

Dual Therapeutic Utility of Proteasome Modulating Agents for Pharmacogeno-Gene Therapy of the Cystic Fibrosis Airway

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Pharmacologic- and gene-based therapies have historically been developed as two independent therapeutic platforms for cystic fibrosis (CF) lung disease. Inhibition of the dysregulated epithelial Na channel (ENaC) is one pharmacologic approach to enhance airway clearance in CF. We investigated pharmacologic approaches to enhance CFTR gene delivery with recombinant adeno-associated virus (rAAV) and identified compounds that significantly improved viral transduction while simultaneously inhibiting ENaC activity through an unrelated mechanism. Treatment of human CF airway epithelia with proteasome modulating agents (LLnL and doxorubicin) at the time of rAAV2 or rAAV2/5 infection dramatically enhanced CFTR gene delivery and correction of CFTR-mediated short-circuit currents. Surprisingly, these agents also facilitated long-term (15-day) functional inhibition of ENaC currents independent of CFTR vector administration. Inhibition of ENaC activity was predominantly attributed to a doxorubicin-dependent decrease in γ -ENaC subunit mRNA expression and an increase in γ -ENaC promoter methylation. This is the first report to describe the identification of compounds with dual therapeutic action that are able to enhance the efficacy of CFTR gene therapy to the airway while simultaneously ameliorating primary aspects of CF disease pathophysiology. The identification of such compounds mark a new area for drug development, not only for CF, but also for other gene therapy disease targets.

Key Words: CFTR, ENaC, gene therapy, cystic fibrosis, adeno-associated virus, AAV

INTRODUCTION

Cystic fibrosis (CF), caused by genetic mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), is the most common lethal disorder in the Caucasian population. CFTR is a chloride channel that localizes to the apical membrane of epithelial cells in many organs such as the lung. The channel is activated by cyclic AMP (cAMP) and regulated by PKA- and PKC-dependent phosphorylation. In addition to functioning as a chloride channel, CFTR has also been shown to regulate several other ion channels at the cell surface [1], including the epithelial amiloride-sensitive Na

channel (ENaC) [2,3], outward rectifying chloride channel [4,5], renal potassium channel [6], and calcium-activated chloride channel [7]. Several mechanisms have been proposed to account for the increased bacterial colonization seen in the CF lung [1]. These include loss of CFTR function leading to dysregulated enhancement of ENaC sodium currents, which is proposed as a predominant mechanism for airway dehydration and poor mucociliary clearance of the CF lung [8,9]. Alternative mechanisms of pathogenesis also include alterations in innate immune defenses in the CF airway as a result of CFTR dysfunction [1,9,10].

Numerous clinical trials for cystic fibrosis have evaluated pharmacologic approaches to correct primary defects associated with CFTR dysfunction. These have included approaches to enhance endogenous mutant CFTR function at the apical membrane [11,12] and normalization of ENaC function using aerosolized amiloride [13–15]. More recently, recombinant adeno-associated virus (rAAV) has been used to deliver a functional CFTR cDNA to the maxillary sinus and/or lungs of CF patients [16–19]. Although recent trials with rAAV2 have demonstrated an impressive safety profile and long-term persistence of vector genomes in the airway epithelia of the lung, successful transduction of airway cells as measured by vector-derived CFTR mRNA has yet to be achieved. Several basic research studies on AAV biology have suggested why such current gene therapy efforts in clinical trials have to date not detected CFTR transgene expression *in vivo* using rAAV2. These include the finding that rAAV2 binds fairly poorly to the surface of polarized human airway cells [20] and the fact that known rAAV2 receptors do not reside on the apical surface of the human airway [21]. Such findings have led to the development and application of alternative serotypes such as rAAV5 that bind to alternative receptors at the airway surface [22] and have enhanced transduction efficiencies [20]. Alternative developments evaluating intracellular barriers to rAAV transduction in the human airway have suggested that both rAAV2 and rAAV5 are susceptible to ubiquitin/proteasome interactions, which modulate the ability of virions to complete their life cycle and efficiently traffic to the nucleus [23]. These studies have led to the application of proteasome inhibitors/modulating agents capable of significantly enhancing transduction of both serotypes from the apical membrane [23,24]. Interestingly, studies comparing rAAV2 directly to rAAV2/5 vectors have suggested that the maximal potential of rAAV2 to transduce airway epithelial from the basolateral membrane is an order of magnitude higher than that of rAAV2/5 infection from the apical or basolateral membrane [24]. These findings suggest the possibility that if apical barriers to infection could be fully circumvented, rAAV2 may emerge as a preferential vector for gene therapy of the CF lung.

Additional limitations to rAAV-mediated gene delivery of CFTR include the limited packaging capacity of this vector (~5 kb) and the relatively large size of the CFTR cDNA (4.5 kb). Several strategies have been used to fit the CFTR cDNA into rAAV vectors, including the use of the ITR as a promoter [25] and the deletion of regions of CFTR thought not to be necessary for *in vivo* function [26,27]. The first strategy is currently being used in clinical trials with rAAV2-based vectors. Although *in vitro* studies have clearly demonstrated the ability of the ITR to function as a promoter [25], expression is much lower than that from heterologous promoters [28]. Hence, the low activity of the ITR as a promoter is

currently thought to be one of the major reasons for lack of detectable vector-derived CFTR mRNA expression in clinical trials [17].

In the present study, we have sought to compare the rAAV2 and rAAV2/5 vector systems directly for their ability to correct CFTR defects in polarized CF airway epithelia. Studies were designed to address several clinically relevant questions. First, as data in mice would predict [29], are type 5 rAAV vectors more efficacious in complementing CFTR chloride transport defects in human airway epithelia compared to type 2 rAAV vectors? It is important to point out in this regard that functional restoration of CFTR chloride transport in polarized CF airway epithelia has yet to be demonstrated with any rAAV vector. Second, in the presence of proteasome modulating agents, which vector serotype has the greatest potential for correcting CFTR abnormalities in CF airway epithelia? Third, is expression from the ITR a limiting factor in the production of vector-derived CFTR mRNA from the current clinically utilized CFTR AAV vector? To this end, we have compared the identical ITR–CFTR vector used in clinical trials for CF (AVtgCF) to a new vector design harboring a minimal 83-bp promoter directing expression of the full-length CFTR cDNA (AVCF83). Both AAV2-based vector genomes were packaged into either type 2 or type 5 capsids and analyzed for their efficiency of transduction in the presence or absence of proteasome modulating agents (LLnL/doxorubicin). Short circuit current measurements, RNA specific real-time reverse transcriptase PCR (RS-PCR), and TaqMan DNA PCR were used to quantify functional correction of CFTR chloride currents, vector-derived mRNA, and vector DNA, respectively, following apical infection of polarized CF airway epithelia. Findings from these studies demonstrated that rAAV2-based vectors were more efficacious than rAAV2/5 at expressing CFTR-derived mRNA and correcting CFTR chloride transport abnormalities in the presence of applied proteasome modulating agents. Interestingly, the application of proteasome modulating agents at the time of infection not only improved the functional conversion of rAAV genomes to expressible forms but also reduced the ENaC hyperabsorption CF phenotype in a manner independent of CFTR gene expression. Quantitative RT-PCR demonstrated that the addition of proteasome modulating agents reduced γ -ENaC subunit mRNA levels in polarized CF airway epithelia by 15-fold. The long-term (15-day) persistence of this effect on ENaC activity appears to be due to doxorubicin-dependent CpG methylation in the promoter and exon 1 region of the γ -ENaC gene. These unexpected findings demonstrate for the first time the identification of a new class of dual therapeutic agents capable of ameliorating a component of primary disease pathophysiology while simultaneously enhancing the efficacy of gene therapy for the disorder.

