Restoring ciliary function to differentiated primary ciliary dyskinesia cells with a lentiviral vector

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INTRODUCTION

Primary ciliary dyskinesia (PCD) is a genetically heterogeneous autosomal recessive disease in which mutations disrupt ciliary function, leading to impaired mucociliary clearance and life-long lung disease. Mouse tracheal cells with a targeted deletion in the axonemal dynein intermediate chain 1 (Dnaic1) gene differentiate normally in culture but lack ciliary activity. Gene transfer to undifferentiated cultures of mouse Dnaic1−/− cells with a lentiviral vector pseudotyped with avian influenza hemagglutinin restored Dnaic1 expression and ciliary activity. Importantly, apical treatment of well-differentiated cultures of mouse Dnaic1−/− cells with lentiviral vector also restored ciliary activity, demonstrating successful gene transfer from the apical surface. Treatment of Dnaic1<sup>flox/fox</sup> mice expressing an estrogen-responsive Cre recombinase with different doses of tamoxifen indicated that restoration of ~20% of ciliary activity may be sufficient to prevent the development of rhinosinusitis. However, although administration of a β-galactosidase-expressing vector into control mice demonstrated efficient gene transfer to the nasal epithelium, treatment of Dnaic1−/− mice resulted in a low level of gene transfer, demonstrating that the severe rhinitis present in these animals impedes gene transfer. The results demonstrate that gene replacement therapy may be a viable treatment option for PCD, but further improvements in the efficiency of gene transfer are necessary.

Much prior effort has been devoted to developing gene transfer as a therapy for other airway diseases, most notably, cystic fibrosis. These studies have identified several challenges to successful gene therapy, including the resistance of well-differentiated (polarized) airway epithelium to apical transduction by viral vectors, the difficulties of obtaining long-term expression and the development of an immune response to many vector components. Although gene therapy of airway diseases has not yet been successful, researchers are continuously making advances in vector design and production. For example, several groups have identified viral envelope proteins useful for targeting lentiviral vectors to the apical membrane of differentiated airway epithelial cells. Our group has recently demonstrated that pseudotyping lentiviral vectors with an avian influenza hemagglutinin (HA) protein allows efficient apical transduction of differentiated airway epithelial cells. The incorporation of endogenous cellular promoters and the use of integrating lentiviral vectors to transduce airway epithelium in animal models have resulted in stable, long-term expression.

To test the hypothesis that gene transfer could restore normal ciliary activity to PCD cells, we utilized an inducible mouse model of PCD we have previously characterized. In this model, activation of an estrogen-responsive Cre recombinase (CreER) by tamoxifen treatment causes the deletion of two exons flanked by loxP sites in the axonemal dynein intermediate chain 1 (Dnaic1) gene. The mouse Dnaic1 protein is 82% identical to the human DNAI1 protein, and mutations in DNAI1 cause about 10% of PCD cases. The deletion disrupts the structure of critical WD40 domains in the Dnaic1 protein and prevents the assembly of the outer dynein arm, resulting in immotile cilia and a PCD phenotype.

Keywords: cilia; airway; lentiviral; bronchiectasis; rhinosinusitis; DNAI1

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Unlike traditional knockout models, the use of an inducible system avoids the complications of neonatal hydrocephalus, heart defects, and other situs abnormalities that frequently occur in PCD mice, allowing us to study adult PCD animals.

In this report, we have tested the ability of an HA-pseudotyped lentiviral vector to restore ciliary activity to both undifferentiated and differentiated PCD cells in vitro. We also utilized our inducible mouse model to estimate the level of gene transfer required to prevent upper airway disease and to investigate the turnover of a ciliary protein, two important aspects that will have to be considered when designing clinical trials for PCD. Finally, we examined the effect of pre-existing rhinosinusitis on the ability of the HA-pseudotyped lentiviral vector to transduce the nasal epithelium of PCD animals.

RESULTS

Construction of lentiviral vectors

A full-length mouse cDNA for Dnaic1 was cloned into a lentivirus gene transfer vector (SIN6.1CB-W) based on the equine infectious anemia virus and the construct was verified by direct sequencing (Figure 1a). The Dnaic1 cDNA was under control of a hybrid promoter consisting of the human cytomegalovirus enhancer followed by the chicken β-actin promoter that is ubiquitously expressed. Additional vectors expressing the reporters enhanced green fluorescent protein (EGFP), firefly luciferase, or β-galactosidase (β-gal) from the same construct were utilized as controls in these studies. Viral particles pseudotyped with influenza HA from fowl plaque virus were produced by transfection of 293T cells as previously described.

