellular trafficking appears to be the major rate-limiting step in rAAV transduction for several other cell types of mouse and human origin (11, 38, 39). However, such rate-limiting features of rAAV transduction in mouse airway epithelia have not yet been studied. Inhibition of



**Figure 3.** Species-specific differences in the polarity of recombinant adeno-associated virus (rAAV) 5 and rAAV2 transduction in murine (MAE) and human (HAE) airway epithelial cultures. Mouse and human airway epithelial ALI cultures were infected with  $2.0 \times 10^3$  particles/cell of rAAV luciferase virus (serotype 2 or 5) from the apical (A and C) or basolateral (B and D) surface. The relative luciferase activity was measured on Days 3 and 15 after infection. Data represent the mean ( $\pm$  SEM) relative luciferase activity (per well) from three independent experiments ( $n = 10$  transwells for each experimental point). (E and F) *In vivo* rAAV transduction in mouse trachea (E) and lung (F) was performed as described in MATERIALS AND METHODS, using  $2 \times 10^{10}$  particles of rAAV vector per mouse. The relative luciferase activity was measured at 2 wk and 3 mo after the infection, and data represent the mean ( $\pm$  SEM) of the relative luciferase activity (per mg protein) from three independent experiments ( $n = 4$  animals for each experimental point).

the proteasome with tripeptidyl aldehyde proteasome inhibitors (such as LLnL) and anthracycline derivative proteasome-modulating agents (such as doxorubicin) can effectively increase rAAV2 and rAAV5 transduction in human airway cell lines and primary human airway epithelial cultures by augmenting the nuclear uptake of virus (11, 16, 18). Given the species-specific differences in the ability of rAAV5 and rAAV2 to transduce human and mouse ALI cultures from the apical membranes, we sought to investigate whether intracellular blocks in transduction associated with the proteasome might also differ for these two serotypes in a species-specific manner. To this end, we compared rAAV2 and rAAV5 transduction at 3 d after infection of ALI cultures of murine and human airway epithelia in the presence and absence of a combined cocktail of proteasome-modulating agents (LLnL and doxorubicin). This combination of proteasome-modulating agents was previously shown to induce rAAV2 and rAAV5 transduction from the apical surface of human ALI cultures 1,000-fold (11). Results from this analysis demonstrated that rAAV2 and rAAV5 transduction of mouse ALI cultures was significantly enhanced (30,000-fold and 2,000-fold, respectively) in the presence of proteasome-modulating agents after apical infections (Figure 4A). As previously observed, these agents also enhanced apical transduction in human airway epithelia for both serotypes (Figure 4B). The level of augmentation seen in human epithelia, in contrast to that of mouse epithelia was similar for both rAAV2 and rAAV5 (~20,000-fold). Interestingly, rAAV2 and rAAV5 transduced both human and murine primary polarized airway epithelia cultures at similar efficiencies in the presence of proteasome-modulating agents. Given our previous reports demonstrating that proteasome-modulating agents do not alter the uptake of virus in human airway epithelia (11, 16), these findings suggest that species-specific differences in intracellular trafficking between rAAV2 and rAAV5 vectors may be the reason for altered serotype efficiencies in transduction from the apical membrane.

#### Analysis of rAAV2 and rAAV5 Binding to Mouse Airway Epithelia and the Localization of Known rAAV Binding Receptors

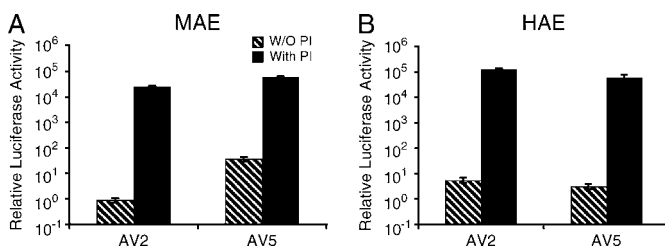
The ability of proteasome-modulating agents to more significantly enhance rAAV2 transduction of mouse ALI epithelia, as compared with rAAV5, suggests that postentry barriers to rAAV2 transduction predominate in mouse airway epithelia. These data would suggest that binding and uptake of these two serotypes are not the major variable accounting for differences in transduction. To this end, we first sought to evaluate the