RESULTS

Proteasome Modulation Agents Enhance rAAV-Mediated CFTR Functional Correction in Polarized CF Airway Epithelia

Current hypotheses for how proteasome inhibitors augment rAAV transduction in polarized airway epithelia suggest that increased functional conversion of single-stranded genomes to expressible forms is facilitated by the increased bulk flow of rAAV into the nucleus [23,24,38]. Based on these findings, we assessed the efficacy of both the current clinically used ITR-driven full-length CFTR rAAV vector (AV2tgCF, also called tgAAV2-CF) and a second-generation vector harboring a short 83-bp synthetic promoter driving expression of a full-length CFTR cDNA (AV2CF83) for their ability to correct CFTR chloride transport in human CF airway epithelia in the presence of proteasome modulating agents. Additionally, since rAAV5 has been suggested to transduce the apical surface of human airway epithelia more efficiently than rAAV2, we also evaluated pseudotyped rAAV2/5 viruses with both types of vector genomes.

We used rAAV2 or rAAV2/5 vectors utilizing the ITR or synthetic promoters to infect polarized CF airway epithelia from the apical surface in the presence or absence of a combined cocktail of LLnL and doxorubicin. Fifteen days following infection, we assessed CFTR-mediated cAMP-sensitive short-circuit current (I_{sc}) after stimulation by IBMX (100 μ M) and forskolin (10 μ M) and

compared it to normal human airway epithelia. In total, we infected samples from three different CF donors (CFB-16, CFB-19, and CFB-26) and analyzed them for CFTR correction. Results from these experiments are shown in Fig. 1. In the absence of proteasome inhibitors, we saw only minimal restoration of cAMP-inducible chloride currents with the minimal promoter vector (AV2CF83) of the type 2 serotype ($0.76 \pm 0.16 \mu\text{A}/\text{cm}^2$) and no significant function correction with any of the other three viruses tested (AV2tgCF, AV2/5tgCF, or AV2/5CF83). However, when we provided proteasome inhibitors LLnL and doxorubicin (Dox) only at the time of infection, AV2CF83 restored CFTR-mediated chloride current upon IBMX/forskolin stimulation in the CF epithelia at the highest level ($2.9 \pm 0.3 \mu\text{A}/\text{cm}^2$), reaching approximately 80% of that seen in normal human airway epithelial ($3.5 \pm 0.8 \mu\text{A}/\text{cm}^2$). Surprisingly, pseudotyped AV2/5CF83 with the same synthetic promoter gave significantly less correction of chloride currents in CF epithelia ($1.0 \pm 0.2 \mu\text{A}/\text{cm}^2$), which was even lower than that seen with the ITR promoter CFTR vector AV2tgCF ($1.9 \pm 0.2 \mu\text{A}/\text{cm}^2$). For each serotype, the addition of the 83-bp synthetic promoter significantly increased ($P < 0.03$) IBMX/forskolin-responsive I_{sc} compared to ITR-driven CFTR vectors.

Functional Activity of Intracellular Viral Genomes is Significantly Enhanced by the Addition of LLnL/Dox at the Time of Vector Administration

To correlate the level of functional correction with the ability of rAAV vector genomes to express CFTR mRNA, we harvested epithelia following functional analysis and quantified vector-derived mRNA and DNA for each sample. We quantified vector-derived mRNA using a previously described [35] RS-PCR method that normalizes the copies of vector-derived and endogenous CFTR mRNAs to the level of β -glucuronidase (GUS) expression (the same method used for analysis of rAAV CF clinical trial samples [17]). Results from this analysis (Fig. 2A) demonstrated near-undetectable vector-derived CFTR mRNA transcripts in all vector groups that did not receive LLnL/Dox at the time of vector administration. These results support the lack of CFTR functional activity seen in these experimental groups. In contrast, LLnL/Dox treatment significantly enhanced the level of vector-derived CFTR mRNA transcripts in both AV2tgCF and AV2CF83 vector groups greater than 150-fold. Although similar levels of induction were seen in AAV2/5 vector groups, the total level of vector-derived CFTR mRNA remained 10- to 40-fold lower than that seen in the AAV2 vector groups. Comparison of transgene-derived CFTR mRNA to endogenous CFTR mRNA levels in the various vector groups also reflected greater relative expression in AV2tgCF and AV2CF83 groups (Fig. 2B) that was equivalent to or slightly greater than endogenous CFTR mRNA levels. Although the fold differences in vector-derived

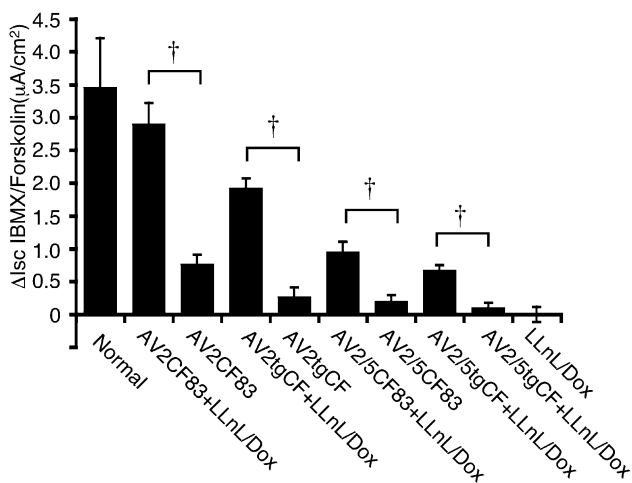


FIG. 1. Complementation of CFTR chloride transport abnormalities in CF airway epithelia using combined CFTR rAAV and proteasome modulation. Results depict the mean \pm SEM ($N = 9$) ΔI_{sc} response to IBMX/forskolin in CF airway epithelia treated under the indicated conditions marked on the x axis. The response from non-CF untreated controls (marked Normal) is given as a reference for fully functional CFTR, while all other treatment groups were CF epithelia. Assays were performed at 15 days postinfection. †Significant difference using the Student t test, $P < 0.001$.

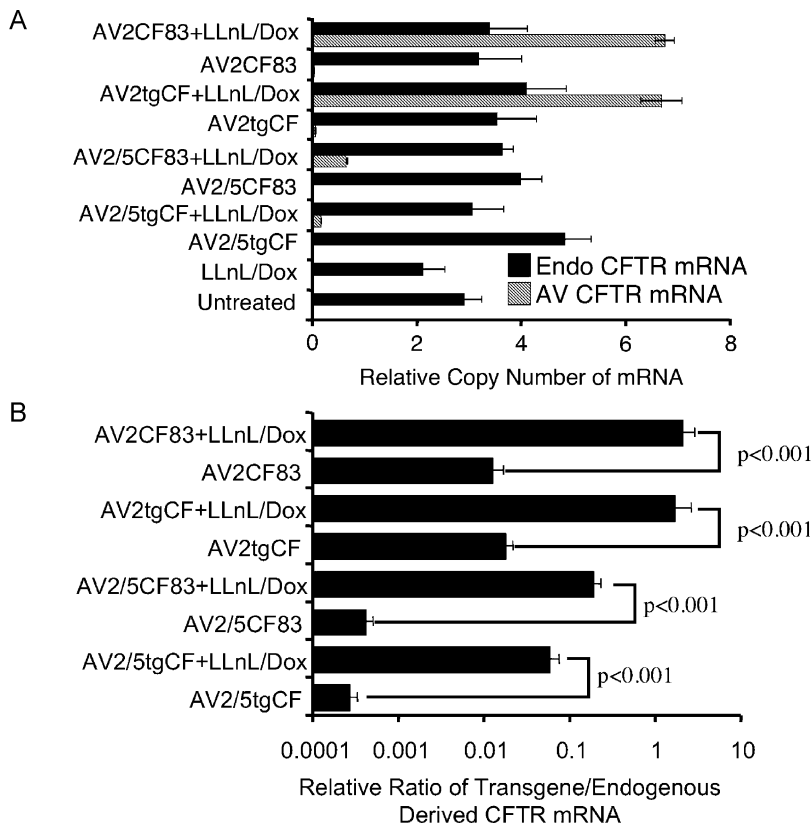


FIG. 2. Expression of transgene-derived and endogenous CFTR mRNA in CF airway epithelia. (A) RS-PCR was used to measure the relative levels of rAAV and endogenous CFTR mRNA in all CF airway epithelial samples analyzed in Fig. 1. Values represent the mean \pm SEM ($N = 9$) relative copies of CFTR mRNA. (B) The relative ratio of transgene-derived to endogenous CFTR mRNA was calculated for each sample individually and plotted as an index of the relative level of correction. Values represent the means \pm SEM ($N = 9$). A relative ratio of 1 reflects approximately equivalent levels of transgene-derived and endogenous CFTR message. P values for various comparisons were calculated using the Student t test.