Transduction of 293 cells with Dnaic1-encoding lentivirus resulted in expression of a protein of the correct size that reacted with a purified monoclonal antibody against human DNAI1 on western blotting (Figure 1b), confirming the vector was expressing full-length Dnaic1.

Gene transfer to undifferentiated PCD cells restores ciliary activity

To test the hypothesis that exogenous expression of Dnaic1 could restore ciliary activity to Dnaic1−/− (PCD) cells, mouse tracheal epithelial (MTE) cells from Dnaic1floxFlox mice that also express CreER were cultured at an air/liquid interface on porous inserts. Cells were treated with tamoxifen to activate CreER and induce the deletion of Dnaic1 and generate PCD cells as previously described. After the cells reached confluence, but before ciliated cell differentiation was visible (day 5 of culture), cultures were transduced with a lentiviral vector expressing EGFP from the chicken β-actin promoter. Each experiment also included cultures that were not treated with tamoxifen or cultures from heterozygous mice (Dnaic1floxFlox; CreER +/−) treated with tamoxifen as a positive control for ciliogenesis. As expected, the percentage of the culture surface area demonstrating active ciliary beating in the PCD cells and PCD cells transduced with the EGFP-expressing control vector was extremely low, averaging <1% in four separate experiments (Supplementary Video S1). Transduction of PCD cells with a Dnaic1-expressing lentivirus resulted in a clearly visible increase in ciliary activity, seemingly in parallel with the development of ciliary activity in the positive control cultures. In four separate experiments, treatment of undifferentiated cultures of PCD cells with lentivirus expressing Dnaic1 increased the level of ciliary activity significantly over untreated PCD cultures. At the time when ciliary activity was maximal in the vector-treated cultures, the level of ciliary activity averaged ~10% of the positive control cultures, compared with ~0.4% for the PCD cultures (P = 0.04 by Student’s t-test, n = 4; Figure 2a). Measurement of ciliary beat frequency (CBF)
using the Sisson-Ammons Video Analysis (SAVA; Ammons Engineering, Clio, MI, USA) system demonstrated that there was no significant difference in CBF between the positive control cultures and the virally transduced cultures (heterozygote = 18.4 Hz; experimental = 18.2 Hz; \( P = 0.42 \) by Students t-test, \( n = 9 \); Supplementary Videos S2 and S3). Figure 2b illustrates the typical time course of the development of ciliary activity in an experiment in which treatment with a lentiviral vector expressing Dnaic1 from the chicken \( \beta \)-actin promoter increased ciliary activity from 0.1 to 8.5%. Ciliary surface activity in the positive control cultures typically reached 40–50%, depending on the culture and the time of measurement. Ciliary activity was maintained in the virally transduced cultures for the remainder of the experiment, usually 2–3 weeks, at which time ciliary activity in the positive control cultures was also diminishing owing to the limited lifespan of primary cultures.

At the end of these experiments, individual cultures were collected for various additional analyses. Qualitative analysis of cellular DNA was performed by PCR amplification using primers specific for deleted exons of \( Dnaic1 \) followed by gel electrophoresis. As the primers span the intron between exons 17 and 18, it is possible to distinguish products derived from the endogenous genomic \( Dnaic1 \) (333 bp) and products derived from the vector-encoded cDNA (223 bp). These experiments confirmed the essentially complete deletion of the endogenous \( Dnaic1 \) in cultures treated with tamoxifen (as shown previously; Figure 3a in Ostrowski et al.\textsuperscript{10}) and the presence of the integrated viral genome in cultures treated with vector (2/2 cultures; data not shown). Treatment of PCD cultures with \( Dnaic1 \)-expressing lentivirus also resulted in easily detectable levels of \( Dnaic1 \) RNA. Quantitative RT-PCR using exon 17–18 specific primers demonstrated that virally transduced cultures expressed \( Dnaic1 \) RNA at levels between 7 and \( \sim 140\)-fold higher than the PCD cultures when analyzed 4 weeks after transduction (\( n = 3 \); data not shown).