abundance of known AAV2 and AAV5 binding receptors in mouse ALI airway epithelia in comparison with mouse trachea. Previous studies have demonstrated that heparan sulfate proteoglycan and 2,3-linked sialic acid are binding receptors for AAV2 and AAV5, respectively (40, 41). As previously seen in human airway epithelium (10, 42), localization of membrane-associated heparan sulfate proteoglycan (HSPG) in mouse ALI and tracheal epithelia gave rise to staining at the basal membrane (Figure 5A). As also seen in human airway epithelia (41), *Maackia amurensis* lectin binding to 2,3-linked sialic acid gave rise to apical membrane staining of both mouse ALI and tracheal epithelia (Figure 5A). However, mouse ALI cultures appeared to have a higher level of 2,3-linked sialic acid secreted into the support membrane of the transwells, whereas only low levels of 2,3-linked sialic acid staining were seen at the basal membrane of native mouse tracheal epithelia. In general, the localization of HSPG and 2,3-linked sialic acid in the ALI cultures correlated with the binding of various rAAV serotypes at 4°C from the apical and basolateral compartments, as determined by Southern blotting for viral DNA (Figure 5B). rAAV2 bound much more effectively to mouse ALI epithelia from the basolateral compartment, where HSPG was most abundant, as compared with the apical compartment, which was devoid of HSPG (Figure 5B, compare lanes 1 and 2). Similarly, rAAV5 bound to epithelia from the apical and basolateral surfaces that both expressed 2,3-linked sialic acid (Figure 5B lanes 4 and 5) with near-equivalent efficiency at 4°C. Similar trends were also seen for both serotypes at 1 h after infection with virus at 37°C (Figure 5C). The polarity of transduction of mouse ALI epithelia with both serotypes also appeared to mirror binding efficiencies to the apical and basolateral membranes, with rAAV2 transducing the basolateral membrane ~100-fold more effectively than the apical membrane, and rAAV5 transducing both membranes with equivalent efficiency (Figures 3A and 3B).

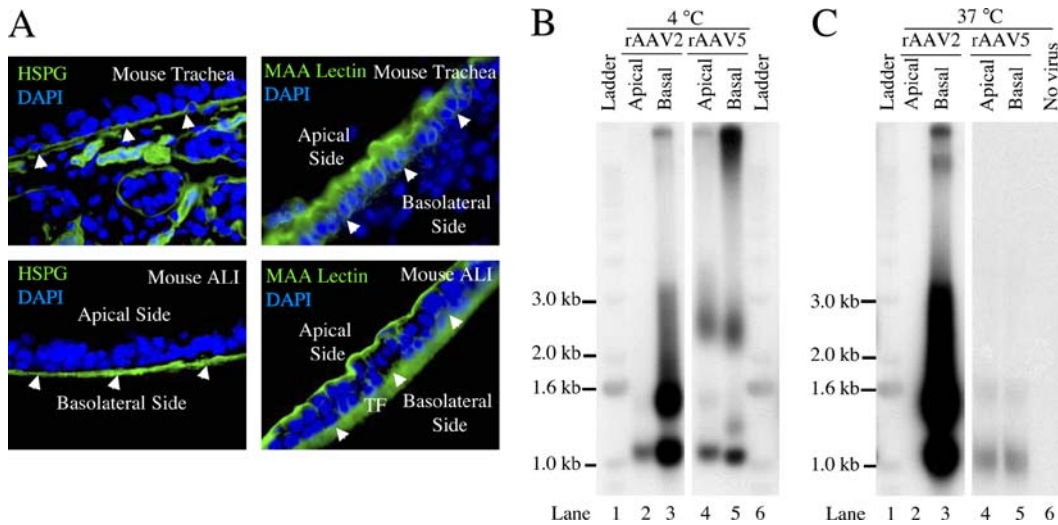
Despite a correlation in viral binding and transduction from apical and basolateral compartment with rAAV2 and rAAV5 serotypes, comparative analysis of binding and transduction from the apical membrane between the two serotypes did not correlate. rAAV2 and rAAV5 both bound to the apical membrane with similar efficiencies, yet rAAV5 transduced (i.e., expressed transgene) the apical membrane of mouse ALI epithelia ~50-fold more efficiently than did rAAV2 vectors (Figure 3A). We also attempted to evaluate the abundance of viral genome conversion products previously associated with transgene expression (circular and linear intermediates) at 3 d and 15 d after infection of mouse ALI cultures, but no conversion products could be detected with either of the two serotypes after apical transduction (data not shown). These findings suggested that a small minority of genomes were giving rise to transgene expression. Given that proteasome-modulating agents normalized transduction with both serotypes to equivalent levels after apical membrane transduction (Figure 4A), these findings suggest that apical uptake of functional viral particles is not substantially different for these two serotypes. Hence, it appears that an alternative non-HSPG binding receptor likely exists on the surface of mouse airway epithelia for rAAV2 that is considerably more susceptible than rAAV5 to an intracellular proteasome-dependent block. A similar apical HSPG-independent uptake pathway for rAAV2 has been proposed in human airway epithelia (16), which is also responsive to proteasome-modulating agents. However, the magnitude of this proteasome-dependent block in rAAV2 transduction appears to be significantly greater in mouse as compared with human airway epithelium.

