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Expression of a Truncated Cystic Fibrosis Transmembrane Conductance Regulator with an AAV5-pseudotyped Vector in Primates

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Gene therapy using recombinant adeno-associated virus (rAAV2) vectors for cystic fibrosis has shown gene transfer and remarkable safety, yet indeterminate expression. A new construct has been characterized with a powerful exogenous promoter, the cytomegalovirus enhancer/chicken β-actin promoter, driving a truncated CF transmembrane conductance regulator (CFTR), pseudotyped in an AAV5 viral coat. Our goal is to demonstrate that airway delivery of a pseudotyped rAAV5 vector results in gene transfer as well as expression in non-human primates. Aerosolized pseudotyped rAAV5-ΔCFTR or rAAV5-GFP (green fluorescent protein) genes were delivered to four and six lungs, respectively. The pseudotyped rAAV5 vector did result in GFP gene transfer (1.005×10^6 copies/μg DNA on average) and quantifiable gene expression. Microscopy confirmed protein expression in airway epithelium. Similarly, the vector also resulted in vector-specific CFTR DNA (1.24×10^5 copies/μg) and mRNA expression. Immunoprecipitation and ^{32}P phosphoimaging were used to demonstrate CFTR protein expression, as qualitatively enhanced beyond the barely detectable endogenous expression in untreated animals. Based on these promising studies, this CFTR minigene construct is a therapeutic candidate.

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INTRODUCTION

Cystic fibrosis (CF), the most common lethal inherited disorder in North America, results from mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) chloride channel.[1] The degree of pulmonary manifestations is the primary determinant of morbidity and mortality in CF; thus, the potential to intervene with the transfer of a normal copy of CFTR to airway epithelia holds therapeutic promise for affected patients.[2,3] Several characteristics of recombinant adeno-associated virus (rAAV) have made it an excellent vector candidate for gene therapy, including its tropism, its ability to transduce differentiated cells and achieve stable integration, and its safety record devoid of a prohibitive inflammatory response.[4]

Clinical trials have made advances in demonstrating feasibility in phase I aerosolized studies.[5–7] Phase I and II trials further established a dose–response relationship with CFTR complementary DNA (cDNA) transfer. In contrast to the success in demonstrating gene transfer, gene expression has been more difficult to verify in most CF gene therapy trials. However, the most recent phase II trial of repeated aerosolized administration of AAVCF resulted in improved lung function in patients with mild CF lung disease:[8] the improved clinical status suggests CFTR expression albeit without detection of mRNA transcripts. Multiple factors contribute to confounding the detection of gene expression. First, the AAV2 serotype, the prototype serotype, has a relative paucity of apical receptors on airway epithelia.[9,10] Altered trafficking due to endosomal processing, as well as enhanced proteasome-mediated degradation of vector and ubiquitination,[11,12] result in relatively inefficient expression. Vectors based on alternative serotypes such as AAV5, AAV6, and now AAV1 (homologous to AAV6) directly target the apical epithelium[11,13] and have shown more efficient cellular uptake and ability to evade some of the previously mentioned barriers to AAV2.[10,14,15] Pseudotyping with alternative AAV capsids to package rAAV genomes will provide not only more efficiently targeted gene transfer for a site-specific organ, but also a strategy to bypass barriers specific to a given serotype and to circumvent an amnestic humoral immune response.

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Secondly, low expression in the clinical trials[16] may reflect the endogenous promoter activity derived from the AAV inverted terminal repeat[17] owing to the size limitations of rAAV (~4.7 kb). The discovery of chicken β-actin (CBA) promoters as potent rAAV promoters for a variety of target organs has challenged the presumed benefit of tissue-specific promoters,[18,19] as well as powerful exogenous cytomegalovirus or Rous sarcoma virus promoters.[15,20] A new pseudotyped rAAV5-CBA-driven CFTR construct has been characterized as being capable of correcting the CF chloride transport *in vitro* as well as weight normalization in a vector-rescue of $CFTR^{-/-}$ mice in a *Pseudomonas* agarose bead airway challenge.[20]

In our current studies, we seek to determine whether this minigene construct can enhance *in vivo* gene transfer and expression for quantifiable vector-derived gene transfer and mRNA expression. We use our well-characterized bronchoscopic model of aerosolized vector delivery to macaques for two purposes: (1) to investigate if the AAV2 construct, packaged in AAV5 capsid proteins, with modifications so beneficial *in vitro*, could result in gene transfer sufficient for uniform transgene expression *in vivo*, as well as (2) to demonstrate vector-specific CFTR expression in a primate model despite a background of endogenous CFTR expression. This latter aim underscores some of the remaining challenges in paralleling the clinical paradigm that has a similar background of endogenous expression.

RESULTS

rAAV5-mediated DNA transfer

A previously described rAAV5-CBA-promoter-driven CFTR minigene vector has been shown to correct the CF chloride defect in patch clamp studies *in vivo* in the CFTR-defective IB3-1 cell line.[20] Sirninger *et al.*[20] designed an rAAV5-CFTR construct consisting of a CBA promoter-driven DNA cassette packaged into an rAAV5 capsid using a functional CFTR coding sequence deleted for the first 264 amino acids, called "Δ264CFTR" (**Figure 1**). This deletion did not alter biologic activity or functional correction of the chloride defect *in vitro*.