Apical gene transfer to differentiated PCD cells restores ciliary activity

Gene transfer to differentiated airway epithelial cells from the apical surface has been shown to be a major obstacle to successful gene therapy of lung diseases, including cystic fibrosis. To determine whether apical delivery of an HA-equine infectious anemia lentivirus encoding wild-type \( Dnaic1 \) to fully differentiated PCD cells could restore ciliary activity, tamoxifen-treated MTE cells from \( Dnaic^{+/-}\) mice were cultured at an air/liquid interface for 19 days. At this time, all positive control (non-PCD) cultures were well differentiated and displayed easily visible ciliary activity covering 35–65% of the culture surface, whereas the PCD cultures had few or no actively beating ciliated cells. Apical treatment of the PCD cultures with \( Dnaic1 \)-expressing lentiviral vectors again resulted in an increase in ciliary activity. The percentage of the surface area of the vector-treated cultures increased to levels as high as 8.8%, whereas the PCD cultures averaged less than 0.1%. The average ciliary activity in treated cultures was 10-fold higher than that in the PCD cultures (6.1% vs 0.6%; \( P = 0.02 \) by Student’s t-test, \( n = 4 \)). Compared with the positive control cultures that had an average of 56% of their surface covered by active ciliated cells, treatment with the lentiviral vector restored \( \sim 10\% \) of the normal level of ciliary activity (Figure 3a). Active ciliated cells appeared in the cultures 5–6 days after vector application (Figure 3b) and increased in number over the next several days. RT-PCR analysis demonstrated full-length \( Dnaic1 \) RNA in differentiated PCD cultures treated with vector, and quantitative reverse transcriptase PCR with primers specific for exons 17–18 demonstrated levels several thousand-fold that was observed in the PCD cultures (\( n = 3 \); data not shown). The amount of intact \( Dnaic1 \) RNA in the cultures transduced after differentiation appeared greater than that found in cultures transduced early in culture; this most likely reflects the increased cell turnover in the cultures transduced early because of the longer time interval between the viral treatment and the RNA isolation in the two groups. Similarly, western blot analysis showed the levels of intact \( Dnaic1 \) protein to be higher in the virally transduced differentiated cultures than in the virally transduced undifferentiated cultures. \( Dnaic1 \) protein was expressed in the late cultures at almost 60% of the level in positive control cultures, whereas no full-length \( Dnaic1 \) protein was detectable in the cultures that were transduced at earlier time points (data not shown).

Turnover of \( Dnaic1 \) protein

In contrast to the cultures treated with vector before differentiation, in which the appearance of ciliary activity roughly paralleled the development of ciliary activity in the positive controls, we noticed a delay of 5–6 days before ciliary activity was observed in the already differentiated PCD cultures following vector treatment (compare Figures 2b and 3b, which are from the same experiment). One hypothesis for the delay in restoration of ciliary activity to the differentiated PCD cells is that the incorporation of the vector-expressed wild-type \( Dnaic1 \) protein into the already fully assembled ciliary axonemes may be rate limiting. Very little is known about the time required for assembly or turnover of axonemal proteins in mammalian systems, and this may be an important consideration when designing or testing treatments for PCD. Therefore, we took advantage of the inducibility of our system to study the turnover of the intact \( Dnaic1 \) protein in cultured tracheal epithelial cells. In these studies, MTE cells from \( Dnaic^{+/-}\) mice were cultured at an air/liquid interface in the absence of tamoxifen until ciliated cell differentiation was well established (14–21 days). The percentage of the culture
Surface area covered with actively beating cilia was measured and the cultures were then treated with tamoxifen (1 μM tamoxifen in media on days 0, 2 and 4) to induce the deletion of exons 17–18 from Dnaic1. Cultures were collected at various time intervals during treatment and the amount of intact Dnaic1 gene and the level of Dnaic1 protein were measured, along with the amount of ciliary activity remaining. As expected, treatment with tamoxifen rapidly induced the deletion of the floxed Dnaic1, decreasing the percentage of intact Dnaic1 to ~60% 48 h after tamoxifen addition and to <25% after 5 days of tamoxifen treatment (Figure 4). Similarly, the amount of intact Dnaic1 protein in the cellular (soluble) fraction of total-cell lysates decreased rapidly over the first 5 days of treatment, whereas the level of deleted Dnaic1 protein increased (Figure 4 and Supplementary Figure S1). In contrast, the level of intact Dnaic1 protein in the axonemal fraction (pellet) decreased at a slower rate, with over 50% of the untreated control level remaining 5 days after initiation of treatment and almost 30% remaining 14 days after initiation of treatment (Figure 4 and Supplementary Figure S1). The high levels of intact Dnaic1 protein remaining in the axonemal fraction were reflected in even higher levels of ciliary activity, with over 90% of the activity of untreated control cultures maintained at 5 days and >70% maintained at 14 days (Figure 4). These results suggest that Dnaic1, and likely other ciliary proteins, are very stable once they are incorporated into the axonemal structure. Further, these results suggest that the delay in the restoration of ciliary activity in well-differentiated PCD cultures may be due to the relatively slow incorporation of the newly synthesized Dnaic1 protein into pre-existing axonemes.