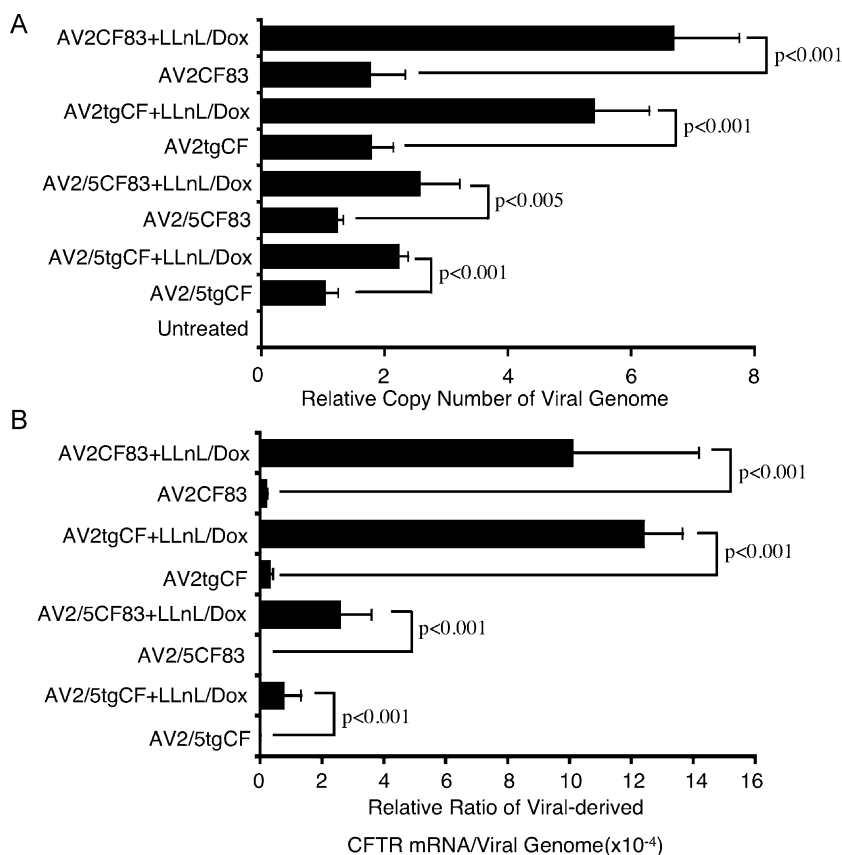
CFTR mRNA between the various vector groups did not quantitatively mirror the fold differences in CFTR correction, the overall trends were similar. All rAAV vectors gave much higher levels of vector-derived CFTR mRNA when proteasome modulating agents were applied at the time of infection, and consistent with CFTR functional correction, AAV2 vectors expressed CFTR mRNA at much higher levels than AAV2/5 vectors.

Current knowledge of the mechanism that proteasome inhibitors use to enhance rAAV transduction from the apical membrane suggests that these agents act on intracellular processes that enhance rAAV movement to the nucleus [23,24]. With this underlying hypothesis, we sought to investigate the level of viral DNA that remained in cells at 15 days posttransduction for each of the various serotypes and treatment conditions. In light of previous studies [23,24], we predicted that LLnL/Dox would predominantly increase the transcriptional activity of viral genomes by virtue of greater nuclear accumulation and conversion to double-stranded intermediates. Furthermore, we predicted that substantial viral DNA would remain in epithelia infected in the absence of LLnL/Dox and that these forms would predominantly be transcriptionally inactive as single-stranded genomes. Indeed, results from DNA analysis support these hypotheses. LLnL/Dox treatment increased DNA persistence only marginally, by 2- to 3-fold (Fig. 3A),

regardless of the vector type. Such findings are consistent with previous work demonstrating that intracellular processing of the virus is the major rate-limiting step in airway transduction. However, when transcriptional activity of viral genomes was assessed by calculating the vector-derived mRNA/DNA ratios, LLnL/Dox treatment significantly enhanced the transcriptional activity of vector genomes by 40- to 50-fold for the AAV2 vector groups (Fig. 3B). Enhancement of vector-derived mRNA/DNA ratios for the AAV5 vector groups was also very large but could not be accurately calculated since mRNA levels in the absence of LLnL/Dox were at background levels. Overall, the effect of proteasome modulating agents on increasing the functional activity of viral genomes was universal for both type 2 and type 5 serotypes. However, in general, rAAV2 vectors performed better than rAAV5 vectors. These findings support the notion that modulation of the proteasome allows for more efficient intracellular processing of rAAV genomes to transcriptionally active intermediates without significantly affecting their overall abundance within cells.

Evaluation of vector-derived mRNA/DNA ratios was also useful in assessing increased efficacy of vectors harboring a short 83-bp minimal promoter. Although the CF83 promoter improved reporter gene expression in IB3 cells 30-fold above that seen with the ITR promoter [32], the effect on gene expression was

FIG. 3. Quantification of vector DNA following rAAV infection of CF airway epithelia. The total DNA fraction (nuclear and cytoplasmic) remaining following mRNA isolation was quantified by TaqMan PCR for the number of vector genomes for the indicated conditions. Samples are identical to those analyzed in Figs. 1 and 2. (A) Values represent the mean \pm SEM ($N = 9$) relative copies of rAAV CFTR vector genomes for each sample. (B) The ratio of vector-derived CFTR mRNA to vector DNA was calculated for each individual sample as an index of vector genome transcriptional activity. Higher ratios represent a greater level of transcription per vector genome for a given condition. Values represent the means \pm SEM ($N = 9$). P values for various comparisons were calculated using the Student t test.



minimal in differentiated airway epithelia. RNA/DNA ratios were not significantly different for AV2CF83 compared to AV2tgCF in the presence of LLnL and Dox, implying no significant augmentation in transcriptional activity of genomes containing the synthetic promoter. However, after the treatment of LLnL and Dox, the RNA/DNA ratios for AV2/5CF83 were approximately 3-fold higher than those for AV2/5tgCF, suggesting that the synthetic promoter may have some beneficial effect on transcription, although not as great as in IB3 cells.