The schematic experimental design to test this minigene vector in macaques is outlined in **Figure 1a**. Our aerosolized gene delivery model is based on a reproducible regional analysis of a lung into nine regions for statistical power.[21] The schematic design was to deliver aerosolized pseudotyped AAV5-GFP (green fluorescent protein) vector at 0.5×10^{14} or 1×10^{14} DRP (DNase resistant particles)/lung in two lungs and four lungs, respectively (**Figure 1a**). The doses were chosen such that the lower dose duplicated those achieved in prior rAAV2 studies and then doubled to test whether a higher achievable dose resulted in higher gene transfer, transduction, or toxicity. In parallel, the aerosolized pseudotyped AAV5-Δ264CFTR vector was delivered at a dose of 1×10^{14} DRP/lung into four lungs to determine vector-specific CFTR transfer and expression. Two of the lungs were contralateral to those treated with the GFP vector that served as an internal control vector. A remaining animal was not exposed to either vector as an untreated control.

Gene transfer of the GFP transgene was reproducibly detected by real-time polymerase chain reaction (PCR). All GFP-treated lung regions were positive for GFP-DNA transfer in 54 regions (**Figure 2a**). The two dosage groups were not statistically different and the medians were 1.04×10^5 and 6.46×10^4 copies/μg of DNA for those dosed with either 0.5×10^{14} or 1×10^{14} DRP/lung, respectively.

Transfer of the Δ264CFTR transgene was reproducibly detected by real-time PCR. All CFTR-treated lung regions were positive for Δ264CFTR-DNA in a total of 36 regions, and the median was 4.66×10^4 copies/μg of DNA (**Figure 3**). The untreated macaque demonstrated no evidence of Δ264CFTR in any lung region, as expected.

rAAV5-mediated RNA expression

To evaluate whether higher achievable gene transfer resulted in gene transduction, we determined the presence of vector-specific

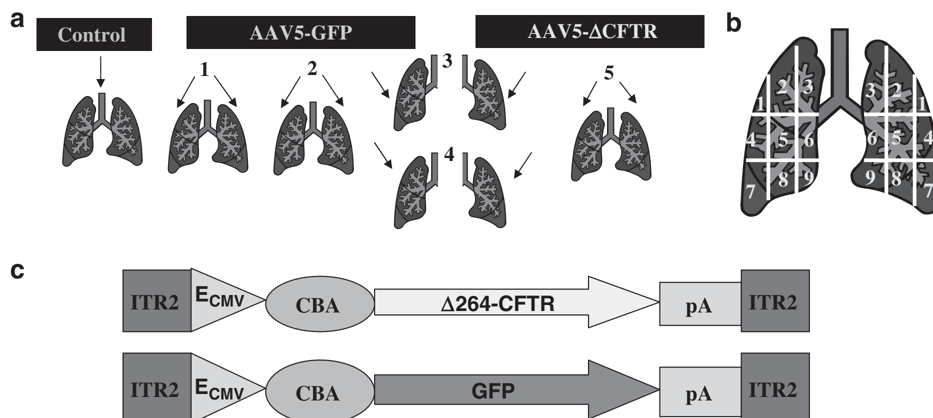

Figure 1 Experimental design. (**a**) Study design. Five macaques were dosed with up to 2×10^{14} DRP of either a pseudotyped AAV5-truncated CFTR or pseudotyped AAV5-GFP gene. One untreated macaque remained as a control. Five macaques were dosed with the following regimen: (1) AAV5-GFP to both lungs ($n = 2$), or (2) AAV5-GFP to the right lungs and AAV5-Δ264CFTR to the left lungs ($n = 2$), or (3) AAV5-Δ264CFTR to both lungs ($n = 1$). The dose of the pseudotyped, Δ264CFTR gene remained unchanged at 1×10^{14} DRP/lung. For the dosing of AAV5-GFP, two lungs received 0.5×10^{14} DRP/right lung (no. 1, no. 3) and four lungs received 1×10^{14} DRP/lung (no. 1, left lung, no. 2, no. 4, right lung). (**b**) Regional analysis of lung. (**c**) Plasmids. Depiction of the plasmids that were co-transfected into 293 cells to form a pseudotyped AAV5 vector. This product was then aerosolized and delivered endobronchially to the lungs of Rhesus macaques using a Microsprayer. DRP, DNAse-resistant particles.