Level of Dnaic1 required for effective mucociliary clearance

The above studies clearly demonstrate that apical treatment with a lentiviral vector can restore ciliary activity to PCD cells in vitro; however, the percentage of cells corrected was only 10–15% of the positive control cultures. To determine whether this level of transfer might be sufficient to improve the outcome of individuals with PCD we treated Dnaic1flox/flox/CreER+ mice with different doses of tamoxifen to induce different levels of Dnaic1 gene deletion. We also included several Dnaic1WT/WT/CreER+ animals that were not treated with tamoxifen, but because of low levels of leakiness (that is, CreER activation and deletion of the target gene in the absence of tamoxifen treatment16), also had varying levels of genomic Dnaic1 deletion. MCC was measured in the nasopharynx as previously described.18 Following the measurement of MCC, the nasal cavity was fixed for histology and the level of intact genomic Dnaic1 remaining was determined in excised tracheal tissue by quantitative PCR. As shown in Figure 5a, the rate of MCC was directly correlated with the level of intact genomic Dnaic1 remaining (R² = 0.7). As noted in our previous study,18 most animals with <20% of intact genomic Dnaic1 remaining had essentially no MCC, although two animals with 16 and 19% Dnaic1 had MCC rates of ~2 mm min⁻¹ and one animal with 22% of the wild-type level of intact genomic Dnaic1 had a MCC rate of 3.8 mm min⁻¹. Examination of the nasal cavity of these animals revealed essentially normal histology in two, whereas the third showed severe mucus accumulation and sinusitis (Figure 5b). In contrast, all animals with lower levels of intact genomic Dnaic1 (<15%) had extensive mucus accumulation in the nasal cavity, in agreement with previous studies of these PCD mice. Together, these studies indicate that successful gene transfer of as little as 20% of the normal level of a ciliary gene may be able to restore a low level of MCC and significantly lessen the severity of disease.

Gene transfer to PCD airway cells in vivo

To determine whether the HA-pseudotyped lentiviral vector could efficiently transduce PCD airway epithelium in vivo, we

![Figure 4](image-url)  
**Figure 4.** Turnover of Dnaic1 protein in ciliated cells. Differentiated cultures of Dnaic1flox/flox CreER+/- cells were treated with tamoxifen (days 0, 2 and 4) and the percentage of intact Dnaic1 DNA (circles), cytoplasmic protein (triangles) and axonemal protein (diamonds) were measured over time, along with the level of ciliary activity (squares). Measurements were taken before treatment (day 0) and at various times after the treatment with tamoxifen was begun. Data shown are from a single experiment. See text for details.