Proteasome Modulating Agents Reduce the Amiloride-Sensitive Sodium Currents in CF Airway Epithelia by Decreasing ENaC Subunit mRNA Levels

ENaC is the major component of baseline short circuit current in CF airway epithelia and is greatly elevated due to a lack of functional CFTR. It has been previously suggested that as little as 6–10% transduction with a CFTR-expressing vector can fully correct CFTR-mediated chloride currents in a polarized airway epithelia due to gap-junctional cell–cell coupling of Cl^- ions in the epithelium [39]. In contrast, normalization of elevated Na^+ current caused by dysregulated ENaC activity requires 100% transduction of a CF epithelium since

CFTR must physically interact with ENaC to regulate Na^+ conductance properly [40]. Consequently, the extent of normalization of elevated amiloride-sensitive sodium currents in each of the CFTR vector-treated groups could be used to infer indirectly the percentage of cells expressing vector-derived CFTR in the epithelia. Assessment of amiloride-sensitive ENaC short circuit current revealed the surprising finding that all vector groups administered with LLnL/Dox demonstrated complete normalization of elevated Na^+ currents at 15 days post-infection (Fig. 4A). Further analysis of this finding demonstrated that this effect on ENaC activity was independent of vector administration and was also seen in mock-infected controls (Fig. 4A). Despite these changes in LLnL/Dox-induced ENaC activity, we saw no difference in transmembrane resistance among all groups (Fig. 4B), suggesting that the epithelium had remained intact throughout the experiment. Furthermore, morphologic analysis of 15-day LLnL/Dox-treated cultures revealed no obvious morphologic changes in epithelial integrity compared to control untreated cultures (Fig. 5). These findings support the notion that down-regulation of ENaC in these studies was independent of CFTR correction and predominantly mediated by the proteasome modulating agents.

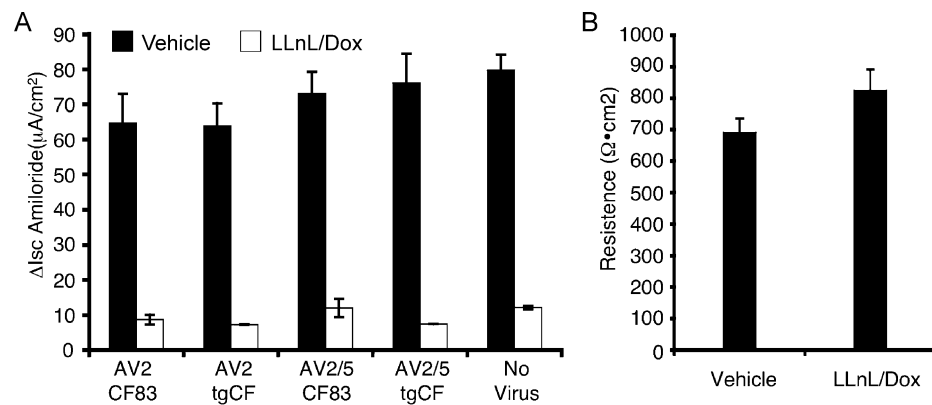


FIG. 4. Proteasome modulation inhibits the function of amiloride-sensitive sodium channels in polarized CF airway epithelia. (A) CF airway epithelia were infected with the indicated viral vectors in the presence or absence of applied LLnL/Dox at the time of infection. Results depict the mean \pm SEM ($N = 9$) of amiloride-sensitive sodium current in CF airway epithelial cells for each of the indicated treatments. Compared to results in Figs. 1 and 2A, the reduction in ENaC activity caused by LLnL/Dox treatment was significant in all groups ($P < 0.001$) and independent of the level of CFTR functional correction. (B) The transepithelial resistance was measured and compared between the vehicle and the LLnL/Dox groups prior to I_{sc} measurements. Results represent the mean \pm SEM ($N = 45$) resistance of combined vehicle-treated or LLnL/Dox-treated groups from (A).

Intrigued by the finding that a single treatment with LLnL/Dox could normalize ENaC currents in CF airway epithelia for 15 days, we hypothesized that the mechanism may involve down-regulation of certain ENaC subunits. To this end, we utilized quantitative TaqMan RT-PCR to determine the mRNA levels of α , β , and γ ENaC subunits in polarized CF airway epithelia following LLnL/Dox treatment (Fig. 6A). Results from this analysis demonstrated that the ratio of γ -ENaC subunit to β -actin

mRNA was most significantly decreased (16-fold) in the LLnL/Dox treatment group (0.014 ± 0.005 , $n = 9$) compared to the untreated control (0.222 ± 0.096 , $n = 12$). Similarly, the mRNA levels of α -ENaC and β -ENaC were also reduced by 2- and 3-fold, respectively, following treatment of proteasome inhibitors. These findings suggested that decreased transcription of predominantly the γ -ENaC subunits might be responsible for the observed inhibition of ENaC currents by LLnL and/or Dox.

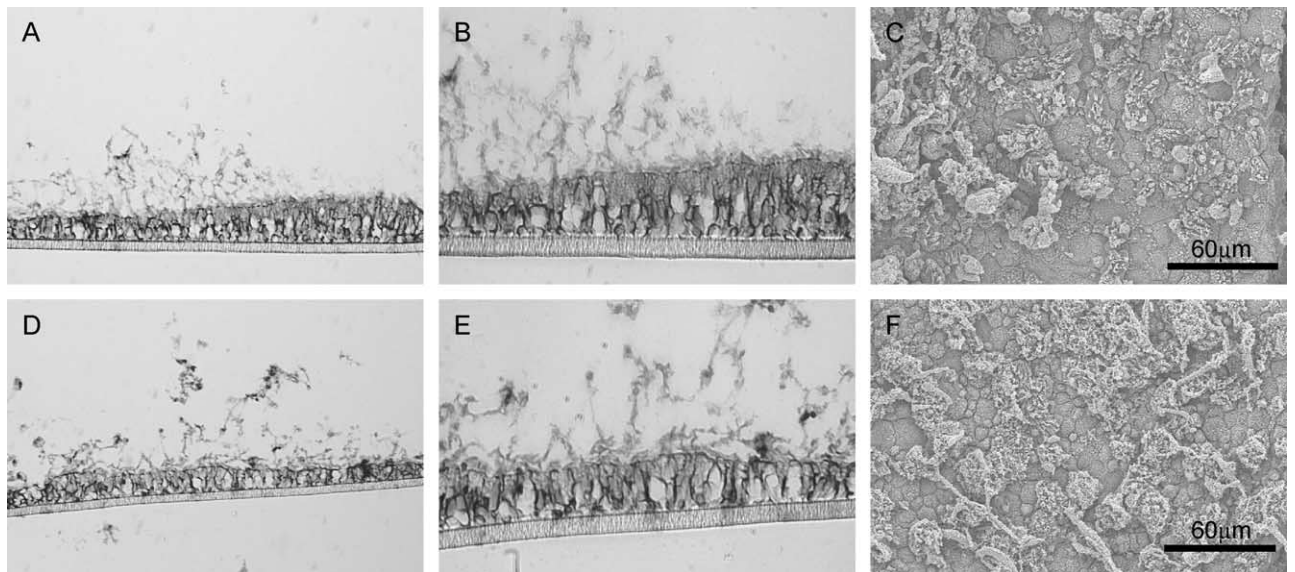


FIG. 5. Morphologic analysis of polarized CF airway epithelia following LLnL/Dox treatment. Polarized CF airway epithelia were treated with (A, B, C) vehicle or (D, E, F) LLnL/Dox for 16 h and morphologically evaluated at 15 days. Photomicrographs of H&E-stained cryosections (A, B, D, E) and scanning EM of the apical surface (C, F) are shown.