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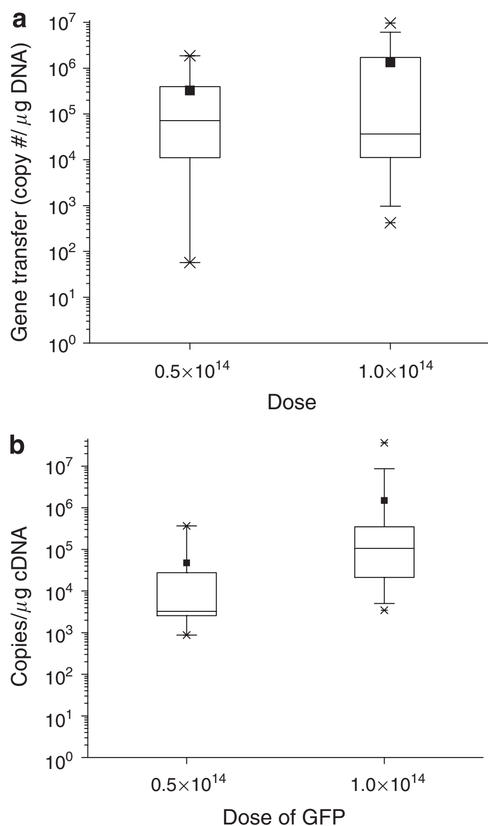

Figure 2 GFP gene transfer and expression. (**a**) Box plot showing GFP dose per lung region versus DNA transfer (copy no./μg DNA). The GFP doses were either 0.5×10^{14} DRP/lung for 18 samples or 1×10^{14} DRP/lung for 36 samples. (**b**) GFP dose per lung region versus RNA transduction (vector copy no./μg cDNA). The GFP doses were dosed at either 0.5×10^{14} DRP/lung for 18 samples or 1×10^{14} DRP/lung for 36 samples. The black squares (■) represent the mean and the line (—) represents the median. The x's ($\times$) represent the 95 and 5% confidence intervals. The enclosed area represents 50% (25–75% confidence interval) of numeric values.

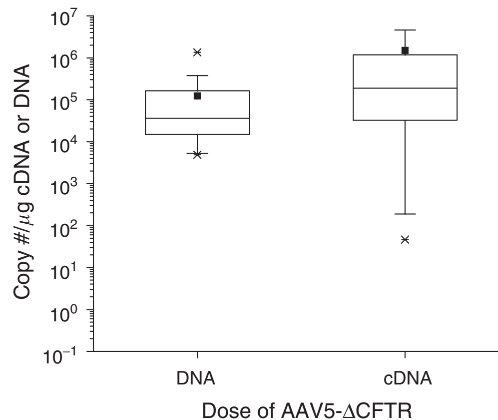

Figure 3 Box plot analysis showing Δ264CFTR gene transfer per lung region (copy no./μg DNA) on the left and Δ264CFTR mRNA transduction per lung region (copy no./μg cDNA) on the right. The black squares (■) represent the averaged mean and the line (—) represents the median. The x's ($\times$) represent the 95 and 5% confidence intervals. The box encloses the 25–75% confidence interval, representing 50% of the values.

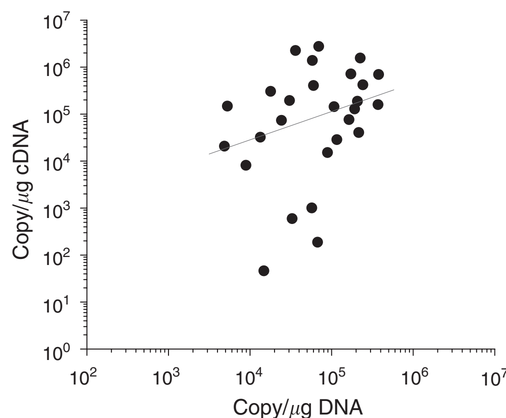

Figure 4 Δ264CFTR gene transfer (copy no./μg DNA) per lung region analyzed versus mRNA transduction (vector copy no./μg cDNA). There is a linear association between the log–log transformed levels of Δ264CFTR DNA transfer and mRNA expression. Increasing mRNA expression is demonstrated at higher levels of Δ264CFTR transfer and the correlation is significant (P-value is still <0.0001, R-value is 0.57).

mRNA expression by quantitative reverse transcriptase–polymerase chain reaction (RT–PCR). GFP RNA expression was present and quantifiable in all treated regions (54/54 regions). The medians of mRNA expression were 4.29×10^3 and 1.10×10^5 copies/μg cDNA, respectively, for the two doses of vector, 0.5×10^{14} and 1×10^{14} DRP/lung and the differences in mRNA expression were statistically significant (**Figure 2b**). So despite the fact that the gene transfer was not significantly different between the two doses, at the higher dose there was a marked biologic effect on mRNA expression, which was significantly increased. The untreated lungs again showed no evidence of either GFP transfer or GFP expression.

To determine vector-specific Δ264CFTR mRNA expression, primers were designed that were directed to regions overlapping the plasmid kozak region and CFTR cDNA. Furthermore, samples were considered positive only if both DNAsed-treated RT^+ samples demonstrated product and both β-actin and vector-specific CFTR were absent in the corresponding RT^- sample. Vector-derived mRNA expression was quantifiable in all 36 treated lung regions, and the median was 1.919×10^5 copies/μg cDNA (**Figure 3**). The untreated lungs showed no evidence of either Δ264CFTR gene transfer or mRNA expression.