![Figure 5](image-url)  
**Figure 5.** (a) Relationship between level of intact genomic Dnaic1 and MCC. Most animals with >20% Dnaic1 remaining had MCC rates >2 mm min⁻¹ and no evidence of disease. (b) Histology of the nasal cavity of animals with 16% (left) and 19% (right) of the wild-type level of Dnaic1 showing the absence (left) or presence (right; arrow) of rhinosinusitis. The maxillary sinus (*) and nasal septum (arrow) are indicated (left). Sections were stained with alcian blue-periodic acid Schiff.
administered vector to groups of PCD and control animals by nasal inhalation. To measure both the level of gene transduction and the stability of expression, we first utilized a vector expressing both firefly luciferase and β-gal from a single chicken β-actin promoter construct. Four days after vector administration, animals were anesthetized and luciferase activity was measured in the nasal cavity as described in Materials and Methods. Clear evidence of gene transfer to the control animals was observed, with an average luciferase activity of approximately eightfold higher than the untreated (no virus) negative control animal (1.6 × 10^6 ± 2.9 × 10^5 photons s⁻¹ cm⁻² (average ± s.e.m., n = 6) vs 2.1 × 10^5 ± 8.0 × 10^4 photons s⁻¹ cm⁻² (average ± s.e.m. of three repeat measures; Figure 6). In contrast, only one of the PCD animals exhibited a substantial level of luciferase activity (1.0 × 10^6 photons s⁻¹ cm⁻²), with the PCD animals together averaging 4.6 × 10^6 ± 1.5 × 10^5 photons s⁻¹ cm⁻² (average ± s.e.m., n = 5); significantly different from controls with P < 0.005 by Student’s t-test. The level of luciferase activity was increased in the control animals at 7 and 12 days after treatment, whereas the level of activity in the PCD animals, although increased, remained significantly lower than the controls. At day 7, the control animals averaged 1.3 × 10^7 ± 2 × 10^6 photons compared with 1.7 × 10^6 ± 8.4 × 10^5 photons for the PCD animals; at day 12 the control animals averaged 2.4 × 10^7 ± 5.9 × 10^6, whereas the PCD animals averaged 2.2 × 10^6 ± 1.2 × 10^5 (significantly different at both days with P < 0.007). After 1 month, the level of luciferase activity declined in both groups, and was no longer significantly different between them, although the average activity remained higher in the controls (Figure 6). To examine the cellular distribution of viral mediated gene transfer, animals were euthanized and tissues were processed for detection of β-gal. Sections were prepared and β-gal-positive cells were enumerated in the nasal cavity and trachea. In agreement with the luciferase data, there were significantly fewer β-gal-positive cells in the nasal cavity of the PCD mice compared with the control animals (2.2 ± 2 vs 116 ± 54, mean ± s.e.m. in three sections per animal; n = 5; P = 0.03 by Student’s t-test). In contrast, there was no significant difference in the numbers of β-gal-positive mice in sections of the tracheas from PCD mice compared with the control animals (174 ± 107 vs 30 ± 24; n = 5; P > 0.11 by Student’s t-test), indicating that the inhibition of gene transfer was limited to the site of disease pathology, that is, the nasal cavity. To further examine the distribution of virally transduced cells in the PCD animals compared with controls, additional groups of PCD and control animals were treated with an HA-pseudotyped lentivirus expressing only β-gal. Animals were again administered vector by nasal inhalation, but were euthanized and examined for positive gene transfer about the time of maximum luciferase expression (Figure 6; 12 days after treatment). In confirmation of our earlier study, control animals displayed a high level of gene transfer to the ciliated epithelium of the nasal cavity. β-gal-positive cells were easily identified along the septum, nasal turbinates and throughout the nasopharynx, whereas olfactory tissue was routinely negative (Figures 7a and c). PCD animals displayed little or no gene transfer to the nasal epithelium (Figures 7b and d). Although control animals averaged over 500 β-gal-positive cells, PCD animals had significantly fewer positive cells (532 ± 42; n = 7 vs 62 ± 31; n = 9; P = 1.3 × 10⁻⁷ by Student’s t-test). In contrast, transfer to the trachea was again similar between the two groups (control = 285 ± 31, PCD = 298 ± 39; P > 0.4 by Student’s t-test; Figures 7e–h). These data clearly demonstrate that the accumulated mucus and/or inflammation in the nasal cavity of the PCD animals prevent efficient gene transfer.

**DISCUSSION**

PCD is a genetically heterogenous disease that occurs with an incidence of ~1:16,000, and is therefore classified as a ‘rare’ or ‘orphan’ disease. Currently, there are no specific treatments available for PCD, and individuals with PCD suffer from repeated respiratory tract infections eventually leading to bronchiectasis. Our laboratories have been investigating the development of gene replacement therapy as a treatment for PCD and other respiratory diseases, including cystic fibrosis. To investigate the pathogenesis and treatment of PCD, we have previously developed an inducible mouse model of PCD that does not develop hydrocephalus or developmental (heart) defects. In this work, we have utilized this model to investigate several aspects of gene therapy as a treatment for PCD.

Our initial studies demonstrated that expression of the wild-type Dnaic1 from a lentiviral vector successfully restored ciliary activity to cultures of undifferentiated PCD cells. Measurements of CBF were not significantly different between the virally transduced cultures and the controls, suggesting that the expression of the wild-type Dnaic1 was sufficient to restore normal ciliary activity to the PCD cells. This is consistent with an earlier study in which a lentiviral vector pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) was used to express DnaI1 in human PCD cells. Although it may be expected that expression of the wild-type protein will also lead to the correct orientation and regulation of ciliary activity required for efficient MCC, further studies are required to test this hypothesis.