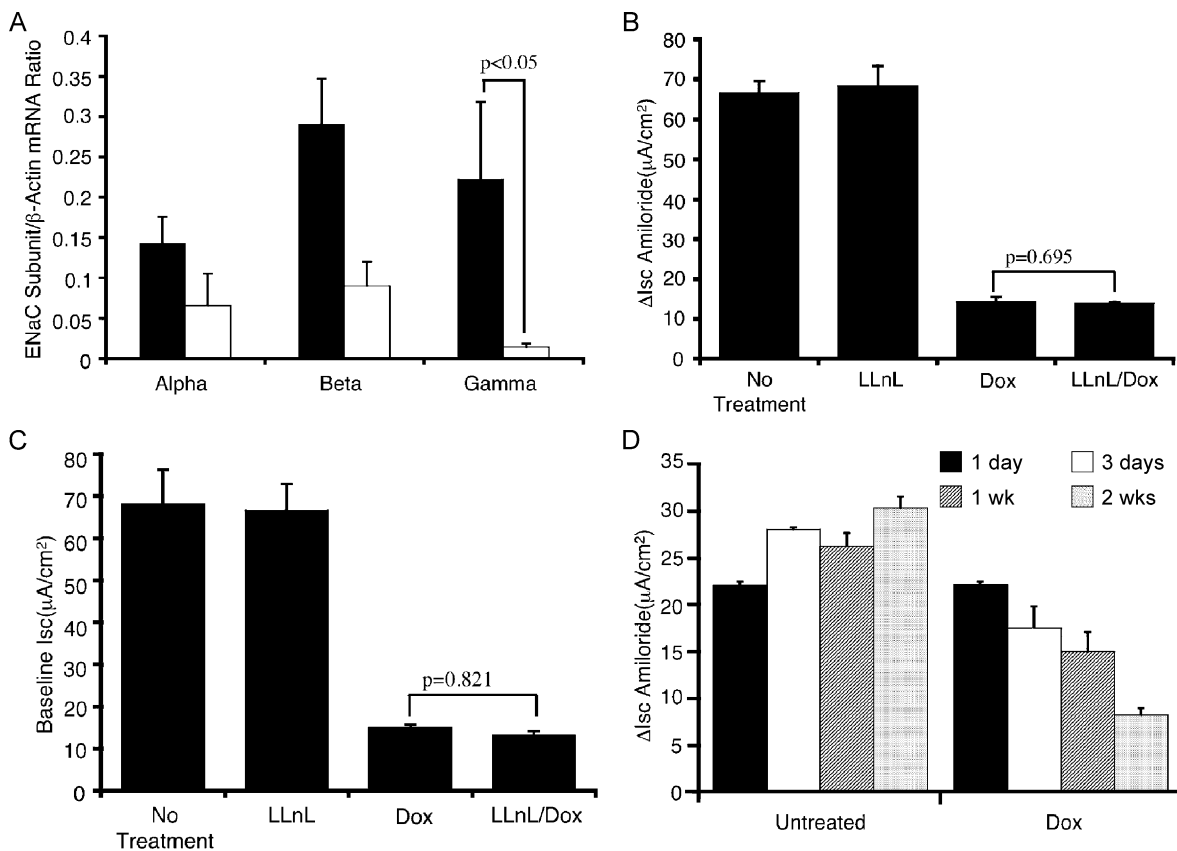


FIG. 6. Doxorubicin inhibits amiloride-sensitive sodium channels in polarized CF airway epithelia. (A) CF epithelia were treated with or without LLnL/Dox for 16 h, and total mRNA was prepared at 15 days posttreatment. The abundance of various ENaC subunit mRNAs was measured by quantitative RT-PCR, and the ratio of a given ENaC subunit to β -actin mRNA was calculated. The results represent the mean \pm SEM mRNA ratio for each ENaC subunit ($N = 12$ for the vehicle group (black bars); $N = 9$ for the proteasome-treated group (white bars)). (B) CF epithelia were treated with LLnL alone, doxorubicin alone, or LLnL/Dox for 16 h. The amiloride-sensitive I_{sc} were measured at 15 days posttreatment. Results depict the mean \pm SEM ($N = 3$) amiloride-sensitive I_{sc} for each group. (C) Comparison of the baseline I_{sc} for each group as shown in (B). (D) Kinetics of doxorubicin inhibition of the amiloride-sensitive sodium channel in CF epithelia. Amiloride-sensitive I_{sc} in polarized CuFi cells was measured at 1 day, 3 days, 1 week, and 2 weeks after treatment with doxorubicin and compared to untreated groups. Results depict the mean \pm SEM of amiloride-sensitive sodium current for each of the indicated treatments ($N = 3$ for each group).

To distinguish whether LLnL and/or Dox was acting to inhibit ENaC function in CF airway epithelia, we analyzed the effects of each compound individually. Results from this analysis demonstrated that Dox alone was responsible for changes in ENaC activity. Amiloride-sensitive I_{sc} was reduced equivalently by treatment with Dox or Dox/LLnL (Fig. 6B). Because ENaC I_{sc} is a major component of the baseline current in CF airway epithelia, the baseline currents were also similarly reduced by treatment with Dox or Dox/LLnL (Fig. 6C). Treatment with LLnL alone resulted in no significant change in either baseline or amiloride-sensitive I_{sc} (Figs. 6B and 6C). With the hypothesis that transcriptional inhibition of ENaC subunit genes was accounting for Dox-dependent decline in ENaC function, we next sought to evaluate the time course of changes in amiloride-sensitive I_{sc} seen in the presence of Dox. Because this

study required a relatively large number of CF samples, we utilized a recently reported transformed CuFi cell model to generate polarized air-liquid interphase CF epithelia [33]. This model has previously demonstrated elevated baseline amiloride-sensitive I_{sc} indicative of CF-associated elevated ENaC activity. Results from this analysis demonstrated a gradual decline in ENaC I_{sc} of Dox-treated CuFi epithelia over the course of 1 to 14 days that was not observed in control untreated samples (Fig. 6D).

Doxorubicin Treatment Increases γ -ENaC Promoter Methylation

Since it has been reported that doxorubicin treatment leads to CpG demethylation of the multidrug resistance gene (MDR-1) promoter and a consequent increase in MDR expression [41], we hypothesized that increased

Dox treatment in a fashion independent of CFTR genotype.

A second candidate CpG island located in exon 1 (-229 to +270 bp) of the γ -ENaC gene was also assessed for methylation in CuFi-1 cells following doxorubicin treatment (Fig. 7C). Southern blot analysis of genomic DNA digestion with *XbaI/MspI* or *XbaI/HpaII* was used to assess the methylation status of five *MspI/HpaII* sites inside this region, three of which localized to the CpG island. Results from this study revealed an ENaC gene hybridizing fragment of approx-

imately 800 bp that was protected from *HpaII* digestion following doxorubicin treatment compared with untreated and *MspI*-digested groups (Fig. 7C). These results suggest that all three *MspI/HpaII* sites inside this CpG island are likely methylated following doxorubicin treatment.

Doxorubicin Promotes Minimal Genome-wide CpG Methylation

The potential therapeutic utility of doxorubicin for rAAV gene therapy of CF will depend on the potential adverse effects of promoting genome-wide methylation. To assess the extent to which doxorubicin changes methylation of the genome, we performed differential methylation hybridization (DMH) assays to assess hypermethylated CpG islands in Dox-treated and untreated CuFi-1 cells. We used amplicons, representing differential pools of methylated DNA in treated cells relative to untreated control cells, as targets for microarray hybridization. Genomic fragments containing hypermethylated sites were protected from digestion with a methylation-sensitive enzyme and amplified by linker-PCR. By comparing the relative hybridization of Cy5-labeled (red), hypermethylated CpG island loci to those observed with Cy3-labeled (green) control amplicons, we could determine the relative extent of methylation changes at 8640 loci. CpG island loci appearing as “yellow” spots ($Cy5/Cy3 = 1$) represented no significant change in methylation. As controls for changes in methylation that occur at the genome level in tumor cells, we also included comparison between a human Daudi lymphoma cell line and normal human lymphocytes. Results from this analysis demonstrated a total of 33 loci with a greater than 1.5-fold change ($Cy5/Cy3$ ratio >1.5 or <0.6) in methylation status following doxorubicin treatment of CuFi-1 cells (Figs. 8A and 8B). This threshold ratio was used effectively to identify hypermethylated CpG islands in breast tumors in a previous study [37]. Using these same criteria, comparison of Daudi cells to normal human lymphocytes demonstrated that 194 loci had a >1.5 -fold change in methylation as a result of transformation. In summary, less than 0.4% of the total 8640 loci tested demonstrated a significant change in methylation following Dox treatment of CuFi-1 cells.