Figure 4 represents an analysis of all regions to determine whether increased gene transfer correlates with a higher level of transduction. In fact, there is a linear association between the levels of Δ264CFTR DNA transfer and mRNA expression. Increasing mRNA expression is demonstrated at higher levels of Δ264CFTR transfer, and the correlation is significant ($P<0.0001$, $r=0.57$).

rAAV5-mediated protein expression

GFP expression via fluorescence was detected by confocal microscopy in the epithelial cells of the airways of all GFP

vector-treated animals (**Figure 5a** and **b**). GFP fluorescence was visualized in the cytoplasm of ciliated epithelial cells along bronchial airways and in submucosal glands in all experimental animals, whereas GFP-dependent fluorescence was absent in the non-treated animals as well as the Δ264CFTR-vector-treated animal, as demonstrated previously.[21,22] This comparison to control animals confirms the visible difference between GFP-dependent fluorescence and background autofluorescence. The arrows delineate the apical border of the airway epithelial cells, shown in the bright-field picture, and illustrate the corresponding absence of intracellular green fluorescence in the control (**Figure 5c** and **d**). Western blot analysis further verified the presence of rAAV5-mediated GFP expression in representative regions from vector-treated animals (**Figure 5e**). The results demonstrate that the gene transfer resulted in rAAV5-mediated GFP expression, confirmed by confocal microscopy *in situ* as well as by Western blot analysis.

CFTR protein expression is well recognized as being difficult to detect *in vivo*. In fact, the level of CFTR expression *in vivo* is too low to detect experimentally by commercially available antibodies in a Western blot analysis, as the typical level of endogenous CFTR RNA expression is approximated at 1 copy/cell in airway epithelial or submucosal cells.[23] Our studies use immunoprecipitation by M3A7, a monoclonal antibody to the human CFTR C terminus, on fresh tissue homogenates followed by *in vitro* phosphorylation by the addition of the catalytic subunit of protein A and γ^{32}P-ATP to detect CFTR protein expression qualitatively by phosphoimaging. Immunoprecipitation of Cos7 cell lysates transduced by a vector with full length CFTR (**Figure 6a**; Lane 1) demonstrates *in vitro* the expected two bands: mature C band (>160 kDa) and barely discernible immature B band (~140 kDa) of full-length CFTR.[24] Notably, endogenous expression is predominately indicated by the presence of the mature C band. Lane 3 demonstrates the product from cell lysates transduced by Δ264CFTR, which has a molecular weight of ~134 kDa, slightly less than that of the immature B band. Of note, when cells are cotransduced by the wild-type CFTR and the truncated Δ264CFTR, again two regions of expression are identified, that of the mature C band and a broad-based band overlapping the immature product as well as the truncated Δ264CFTR product, as all three products are detected by the monoclonal antibody (Lane 2). Immunoprecipitation by M3A7 of fresh tissue lysates followed by phosphorylation using γ^{32}P-ATP further verified the presence of rAAV5-mediated CFTR protein expression in representative regions from vector-treated animals (**Figure 6b**; top panel) in comparison to those control animals treated with the GFP vector as an internal control (**Figure 6b**; lower panel). Notably, the presence of the Δ264CFTR product is demonstrated in Lanes 4–6 (**Figure 6b**; top panel); likewise, the presence of both the mature and immature bands are qualitatively more expressed than the barely detectable bands in the control-animal tissue lysates. The results demonstrate that the gene transfer resulted in vector-specific