#### DISCUSSION

Although *in vitro* polarized airway epithelia lack the cellular and structural complexity of intact proximal airways in the lung,



**Figure 4.** Proteasome-modulating agents augment rAAV-mediated transduction in mouse and human airway epithelial cultures. The primary mouse (MAE) and human (HAE) airway epithelial cultures were infected with  $2.0 \times 10^3$  particles/cell of rAAV luciferase (serotype 2 or 5) viral vectors from the apical surface in the presence and absence of proteasome inhibitors (PI). The relative luciferase activity was measured on Day 3 after infection. Data represent the mean ( $\pm$  SEM) relative luciferase activity (per well) from three independent experiments ( $n = 10$  transwells for each experimental point).



**Figure 5.** Immunostaining for AAV binding receptors and Southern blot analysis of proviral DNA after infection of murine tracheal ALI culture. (A) Heparan sulfate proteoglycan (HSPG) and *Maackia amurensis* (MAA) lectin immunofluorescent staining on sections of mouse trachea and murine ALI cultures was conducted as previously reported (10, 41). Panels were processed as marked with 4',6-diamidino-2-phenylindole staining of nuclei (blue) and FITC staining of the specific antigen (green). Arrowheads mark the basal membrane of the epithelia. Note: The dense staining of the transwell filter

(TF) with MAA is specific, and did not occur when empty collagen-coated transwell filters were stained (data not shown). (B and C) Hirt DNA from rAAV luciferase (serotype 2 or 5)-infected or mock-infected murine ALI cultures were extracted for Southern blotting against a [ $^{32}$ P]-labeled luciferase probe. Hirt Southern blots after apical and basolateral infection for (B) 1 h at 4°C and (C) 1 h at 37°C. Note: different exposure times were used in (B) and (C).

they have been extremely useful intermediate models for studying airway epithelial cell biology, electrophysiology, and gene delivery. From the standpoint of CF, CFTR KO and mutant mice have also been extremely useful in understanding CFTR biology and function in the nasal and intestinal epithelia (26, 31, 32, 35, 43–46). Furthermore, a lung disease phenotype has been reported in the CFTR<sup>mlUnc</sup> line on the C57BL/6 background (47), which can be further induced by bacterial loading with agarose beads (48, 49). However, most of the current CF mouse models appear to lack the spontaneous CF lung disease phenotypes seen in human patients with CF, which is thought to be due to enhanced expression of a Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels in mouse (as compared with human) airway epithelial cells (26, 50).

In the present study, we sought to use polarized mouse airway epithelial models to study both the biology of rAAV transduction and functional complementation of CFTR using rAAV-mediated CFTR gene delivery. Although mouse ALI culture models were useful for studying the biology of rAAV transduction, they could not be used to study CFTR complementation at the channel level because of alternative cAMP-inducible Cl<sup>-</sup> currents that were not associated with CFTR function. In our studies, forskolin-activated Cl<sup>-</sup> current was observed in the polarized murine CF tracheal epithelia generated from  $\Delta$ F508 CFTR mutant (CFTR<sup>mlKth</sup>) mice on a C57Bl/6xBalB/c F1 background (33). These bioelectric data are consistent with previous studies using  $\Delta$ F508CFTR<sup>mlEu</sup> mice (FVB/129 and FVB congenics) and CFTR<sup>mlCam</sup> (C57Bl/6/129) backgrounds, which suggested that an alternative Cl<sup>-</sup> channel exists in the tracheal airways of these strains of mice that can be induced by cAMP pathways (50). Previous studies have demonstrated that murine airway epithelial cells have high levels of an alternative Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel that is induced in CF murine tracheas (31, 35, 44, 51). We also observed that cAMP-inducible Cl<sup>-</sup> currents were enhanced in ALI cultures derived from  $\Delta$ F508CFTR mutant (CFTR<sup>mlKth</sup>) mice. Hence, these species-specific differences in the regulation of chloride transport make *in vitro* mouse airway epithelia poor models for evaluating the functional correction of CFTR. To our knowledge, this is only the second report evaluating chloride channel activity in CF and non-CF mouse ALI airway epithelial cultures. However, our results dif-