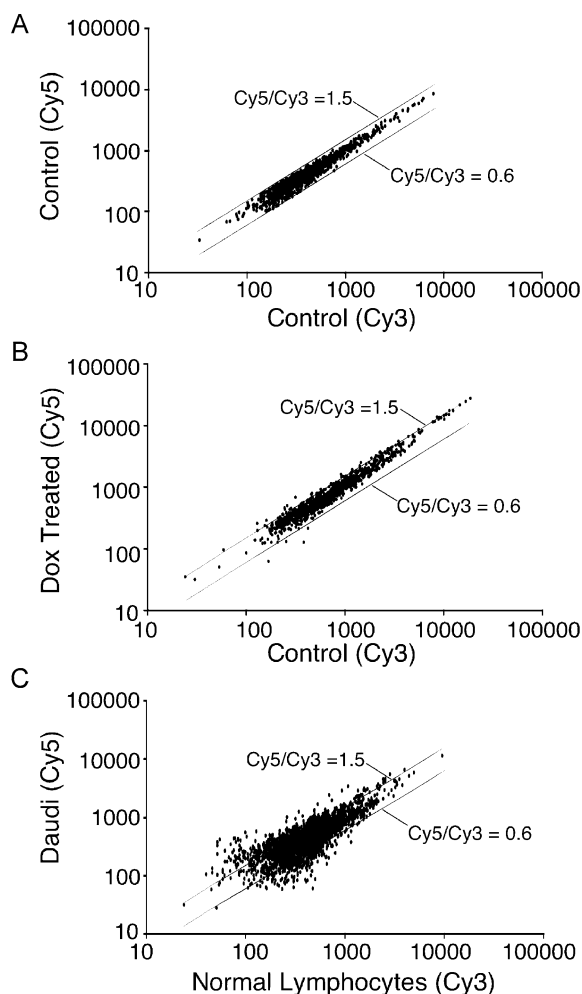


FIG. 8. Analysis of doxorubicin-induced changes in genome-wide methylation using methylation microarrays. (A) Self-hybridization control analysis using Cy3- and Cy5-labeled amplicons prepared from the control untreated CuFi-1 cells and hybridized to the CpG island microarray. (B) Comparative hybridization analysis using a Cy5-labeled Dox-treated amplicon and Cy3-labeled untreated amplicon derived from CuFi cells and hybridized to the CpG island microarrays. (C) Similar analysis was performed using Cy5- and Cy3-labeled amplicons from Daudi lymphoma cells and normal lymphocytes, respectively. $Cy5/Cy3$ ratio limits of 1.5 (increased methylation) and 0.6 (decreased methylation) are marked on each graph.

DISCUSSION

Current knowledge regarding rAAV transduction biology suggests that both receptor abundance and/or intracellular barriers that affect the movement of virus to the nucleus are significant determinants that influence efficiency of this vector for gene therapy in a given tissue target. Currently gene therapy trials for CF lung disease have demonstrated an impressive safety profile for rAAV2, but have failed to demonstrate CFTR transgene expression [17]. The source of this shortcoming

can be due to several aspects of rAAV biology and/or vector design. First, since the CFTR cDNA is very large, low-level expression in current trials may be due to low promoter activity of the ITR used to drive expression of the CFTR gene. Second, studies in mice have demonstrated that rAAV5 much more efficiently infects airway epithelia [20,29]. Hence, the choice of rAAV2 as a therapeutic platform for lung gene delivery may be suboptimal. Third, studies have also demonstrated that intracellular processes controlled by the ubiquitin/proteasome system significantly influence rAAV transduction with both type 2 and 5 serotypes [23,24]. This current study was designed to evaluate directly which of these three parameters has the greatest influence on the efficacy of current CF lung gene therapy efforts. As such, we compared the current clinically used CFTR rAAV2 vector with both rAAV2/5 pseudotyped virus and a new vector design that incorporates a minimal 83-bp promoter upstream of CFTR.

An important initial reference to current clinical trials was the assessment of CFTR delivery and expression in the absence of applied proteasome inhibitors. In this context, ITR promoter rAAV2- and rAAV2/5-based vectors gave no significant CFTR functional correction or mRNA expression, but were capable of delivering a significant number of viral genomes into airway epithelia that persisted for 15 days. For the AV2tgCF vector, this finding is reminiscent of past clinical trial data demonstrating efficient and persistent delivery of viral vector DNA but a lack of detectable transgene-derived mRNA [17]. Coadministration of proteasome modulating agents significantly improved the ability of both rAAV2 and rAAV2/5 vectors to correct CFTR chloride transport abnormalities. Importantly, this is the first demonstration of CFTR functional correction in polarized human CF airway epithelia. In contrast to findings in mouse lung [29], rAAV2 infection of human polarized airway epithelia gave significantly higher levels of CFTR functional correction and mRNA expression compared to rAAV2/5 vectors. Additionally, the addition of the CF83 minimal promoter demonstrated only marginal improvement in functional correction and/or CFTR mRNA expression.

It is interesting to note that differences in the level of functional correction between certain vector comparisons was not linear with changes in mRNA levels. For example, treatment of airway epithelia with LLnL/Dox for the AV2CF83-infected group enhanced mRNA abundance greater than 2 orders of magnitude. However, functional CFTR correction with AV2CF83 was enhanced only three- to fourfold by LLnL/Dox treatment. It is currently unclear if limits in the ability of *in vitro*-polarized airway epithelia to process functional CFTR are the cause of this discrepancy. Furthermore, the percentage of cells transduced and types of cell infected with each serotype may also influence the functional

complementation profile. Previously, it was demonstrated that expression of CFTR in 6–10% of cells in an intact polarized CF epithelia using a strong promoter and retroviral vector could achieve full function correction of chloride secretion [39]. This was possible due to lateral gap-junctional transfer of chloride between CFTR-expressing and mutant cells in the intact polarized epithelia. It is currently unclear if all cell types within polarized airway epithelia have the capacity to pass chloride through gap junctions. Certainly, such a complementation mechanism could also be affected by cell type-specific infection profiles of AAV2 and AAV5 serotypes. It is also unclear if proteasome inhibitor treatment may also affect gap junction permeability. The implications of these findings may be very important when considering mRNA as a surrogate endpoint for gene therapy trials. However, more *in situ* analysis of the types and percentages of cells infected with the various serotypes with and without proteasome inhibitors is needed to understand the current functional complementation profiles as they relate to transgene-derived mRNA expression.

One of the most surprising findings from the current analysis of proteasome modulating agents to enhance rAAV-mediated CFTR delivery to the CF airway was the observation that these same agents simultaneously normalized CF-associated ENaC hyperactivity through an independent mechanism. Such a finding suggests that proteasome modulating agents may have dual therapeutic utility as pharmacologic agents to treat primary pathology and enhance gene therapy for CF lung disease. The amiloride-sensitive ENaC controls sodium transport across many types of epithelia, including airway, kidney, and colon. In the CF airway, the loss of CFTR function results in excessive ENaC activity, which in turn has been hypothesized to dehydrate the airway surface and diminish cell surface clearance in the airway [43], leading to severe recurrent airway infections in CF patients [1,9]. In the current study, the combined administration of LLnL and Dox inhibited the enhanced ENaC activity seen in CF epithelial in a manner that was independent of CFTR complementation. This effect on ENaC activity appears to be predominantly due to altered gene transcription of the γ -ENaC promoter leading to reduced levels of γ -ENaC mRNA.