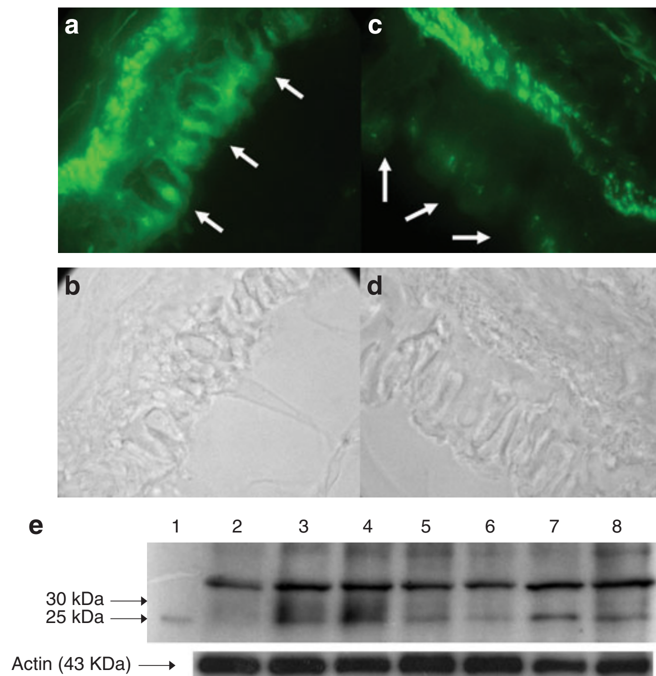

Figure 5 GFP expression. (**a–d**) Fluorescent GFP expression is shown upon microscopy in bronchial epithelium 3 weeks after dosing with the vector. The lung was sectioned into nine regions per lung and dissected at the level of segmental branching and underwent frozen sectioning before being analyzed for GFP fluorescent expression by microscopy. (**a**) GFP expression is shown in the cytoplasm of ciliated airway epithelial cells in a pseudotyped AAV5-GFP-treated macaque and (**c**) there is an absence of GFP-specific expression in pulmonary sections from a control macaque. **b** and **d** are corresponding bright fields of the corresponding sections. Original magnification ×1,000. (**e**) Western blot analysis of GFP (top) and β-actin (bottom) is shown. The first lane contains GFP as a positive control (27 kDa). Lane 2 is a negative animal control and Lanes 3–8 contain samples from different lung regions of an experimental animal, of which all demonstrated GFP expression. β-Actin (43 kDa) controls are depicted in the bottom panel.

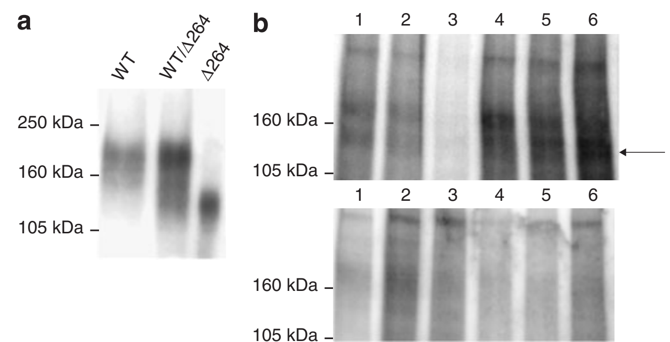

Figure 6 Immunoprecipitation and protein phosphorylation of CFTR: CFTR expression is assessed by *in vitro* phosphorylation. (**a**) Cell lysates: Cos cells were co-transfected with full-length CFTR (Lane 1), Δ264CFTR (Lane 3), or both full-length and Δ264CFTR (Lane 2) to demonstrate the product of each vector *in vitro* in a cell line. The size of the mature C band of full-length CFTR (~160 kDa) and the immature B band (~140 kDa) is shown. (**b**). Tissue lysates: immunoprecipitation by M3A7 of fresh tissue lysates followed by phosphorylation using γ^{32}P-ATP. Lanes 1–6 are representative regions of lung tissue analyzed with 5 μg of monoclonal antibody M3A7 and represent regions from experimental macaques in the top panel versus control macaques in the bottom, respectively. The top panel shows presence of rAAV5-mediated CFTR protein expression in representative regions from vector-treated animals in comparison to the control animals treated with the GFP vector as an internal control (bottom panel).

CFTR expression, confirmed by immunoprecipitation analysis with *in situ* phosphoimaging.

Absence of inflammation

All macaques tolerated bronchoscopies and anesthetics without adverse sequelae or signs of respiratory distress or acute upper respiratory infections. Their clinical course was evaluated twice a day and after 72 h was evaluated on a daily basis. Upon comprehensive review of frozen tissue sections from each lung by a pathologist blinded to the experimental design, there was no evidence of acute or chronic inflammation along the airways, interstitial, or alveolar spaces in experimental animals as compared to the control animal (**Figure 7**). A review of hematoxylin-and-eosin-stained sections from each lung revealed no identifiable difference in the histology between the experimental and control animals. Specifically, there was no evidence of destructive acute changes such as eosinophilia, enlarged bronchial lymphoid aggregates, or inflammatory infiltrates, or chronic changes such as chronic bronchiectasis obliterans or bronchiectasis in the lungs after administration of the rAAV5 vectors. These findings were compared with baseline findings established in non-treated controls, and no evidence of pathologic sequelae was detected.

DISCUSSION

Gene therapy remains a potential therapy for primary lung diseases, such as CF and α1-antitrypsin, as non-invasive inhalational aerosols can target airway epithelial cells and the rapid expansion of vector biology has characterized numerous serotypes directed to apical airway receptors. The original AAV2-based vectors demonstrated safety as well as gene transfer in clinical trials. Thus, these landmark studies demonstrated the feasibility of gene therapy for CF. It is the lack of an easily defined clinical end point that confounds outcome measures, as well as the low level of *in vivo* expression that potentially is clinically adequate but difficult to detect. Actually, this low level of expression may potentially be comparable to the level of endogenous expression typified in a normal individual, as well as being ascertainable by gene therapy. Transfection of even a low percentage of 8% of normal transcripts has preserved normal function.[25] Thus, the aim of expressing CFTR to a level sufficient for maintaining a normal lung phenotype by gene therapy is conceivable.