Importantly, for delivery of a gene transfer vector to the airways by inhalation, the vector must be able to transduce the airway epithelium via the apical surface. We have recently reported that influenza HA-pseudotyped vectors can mediate efficient gene transfer to murine airways. In this study, we found that apical administration of influenza HA-pseudotyped vector expressing Dnaic1 to fully differentiated cultures of PCD cells restored ciliary activity to a similar level as did treatment of undifferentiated cells. In cultures that were examined at the end of the experiment, we observed higher levels of Dnaic1 RNA and protein in cultures that were transduced late in culture compared with those transduced early. Although we think it is likely this difference is due to the longer time period between transduction and analysis allowing for greater turnover (loss) of the transduced cells in the cultures treated early, other possibilities need to be considered. For example, it is possible that in the late-stage cultures, the PCR analysis had been detecting residual vector RNA. In both conditions, the maximum level of correction achieved was ~10%; it is unclear at this time what the rate-limiting step for correction is. Interestingly, the appearance of ciliary activity in apically transduced differentiated cultures required several days, whereas expression of a reporter protein (for example, EGFP) is typically observed in ~72 h. This observation prompted us to examine the stability of the Dnaic1 protein by taking advantage of the inducible nature of our model. Unlike Chlamydomonas, an
organism that rapidly sheds and regrows its flagella, there is little known about the turnover of respiratory ciliary proteins. Our studies indicated that although the Cre-mediated deletion of Dnaic1 and the decline in the level of cytoplasmic Dnaic1 protein occurred rapidly after tamoxifen treatment, the level of axonemal Dnaic1 protein and ciliary activity declined at a much slower rate. These studies demonstrate that once ciliary proteins are assembled into axonemal structures they exhibit an increased stability. These results also suggest that the process of Dnaic1 assembly into outer dynein arms and the incorporation of sufficient outer dynein arms into the existing ciliary axonemes to restore motility requires several days. Alternatively, it is possible that the vector is transducing undifferentiated or pre-ciliated cells that are then undergoing ciliated cell differentiation. In our previous study, 80% of the cells transduced by the HA-pseudotyped vector were identified as ciliated cells, making this seem less likely. Additional studies using a vector expressing both a reporter and the Dnaic1 protein are necessary to distinguish between these two possibilities.

Although the studies reported here demonstrate that ciliary activity can be restored to individual PCD cells by gene transfer, it is not known what level of gene transfer will be necessary to prevent or reduce the disease symptoms caused by PCD. Efficient MCC in the airways requires the continuous transport of mucus, from the bronchioles to the larynx, and so the distribution as well as the number of cells transduced will be important determinants of success. To investigate the level of gene transfer required, we again took advantage of the inducible nature of our model system to generate individual animals with varying levels of Dnaic1 deletion. Measurement of MCC rates in the nasopharynx and examination of nasal histology showed that animals with 20% of the wild-type level of intact genomic Dnaic1 remaining had some MCC remaining and most were free of obvious disease. Although this number (20%) is clearly an approximation, we have

Figure 7. Gene transfer in the nasal cavity (a–d) and trachea (e–h) of control (a and c, e and g) and PCD (b and d, f and h) mice. HA-pseudotyped lentivirus expressing β-gal was administered to mice by nasal inhalation, and 12 days later tissues were stained for the expression of β-gal and paraffin sections were evaluated. Both the nasal cavity and the trachea exhibited positive β-gal staining in the control mice. In PCD animals the level of gene transfer in the nasal cavity was greatly reduced (note the mucus (*) in b and d; compare with Figure 5), while gene transfer to the trachea was not impeded. Nasal sections (a–d) are from ~level 23.26 Arrowheads pointing at the nasal septum in a and b indicate the location of the higher magnification images in c and d. Sections were counter-stained with neutral fast red. Scale bars = 50 μm in c, d, e, f, g, and h.
successfully transduced up to 40% of the surface epithelial cells in the trachea and large airways of mice using HA-pseudotyped lentiviral vector,16 suggesting that improvements in vector design may make gene therapy for PCD possible.

An additional barrier to gene transfer to the airways of adult individuals with PCD, cystic fibrosis or other diseases is the existence of disease-associated pathology, including the presence of excess mucus and/or the presence of inflammatory cells and mediators that may reduce or prevent gene transfer. Mice with a deletion of Dnaic1 invariably develop rhinosinusitis with an accumulation of mucus and inflammatory cells in the nasal cavity and sinuses. Administration of an HA-pseudotyped vector into groups of control and PCD mice by nasal inhalation revealed a profound inhibition of gene transfer to the nasal cavity because of the presence of rhinosinusitis. In contrast, gene transfer to the trachea and lower airways of PCD mice was not significantly affected, demonstrating that the vector was able to successfully transduce regions of the airways lacking the excess mucus and inflammation present in the nasal cavity. However, additional strategies will be required for successful gene transfer to the airways of individuals with pre-existing disease. These may include pretreatment to reduce the level of mucus accumulation and/or inflammation before administration of the vector. Alternatively, administration of vector into affected individuals early in the course of the disease, perhaps prenatally,30 may allow successful gene transfer before the development of severe symptoms.

In summary, the results presented here demonstrate that restoration of ciliary activity to PCD cells by gene transfer to the apical surface of airway epithelial cells is possible using an HA-pseudotyped lentiviral vector, and that successful gene transfer of ~20% of the normal endogenous level of the mutated gene may be sufficient to prevent or mitigate the consequences of disease. Using an inducible mouse model of PCD that allows studies of adult animals, these studies have also demonstrated a significant inhibition of gene transfer to the nasal cavity as a result of pre-existing rhinosinusitis.