ENaC activity can be regulated by altering the open probability of the channel, the conductance of the channel, or the number of functional ENaC molecules on the cell surface. Previous studies have demonstrated a link between the ubiquitin–proteasome proteolytic system and regulation of ENaC turnover at the cell surface. ENaC consists of three subunits (α , β , γ). Each has a proline-rich region (PPXY) at its C-terminal end. The ubiquitin E3 ligase, Nedd4, interacts with ENaC through this PPXY region and adds ubiquitin chains to ENaC subunits. Ubiquitin addition to ENaC serves as signal for

the degradation of ENaC by the proteasome. Mutation of a group of lysine residues at the N-terminus of the α and γ subunits leads to inhibition of ubiquitination and increased ENaC activity [44]. Additionally, inhibition of proteasome activity by carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) has been shown to increase the level of ENaC subunits at the membrane and ENaC activity [45]. Taken together, all previous descriptions suggest that inhibition of the ubiquitin–proteasome system would result in increased ENaC activity. These findings are in stark contrast to our observations that doxorubicin inhibits long-term ENaC activity through increases in γ -ENaC promoter CpG methylation and potentially also other ENaC subunit promoters. Furthermore, the prolonged effects of a single administration of LLnL/Dox that lasts for 15 days support an inhibitory mechanism involving transcriptional regulation that is likely different from the short-term regulation of ENaC by Nedd4 ubiquitin ligase. Interestingly, doxorubicin has been shown to alter methylation of the MDR-1 promoter and increase transcriptional activity of MDR-1 [36,41,46]. Although the net effects of doxorubicin on MDR-1 and γ -ENaC promoter methylation are opposite, the process controlling changes in CpG methylation may be similarly regulated.

Although doxorubicin-induced changes in methylation appear to affect transcriptional activity of the γ -ENaC gene significantly, doxorubicin treatment of CuFi airway cells resulted in fewer genome-wide changes in methylation as deduced by DMH in comparison to tumorigenic changes that occur in Daudi lymphoma cells. Additionally, Dox-induced changes in methylation occurred within a range of ratios between 1.5 and 0.6 and were much less in magnitude than those seen in Daudi cells (0.1 to 7.6). However, this somewhat surprising result must be interpreted with caution. Since the arrays used for analysis did not have internal control MDR-1 and γ -ENaC gene segments known to undergo changes in methylation following doxorubicin treatment, it is currently difficult to compare the extent of change in methylation between the DMH assay and Southern/PCR assays. Therefore, it is possible that the DMH assay used has inadequate sensitivity to detect the type of changes in methylation induced by doxorubicin that lead to functional changes in transcription. Nonetheless, the level of change in genome methylation that occurs following tumor cell transformation is far greater than that seen following doxorubicin treatment of airway epithelia. Further investigation of genome-wide transcriptional effects of doxorubicin treatment caused by changes in methylation will certainly be an important issue for future clinical applications in CF.

The finding that proteasome modulating agents alter baseline ENaC activity in CF airway epithelia may have practical therapeutic applications outside their combined ability to also enhance rAAV transduction. Given the fact

that ENaC hyperactivity is thought to dehydrate the surface airway fluid layer in CF patients and decrease airway clearance of bacteria, inhaled proteasome modulating agents that inhibit ENaC activity could be applied as aerosolized compounds to the lungs of these patients to enhance airway clearance. Although previous attempts to inhibit ENaC using aerosolized amiloride (a direct channel blocker of ENaC) have shown little functional benefit in CF clinical trials [13,14], it is possible that such earlier approaches failed due to the short half-life of the inhibitory compound. Since doxorubicin demonstrates a much longer inhibitory effect on ENaC activity, its efficacy may be significantly improved. Doxorubicin is currently used clinically as an anti-cancer chemotherapeutic agent. However, the potential application in CF gene therapy to the lung requires an in-depth look at several safety concerns, most notably the fact that doxorubicin accumulates in cardiac muscle and leads to cardiac toxicity over time. Although liposome formulations of doxorubicin have been shown to reduce significantly cardiac toxicity, very little is known about the pharmacokinetics of doxorubicin or its lipid formulation following lung delivery.

In summary, our findings suggest that the current clinically used rAAV2 CFTR vector may possess substantial therapeutic utility for gene therapy of CF lung disease if proteasome modulating agents can be simultaneously administered at the time of infection. The dual therapeutic utility of pharmaco-gene therapy agents to treat primary pathophysiologic defects while simultaneously enhancing the efficacy of gene therapy represents a new area for drug development.

METHODS

Primary cultures of human CF bronchial epithelia and rAAV infection.

Airway epithelial cells isolated from bronchial tissue obtained from CF or non-CF patients were seeded onto collagen-coated, semipermeable membranes (0.6-cm² Millicel-HA; Millipore, Bedford, MA, USA). All CF samples used in these studies were homozygous $\Delta F508/\Delta F508$ genotypes. Each tissue sample was evaluated in a separate experiment, and data generated from at least three independent tissue donors were combined for analysis. Methods to generate these air–liquid interface cultures and the medium used were as previously described [30]. Four viral vectors, AV2CF83, AV2tgCF, AV2/5CF83, and AV2/5tgCF, were used in the present studies to infect polarized airway epithelial cells from the apical membrane. All vectors harbored AAV2 ITRs and were packaged into either type 2 or type 5 capsids using a triple plasmid transfection technique and purified by ion-exchange chromatography as previously described [31]. AV2tgCF is the current clinically used AAV2-based full-length CFTR vector in which expression of CFTR is driven off the ITR [16,18]. AV2/5tgCF virus has the same proviral structure as AV2tgCF, but is pseudotyped into AAV5 capsid. AV2CF83 and AV2/5CF83 viruses have an additional 83-bp minimal promoter [32] inserted into the AV2tgCF proviral genome to increase gene expression and are packaged into AAV2 and AAV5 capsids, respectively. The airway epithelial cultures were infected with 75 μ l of virus-containing medium applied to the apical surface of the epithelia at an m.o.i. of 10^4 particles/cell in the presence or absence of 40 μ M LLnL (Boston Biochem, Inc., Cambridge, MA, USA) and 5 μ M doxorubicin (Calbiochem, San Diego, CA, USA). The cells were

incubated at 37°C for 16 h before apically loaded virus was removed and the epithelia were returned to 0.6 ml of basolateral medium and air at the apical interface in the absence of proteasome modulating agents. Cultures were then incubated for an additional 15 days (changing basal medium every 2 days) prior to electrophysiologic and molecular studies. Transepithelial resistance was monitored prior to viral infection and after the 15-day postinfection incubation to confirm epithelial integrity. A transepithelial resistance of >500 ohms was used to indicate that the electrical integrity of the epithelium had not deteriorated over the course of the experiment. In addition to the above primary CF airway model system, polarized CF airway epithelia were similarly generated and infected using the transformed CuFi cell line as previously described [33,34].

Short-circuit current measurement in polarized airway epithelia. Transepithelial short-circuit currents were measured using an epithelial voltage clamp and a self-contained Ussing chamber as previously described [30]. The basolateral side of the chamber was filled with Ringer's buffer solution containing 135 mM NaCl, 1.2 mM CaCl₂, 2.4 mM KH₂PO₄, 0.2 mM K₂HPO₄, 1.2 mM MgCl₂, and 5 mM Hepes, pH 7.4. The apical side of the chamber was filled with a low-chloride Ringer's containing 135 mM sodium gluconate, 1.2 mM CaCl₂, 2.4 mM KH₂PO₄, 0.2 mM K₂HPO₄, 1.2 mM MgCl₂, and 5 mM Hepes, pH 7.4. During the experiment, the chamber was maintained at 37°C and aerated with 100% O₂. Transepithelial voltage was clamped at zero, and the resulting *I*_{sc} was measured and recorded following the sequential addition of the following channel antagonist and agonists: (1) 100 μM amiloride (apical), (2) 100 μM DIDS (apical), (3) 100 μM IBMX/10 μM forskolin (apical), and (4) 100 μM bumetanide (basolateral). Voltage was referenced to the apical compartment. The series resistance of the Ringer's solution and transwell membrane was electrically compensated before starting the experiments. All chemical agonists and antagonists were added to either the apical or the basolateral side of the monolayer by direct injection and mixed by aerating the Ringer's. After experiments, membranes were harvested and stored at -80°C for future molecular studies.