fer from those reported by Boucher and colleagues (32), who demonstrated defected cAMP-inducible Cl<sup>-</sup> currents in CFTR-null, as compared with wild-type, murine ALI cultures.

Despite the fact that CF mouse ALI cultures cannot be used to study CFTR complementation, they did prove useful in addressing species-specific aspects of rAAV transduction in comparison with humans. In the context of developing gene therapies for CF and other lung diseases, *in vivo* murine lung and human airway epithelial ALI models are widely used to evaluate the efficacy of gene delivery (11, 12, 16–18, 28, 52, 53). However, it is currently unclear how well *in vitro* ALI culture models of the airway reproduce the transduction biology of *in vivo* airways. Our present study used the known transduction preference for two known serotypes of rAAV in mouse airways (rAAV5 >> rAAV2) to investigate this question. Findings from our studies demonstrate that ALI cultures of murine airway epithelia retain a preference for apical membrane rAAV5, as compared with rAAV2, transduction as seen *in vivo* in mouse lung and trachea. Interestingly, both serotypes equally transduced human primary polarized airway epithelia. These findings suggest that enhanced transduction with rAAV5 seen in mouse lungs is a feature of divergent airway cell biology in comparison with humans. Additional differences included a lack of polarity preferences for transduction (apical versus basolateral) with rAAV5 in murine ALI cultures that was observed in human ALI cultures. However, both species demonstrated similar polarity of transduction (basolateral → apical) for rAAV2. These findings also highlight differences in the conservation of serotype-specific rAAV transduction biology between these two species.

Despite the differences in transduction with rAAV2 and rAAV5 in murine and human airway epithelia, both species shared a similar responsiveness to proteasome inhibitor enhancement in rAAV transduction. Previous studies have suggested that inefficient trafficking of rAAV from the cytoplasm to the nucleus is a major barrier for rAAV transduction of airway epithelia and other cell types (16, 38, 39, 54). Proteasome-modulating agents enhance nuclear uptake of rAAV2 and rAAV5, leading to significantly improved transduction (11). Proteasome-modulating agents enhanced transduction of rAAV2 and rAAV5 in human and mouse primary polarized airway epithelia

cultures after apical infection, but the extent of augmentation in mouse airway epithelia was significantly greater for rAAV2 as compared with rAAV5. In contrast, proteasome-modulating agents enhanced transduction with rAAV2 and rAAV5 from the apical membrane of human airway epithelia with equivalent efficiencies. Given that both serotypes appear to bind with similar efficiencies to the apical membrane of mouse (Figure 5B) and human (16) airway epithelia, we conclude that proteasome-dependent barriers to rAAV2 transduction are enhanced in mouse as compared with human airway epithelia.

In conclusion, the demonstration of species-specific differences in the tropism of rAAV between murine and human airway epithelia using the same model system highlights the difficulties of using *in vivo* gene delivery to the lungs of mice as a surrogate for determining the most appropriate adeno-associated virus serotypes for clinical trials in humans. Although such differences present challenges from the standpoint of developing clinical gene therapies with rAAV for CF using surrogate mouse models, they also present opportunities for dissecting the biology of molecular mechanisms that control the efficiency of rAAV transduction in the airway.

**Conflict of Interest Statement:** X.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Z.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.F.E. currently holds a sponsored research grant from Targeted Genetics Inc. (2005–2007) on the subject matter of the manuscript, as noted on the title page of this article. He receives no direct income from Targeted Genetics, and does not have stock in or consult for the company.

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