MATERIALS AND METHODS

Generation of animals

The generation and breeding of the Dnaic1fl×/fl× mice24 expressing CreER from the ROSA promoter has been described.18 These mice are maintained on a mixed background (C57Bl/6J129) and when treated with tamoxifen provide a long-lived animal model of PCD. All procedures using animals were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill, and were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health.

Cloning of Dnaic1

The cloning of mouse Dnaic1 into the lentiviral vector was performed using standard molecular biology procedures and reagents. RNA was isolated from cultured MTE cells and a full-length cDNA for mouse Dnaic1 was amplified using Puultra polymerase (Stratagene, Santa Clara, CA, USA) and primers 5′-ACAagctCTGAATGCCTCAGGAAAG-3′ (sense) and 5′-TTTctagACTGGGACTATCTCAGGTTT-3′ (anti-sense). The product was cloned into the TOPO vector (TOPO-TA cloning kit; Invitrogen, Carlsbad, CA, USA) and sequenced. A clone with the correct sequence was transferred to the lentiviral vector SIN6.1CB-W, sequenced and digested, ligated into the lentiviral vector SIN6.1CB-W, sequenced and used for production of vector.

Production of lentiviral vectors

Recombinant equine infectious anemia virus-based lentiviral vector stocks were generated by transient transfection of 293T-based TAB22 cells as previously described.16,31 Briefly, lentiviral plasmids were transfected into 293T cells using the standard calcium phosphate method. After a 48-h incubation, cell supernatant containing lentiviral vectors was collected, clarified by low-speed centrifugation (100g, 10 min) and filtered through 0.2-μm polyethersulfone membranes. Vector was concentrated by high-speed centrifugation at 5000 g (Beckman Avanti J-E centrifuge, JS-5.3 rotor, Beckman Coulter, Inc., Atlanta, GA, USA) for 21 h at 8 °C. Vector pellets were suspended in vector formulation buffer (5 mM 2-(2-hydroxyethyl)piperazin-1-yl)ethanesulfonic acid (pH 7.4), 37 mM NaCl and 40 mM lactate) and stored at –80 °C. Recombinant lentiviral vector stocks were titered on 293T cells by serial dilution as previously described.16 Titers for these experiments ranged from 6 to 10 × 106 infectious units per ml.

Western blotting of Dnaic1

Detection of Dnaic1 protein by western blotting was performed using a mouse monoclonal antibody generated against a synthetic peptide from the human Dna1 protein16 using standard procedures. For analysis of MTE cells, total-cell lysates were prepared in Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Ciliary axonemes were isolated from cultured MTE cells as previously described25 and prepared in gel-loading buffer. For studies of protein turnover, both soluble (cytoplasmic) and pellet (axonemal) fractions from individual cultures were analyzed. Protein samples were fractionated on 4–12% Bis-Tris gradient gels (Invitrogen), transferred to nitrocellulose membranes and probed using Amersham ECL Plus reagents (GE Healthcare, Buckinghamshire, UK), all according to the manufacturer’s instructions. For normalization of loading, western blots of cytoplasmic extracts were reprobed with an antibody to GAPDH (Sigma, St Louis, MO, USA) and axonemal pellets were reprobed with mouse anti-acetylated α-tubulin (Invitrogen). Quantification of signals was performed on an Odyssey Imaging System (Li-Cor, Lincoln, NB, USA).

Culture, tamoxifen treatment and transduction of MTE cells

Procedures for the isolation and culture of MTE cells have been described in detail previously.16,35 To generate PCD cells for gene transfer studies, MTE cells from Dnaic1fl×/fl×, CreER þ/+ mice were seeded on collagen-coated Millicell Cell Culture Inserts (12 mm; 0.4 μm; EMD Millipore, Billerica, MA, USA) and treated with 1 μM tamoxifen for the first 5–7 days of culture. As we have observed no difference between heterozygous and wild-type cultures, mice from both genotypes were used as controls. Lentiviral vectors with a titer of 6–10 × 107 infectious units per ml (33 μl) were applied to the apical surface of washed cultures (day 5 or 19) and allowed to incubate for 3–4 h before the supernatant was removed. Cultures were washed and refed 48 h after infection.

For Dnaic1 turnover studies, differentiated cultures of MTE cells from Dnaic1fl×/fl×, CreER þ/+ mice were cultured in the absence of tamoxifen until ciliated cell differentiation was apparent (day 14–21). Experimental cultures were then treated with 1 μM tamoxifen in media on days 0, 2 and 4, whereas positive control cultures received only media. Ciliary activity was measured and parallel cultures were collected for DNA, RNA and protein analysis. Four separate experiments were performed with similar results, although not all data were collected from each experiment.