The initial rAAV2-based studies highlighted numerous barriers to effective airway gene therapy. The size constraints of AAV vectors require an optimal insert size for packaging at ~4.5 kb, which is prohibitive for large coding sequences such as CFTR (4.45 kb) with the promoter adjuncts essential for expression. Various strategies have been utilized to circumvent the packaging limits of AAV viruses, including using only the endogenous inverted terminal repeat internal promoter,[26] proteosome inhibitors to drive expression,[27] R domain deletions, and others.[28] Likewise, there are multiple barriers to AAV2 vectors owing to a relatively low number of apical receptors,[10,14] intracellular processing[11] and low integration, and slow conversion to transciptionally active genomes.[29,30] Thus, a new generation of rAAV-CFTR vectors utilizing other serotypes, such as AAV5 or AAV6, may better circumvent cell entry and intracellular trafficking barriers known to AAV2. The concept of a more powerful expression cassette with previously reported CFTR minigene constructs demonstrated correction of chloride transport defect *in vitro*,[31] even with a single pseudotyped AAV5 CFTR minigene.[28] Recent studies have characterized a pseudotyped rAAV5-CFTR minigene construct with a truncated CFTR cDNA and a more effective CBA promoter to enhance expression,[20] incorporating the advances in using AAV5 over AAV2 to enhance gene uptake.[10] The endogenous CFTR promoter is known to be weak, producing 1–2 transcripts per cell.[23]

The extension of these studies to non-human primates is critical for safety considerations and to determine the sufficient level of expression similar to endogenous levels. The gene transfer in the GFP and Δ264CFTR studies is comparable at

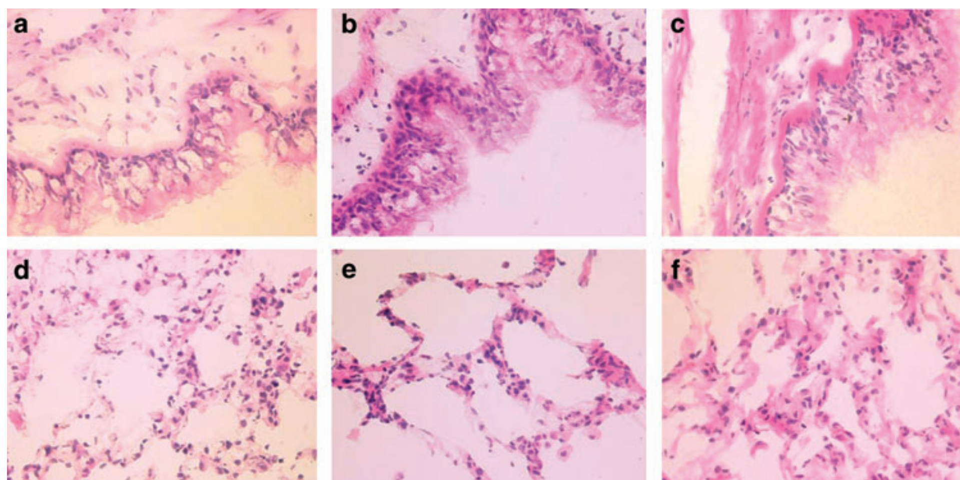

Figure 7 Histology of vector-treated animals in contrast to controls. Hematoxylin-and-eosin-stained lung sections from frozen tissue of (**a** and **d**) animals treated with AAV5-GFP, (**b** and **e**) animals treated with AAV5-Δ264CFTR, and (**c** and **f**) the untreated control, demonstrate the overall absence of pathologic inflammation (**a**, **b**, and **c**) near the proximal airways and airway epithelium as well as (**d**, **e**, and **f**) in the distal alveoli. Original magnification ×20.

10^5 copies/μg genomic DNA, given by an aerosolized method at high titers. These advances reflect the directed delivery of bronchoscopic microspraying that minimizes oropharyngeal loss[21] and the enhanced cellular entry of AAV5 with apical airway receptors. Vector-specific mRNA expression is also confirmed and quantitative: in fact, vector-specific transduction increases in a linear manner with increasing gene transfer. Previous data have suggested that AAV2-mediated reporter gene expression increased when regional doses exceeded a minimal threshold.[21] In these studies, doubling the dose to ensure a higher cellular delivery translated into higher gene transduction. In fact, the current studies show quantifiable mRNA expression in all regions, suggesting that the minimal threshold has been exceeded; despite the similarity of levels of gene transfer, biologically the level of gene expression was a log greater in the higher-titer group (**Figure 2b**). In contrast, the historical AAV2 studies demonstrated an average gene transfer of 1.89×10^4 as opposed to 3.27×10^5 copies/μg of DNA in the current AAV5 studies for identically dosed macaques (**Figure 8**). This difference is approximately a 20-fold increase in gene transfer which is commensurate with the reported 30-fold increase seen in mice, owing to the efficiency of AAV5 infectivity.[10] This log difference in gene transfer resulted in RNA expression in all regions and at a quantifiable level which had not been previously accomplished in prior studies. More importantly, in prior AAV2 studies RNA expression was only detectable qualitatively by nested RT–PCR and not even detected in all regions.

Furthermore, these studies demonstrate that CFTR expression in treated macaques can be detected. This current study substantiates the feasibility of gene transfer by using pseudotyped vectors that can drive expression sufficient for both gene transduction and protein expression. Further development of this mini CFTR construct is promising for clinical application particularly in the absence of an immune response, pathology, and expression comparable to endogenous expression.

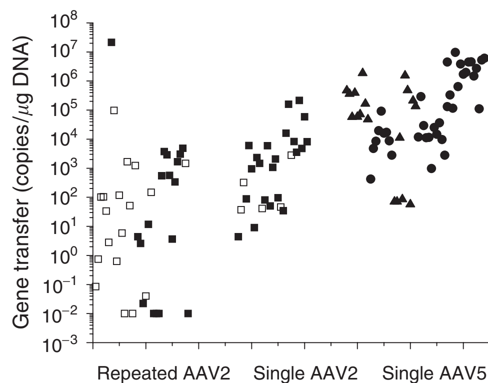

Figure 8 Increase of GFP transfer and transduction. GFP DNA transfer was quantified by real-time PCR and expressed as copies/μg DNA and compared to the transfer previously quantified in the prior single and repeat dosing studies of AAV2 (all doses $= 0.5 \times 10^{14}$ DRP/lung). Also shown in the far right panel is our current study of single doses (of 0.5×10^{14} DRP/lung (▲) or 1.0×10^{14} DRP/lung (●)) of pseudotyped AAV5-GFP. Also shown is the increase in number of samples positive for RNA expression indicated by the filled symbols (■,▲,●), whereas the open squares (□) reflect the absence of RNA expression for given lung section. Of note, the absence of RNA expression is only detected in the prior AAV2 studies. Repeated dosing studies with AAV2-GFP resulted in 18/36 regions (left panel), and single dosing with AAV2-GFP resulted in 22/27 positive RNA regions (middle panel), whereas single dosing with pseudotyped AAV5-GFP resulted in regions positive for RNA transduction in all regions (right panel).

MATERIALS AND METHODS

Animals. Young adult Rhesus macaques (five experimental, one untreated control) were from the Johns Hopkins University breeding colony and housed according to Animal Care and Use Committee guidelines as described previously.[22]

Study design: GFP study. This design was constructed to test if gene transfer by an rAAV5-pseudotyped vector in **Figure 1b**, either an rAAV5-truncated CFTR vector, called Δ264CFTR, or an rAAV5-GFP vector, could affect transgene levels sufficient for gene expression. The experimental design is outlined in **Figure 1a**: a total of six lungs received either 0.5×10^{14} or 1×10^{14} DRP/lung of AAV5-GFP. In an identical manner, four lungs were treated with a 1×10^{14} DRP of Δ264CFTR; thus, the GFP-treated lungs were the internal controls of the lungs experimentally treated with the Δ264CFTR. The remaining two lungs represent the untreated control animal. All treated animals received aerosolized pseudotyped rAAV5 vectors endobronchially with a PennCentury Microsprayer delivered during spontaneous breathing as described previously.[21] Three weeks following vector administration, the animals were autopsied and lungs harvested for analysis. The indicated lung regions are defined as nine per lung (18 regions per animal) biplanar, non-anatomic sections for regional analysis, previously characterized by scintigraphy and described in this airway delivery model[21,22] (**Figure 1b**). Each region (totaling 108: 54 regions for GFP, 36 regions for CFTR, 18 regions for the control) was analyzed for vector-specific DNA transfer, mRNA transduction, and protein expression.

Vectors. The vectors were produced in the Vector Core at the University of Florida and packaged using pseudotyping techniques as described previously.[32] A double plasmid technique used the AAV2*rep*/AAV2*cap*5 helper plasmid, pXYZ5, constructed into an adenoviral plasmid backbone containing AD E4 genes. The recombinant AAV vector constructs were assembled on the pTR-UF backbone (see **Figure 1c**). Vector preparations were from a single pooled stock with 99% purity and titers of 3.18×10^{13} DBP/ml and 6.22×10^{13} (DRP)/ml for the pseudotyped AAV5-Δ264CFTR and pseudotyped AAV5-GFP, respectively.

Aerosol delivery by Microsprayer. rAAV5-pseudotyped (rep2 cap5) vectors were delivered directly to the specified mainstem bronchus using a Microsprayer (PennCentury, Philadelphia, PA) inserted through a 3.5 mm flexible fiberoptic bronchoscope (Olympus, Melville, NY). The macaques were sedated and received aerosolized vector as described previously.[21,22]

DNA and RNA analysis. At necropsy (day 21), the lungs were harvested and infused with 60 cc of saline and divided into a nine-region grid as described previously.[21,22] Approximately 90% of each lung region was flash frozen and prepared for RNA, DNA, and protein extraction. The remaining 10% was frozen in optimum cutting temperature compound (Sakura Finetek, Torrance, CA) and sectioned onto slides for histological analysis. All quantitative PCR and RT–PCR were performed in triplicate using the LightCycler (Roche Molecular Biochemicals, Indianapolis, IN). The product was amplified from either 100 ng of genomic DNA or 360 ng cDNA. Qualitative PCR and RT–PCR cycling for controls was conducted with the GeneAmp PCR System 2400 (Perkin Elmer,

Wellesley, MA). RT^- samples were used as negative controls and underwent PCR to detect β-actin, GFP, and vector-specific CFTR. RT^+ samples were also controls for β-actin using sequence-specific primers, as described previously.[21,22] All RT reactions consisted of treating RNA samples with DNase and reverse transcriptase before undergoing cDNA synthesis using the First-strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ); RT^-samples were not treated with reverse transcriptase. Then, 360 ng of that cDNA was used in quantitative PCR for GFP or CFTR vector-specific detection.