Measurement of ciliary activity

The extent of ciliary activity and measurements of CBF were performed essentially as previously described17 using a Nikon Eclipse TE2000 microscope (Nikon Instruments Inc, Melville, NY, USA) and the SAVA software package.18 Briefly, for measurements of the percentage of active ciliated surface area, nine different low-magnification fields (one central and eight peripheral) from each insert were measured. Typically, two to three inserts were measured and averaged at each time point. As the absolute level of cilogenesis varied between cultures, data are also expressed as the percentage of positive control cultures. For comparison between vector-treated and untreated cultures, data shown in Figures 2a and 3a are taken from the time point when vector-treated cultures showed maximal ciliary activity as a percentage of positive control. For determining CBF, positive control (heterozygous) cultures and PCD cultures treated with vector on day 5 were washed free of mucus and debris, equilibrated at 37 °C and nine random fields were analyzed on day 25 (20 days after vector treatment).

Measurement of Dnaic1 RNA and DNA

RNA and DNA were isolated from MTE cell cultures or tail snips using RNAeasy or DNeasy reagents and kits (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg RNA using SuperScript III (Invitrogen, Carlsbad, CA, USA) and random primers. PCR amplification was performed on a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA). For cDNA, amplicons were digested with enzymes recognizing unique restriction sites (5′-TTTgcggccgCTGGGCCATACTCTCAGGTTTT-3′ (anti-sense). The product was cloned into the TOPO vector (TOPO-TA cloning kit; Invitrogen, Carlsbad, CA, USA) and sequenced. A clone with the correct sequence was transferred to the lentiviral vector SIN6.1CB-W, sequenced and used for production of vector.

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to the manufacturer’s protocols. DNA and RNA concentrations were determined by spectrophotometry. Qualitative PCR analysis of samples was performed by using primers 5’-TGTCCTCAAATCTACTCCAG-3’ and 5’-AACACAGTTGAAAAATGACTGG-3’ that are internal to the deleted region of Dnaic1 to amplify cellular DNA followed by gel electrophoresis using standard procedures. The primers produce a 323-bp product with intact genomic DNA, no product from the deleted genomic DNA (tamoxifen-treated) and a 223-bp product from the vector-encoded cDNA. Quantitative PCR to determine the amount of intact Dnaic1 in various samples was performed using LightCycler SYBR Green reagents (Roche Applied Science, Mannheim, Germany) and a Roche LightCycler using the same primers as previously published. Each sample was analyzed in duplicate. Relative changes in the level of Dnaic1 were calculated from the efficiency of the PCR reaction and the cross-point deviation between samples. For experiments in which the level of Dnaic1 was being quantified, signals were normalized to the single-copy reference gene [I-ENaC (Sca11)];18 for the estimation of levels of Dnaic1 mRNA, signals were normalized to the level of the ciliated cell-specific gene dynein axonemal heavy chain 5 using primers 5’-GTCTGGAGTGSCGCATGCA-3’ and 5’-ATCCTGTACCCCTCCGAGCTCA-3’.

Tamoxifen treatment of mice

To generate PCD mice, Dnaic1flox CreER-/- mice, 6–9 weeks old, were treated with tamoxifen with intraperitoneal injections of 75 µg g-1 body weight18. For the experiment shown in Figure 5, animals were treated with two or three injections of tamoxifen to generate mice with varying levels of intact genomic Dnaic1 remaining. Additional Dnaic1flox CreER-/- mice with varying levels of intact genomic Dnaic1 (due to the spontaneous activation of CreER) and Dnaic1floxt/mice were included in the study. After allowing sufficient time for ciliated cell turnover (>6 mos),18 MCC was measured in the nasopharynx, the nasal cavity was fixed in formalin for 24 hours and stained (blue) cells were counted. To quantify transduction in the nasal cavity, a single section the length of the trachea was evaluated; both sides of the longitudinal section through the trachea were counted. Data are reported as mean ± s.e.m.

Statistical analyses

Results between PCD and vector-treated groups were compared using a Student’s t-test with a P-value of ≤0.05 accepted as significant. In experiments where more than two groups were being compared, an analysis of variance was first performed to determine significance. For the data shown in Figure 5a, the coefficient of determination (R2) was determined by linear regression.

CONFLICT OF INTEREST

Dr JCO is a named inventor on patents involving equine infectious anemia virus (EIAV)-based gene transfer technology and has received royalties for the use of EIAV-based vectors. The remaining authors declare no conflict of interest.

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