The GFP-specific product was amplified using the same methodology, primers, and controls as described previously.[22] Standard controls were 10^5, 10^4, 10^3, 10^2, and 10^1 plasmid copies per reaction. The lowest standard concentration, 10^1 plasmid copies per reaction, represents the lower limit of detection. The vector-specific CFTR-DNA primers were designed to span CFTR cDNA, the synthetic polyA site, non-coding AAV2 sequence, and the right-hand AAV2 inverted terminal repeat (395 bp product). The sequences are 5′-TGCTGATTGCACAGTAATTC-3′ and 5′-AGTGGCCAACTCCATCACTA-3′. The vector-specific CFTR RT–PCR primers had a product size of 227 bp and spanned the kozak consensus sequence and a portion of the CFTR cDNA insert. The sequences are 5′-ACTAGTGCCGCCACCATGA-3′ and 5′-TTCCGGAG GATGATTCCTT-3′. Standard control concentrations were 3.2×10^6, 3.2×10^5, 3.2×10^4, 3.2×10^3, 3.2×10^2, and 3.2×10^1 plasmid copies per reaction. The lowest standard concentration was used to represent the lower limit of detection. Qualitative PCR and RT–PCR were performed on β-actin and vector-specific CFTR, as controls, using the GeneAmp PCR System 2400 (Perkin Elmer, Wellesley, MA), using the same method as described previously.[21]

Protein analysis. *GFP Western blot analysis*: Sixty micrograms of lung protein was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were then blocked, washed, and incubated with a 1:4,000 dilution of primary antibody to GFP, Ab 6556 (Abcam, Cambridge, UK), and a 1:10,000 dilution of secondary antibody (donkey anti-rabbit horseradish peroxidase-conjugate, Amersham Life Science, Arlington Heights, IL) in the same manner, as described previously.[22,33]

Microscopy: Multiple transverse 5 μM frozen sections were made through each of the nine regions perpendicular to visible bronchi or bronchioles when possible. These frozen sections were analyzed by a pathologist blinded to the schematic design for detection of abnormal cellular architecture, infiltrates, and pathology. We examined unstained sections for GFP green fluorescence using a Zeiss Axiskop (Thornwood, NY) upright epi-fluorescence microscope equipped with a GFP-specific filter set (excitation HQ470/40x, emitter HQ 515/30M) microscope.

Immunoprecipitation and protein phosphorylation of CFTR: Biochemical analysis of CFTR was performed by immunoprecipitation with a monoclonal antibody to human CFTR C terminal (R&D Systems, Minneapolis, MN) followed by *in vitro* phosphorylation using protein kinase A and γ^{32}P-ATP. Lung tissue samples were rinsed twice with phosphate-buffered saline (Sigma, St Louis, MO) and homogenized in lysis buffer as described previously.[33] The protein was extracted, prepared for immunoprecipitation and phosphorylation, then separated on a 5% sodium dodecyl sulfate–polyacrylamide gel (BIO-RAD, Hercules, CA) and prepared for autoradiography as described previously.[33] Exposure time was 30–60 min at −80°C. Control cell lysates were from Cos7 cells transiently transfected with wild-type CFTR or Δ264CFTR (or both co-transfected) and grown in T75 flasks. Transfections were performed with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) per the manufacturer's instructions.

Statistical analysis. Data are reported as means with median, as the data are non-Gaussian. S-Plus 6.2 (Insightful, Seattle, WA) was used to perform a Kruskal–Wallis rank sum test to assess the statistical significance of vector transfer and transduction (copy no./μg DNA or cDNA). Non-parametric tests were used because the data were not normally distributed. Box plots of statistical distributions were generated in Origin 7.5 (OriginLab, Northampton, MA). *P*-values were deemed significant at ± 0.05. From consideration of the design effect calculated from variance, number of animals, lung regions, and standard means of experimental results from previous dosing experiments, we determined that we have statistical power to determine differences in gene transfer between experimental maneuvers such as dose, vector serotype, and promoter strength. Calculations were based on an experiment with four animals per maneuver, with nine regions per lung for a total of 72 lung regions. We realize that some of the statistical power is lost because we are comparing multiple lung regions in a limited number of animals. However, the intraclass correlation of the within-monkey effect with respect to copy number per lung region is low: 0.07 for the single-dose and 0.00132 for the multiple-dose experiments. This gives us an effective sample size of at least six from each set of nine samples from one lung.

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