RNA processing and RNA-specific PCR. Transgene-derived recombinant CFTR mRNA and endogenous CFTR mRNA were quantified using an RS-PCR method recently described and currently used in the Targeted Genetics, Inc., clinical trials for CF [35]. Total RNA was isolated from cells growing on Millicel-HA membranes using the RNeasy column purification method (Qiagen, Valencia, CA, USA). Specifically, 350 μl of RNeasy lysis buffer (RLT + β-mercaptoethanol) was added directly to harvested membranes in a microfuge tube and samples were vortexed for 15 s. The lysate was then removed and passed through a Qias shredder (Qiagen) after which the standard minicolumn protocol was followed and RNA was eluted in 30 μl of 10 mM Tris, pH 8, 1 mM EDTA. Quantitation of RNA was performed by absorbance at 260 nm. The RS-PCR was performed as previously described [35] and all cDNA samples were tested in duplicate for rAAV transgene-derived CFTR, endogenous CFTR, and GUS. Both rAAV and endogenous CFTR expression was normalized to the GUS endogenous control. This method allows direct relative comparison of the level of rAAV CFTR expression to endogenous CFTR expression [35].

DNA processing and real-time DNA PCR for viral genomic DNA. Cellular DNA was recovered by ethanol precipitation from a pool consisting of the RNeasy column load flowthrough fraction and the first column rinse (from RNA processing). This allowed for a direct comparison of vector DNA and RNA for a given sample. The recovered DNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated in 2.5 vol of ethanol, and quantitated by absorbance at 260 nm. Seventeen of the 90 DNA isolations were chosen at random and screened for matrix inhibition by evaluating DNA spike recovery; there was no evidence of matrix inhibition (data not shown). All test samples were analyzed in a real-time quantitative TaqMan PCR assay targeting AAV-CFTR (vector-specific) sequences. Each 20-μl reaction contained 200 ng of genomic DNA and was run in triplicate in a 384-well format using an ABI Model 7900

sequence detection system (Applied Biosystems, Foster City, CA, USA). Standards consisted of the plasmid pTgAAVCF (containing the AAV-CFTR sequence) diluted into a background of normal human lung DNA (Clontech/BD Biosciences, Palo Alto, CA, USA) and ranged from 8 × 10⁶ to 8 × 10¹ copies per PCR. AAV-CFTR-specific PCR primers and TaqMan probe were as follows: forward 5'-TGCTGCTCTGAAAGAGGAGAC-3', reverse 5'-GATCGATGCATCTGAGCTCTTTAT-3', probe 5'-(FAM)-TGCTGCTCTCTAAAGCCTTGATCTTGACC-(TAMRA)-3'.

Quantitative RT-PCR for different ENaC subunits. Following CFTR mRNA quantification by RS-PCR, total RNA samples were used to generate cDNA (Invitrogen). The following primers and probe sequences were used for TaqMan PCR quantification of ENaC subunits: α-ENaC subunit, forward 5'-CCTCAACTCGGACAAGCTCG-3', reverse 5'-GAGAGTGGTGAAGGAGCTGATTTG-3', probe 5'-(FAM)-ACCCTCAATCCCTACAGGTACCAGGAAAT-(TAMRA)-3'; β-ENaC subunit, forward 5'-GGAACCACACACCCCTGG-3', reverse 5'-CAAAGAGATCAAGGACCATGGG-3', probe 5'-(FAM)-CCTTATTGATGAACGGAACCCACC-(TAMRA)-3'; γ-ENaC subunit, forward 5'-GCTGGATTTCTGCAGTCAC-3', reverse 5'-CAGGGCCTCTCTGGTCTCTCT-3', probe 5'-(FAM)-AACATCAACCCCTACAAGTACAGCACCGTTC-(TAMRA)-3'. Copies of ENaC subunit mRNA were normalized to the number of β-actin mRNA copies in each sample using commercially available primer sets (Applied Biosystems).

Analysis of γ-ENaC promoter CpG methylation. The methylation status of a CpG island beginning at approximately -1.8 kb of the γ-ENaC promoter was analyzed using a previously described PCR method [36]. Briefly, polarized CF airway epithelia were treated with or without 5 μM doxorubicin for 16 h, followed by continued growth for 1 to 3 days in the absence of doxorubicin. Cells were harvested at various time points and genomic DNA was isolated and digested overnight with *Mbo*I, *Mbo*I/*Msp*I, or *Mbo*I/*Hpa*II. PCRs were then performed using primers that flank the *Msp*I/*Hpa*II sites in this region. The relative positions of the CpG island, restriction sites, and primers are shown in Fig. 7A. Primers used were forward 5'-TTGGAACCGAAAGGTGAGTT-3', reverse 5'-TGAAACGGCGCTGGGCGGAG-3'. The methylation status of a second CpG island close to exon 1 was analyzed by Southern blotting of DNA from early passage transformed CF airway CuFi-1 cells following treatment with or without 5 μM doxorubicin for 16 h and continued growth in culture for 1 to 3 days. Genomic DNA from ~10⁷ CuFi-1 cells was then isolated and digested overnight with *Xba*I, *Xba*I/*Msp*I, or *Xba*I/*Hpa*II. Southern blotting of samples from a 1% agarose gel was then performed using an ENaC gene probe (-631 to +401 bp) internal to the *Xba*I sites (-682 and +438 bp) that flank the exon 1 region (Fig. 7A). The probe was prepared by a nested PCR amplification using the following primer sequences: first-round primers, forward 5'-GAGCTTGACTGTAAGAGAAGAAAC-3', reverse 5'-CAC-ATTTGTAGGCTGGAATCGACC-3'; second-round primers, forward 5'-TTTCACCTATAATAAGCCTCAGAG-3', reverse 5'-GCCAGTGTGTCAC-TTTCGGGAG-3'.

DMH assay. Methylation amplicons were prepared essentially as previously described [37]. Briefly, genomic DNA (1–2 μg) from doxorubicin-treated and control CuFi cells was digested with *Mse*I (a 4-bp TTAA cutter that confines the bulk of DNA to <2000-bp fragments but retains GC-rich fragments). The 3' overhangs of the digest fragments were used to ligate PCR linkers H-24/H-12 (5'-AGGCAACTGTGCTATCCGAGGGAT-3' and 5'-TAATCCCTCGGA-3'). The samples were further digested with the methylation-sensitive endonucleases *Hpa*II and *Bst*UI. PCR was then performed to amplify preferentially the methylated GC-rich fragments or fragments containing no internal *Hpa*II or *Bst*UI sites using the flanking linker H24 as a primer [37]. After PCR, the control amplicon was labeled with Cy3 (green) fluorescence dye, whereas the doxorubicin-treated amplicon was labeled with Cy5 (red). The labeled samples were cohybridized to a panel of 8640 short CpG island tags arrayed on Corning UltraGap slides. Hybridization and posthybridization wash were conducted using a Pronto Universal hybridization kit as suggested by the manufacturer (Corning). Signal intensities of hybridized spots were analyzed with the GenePix 5.1 software program (Axon). Because Cy5 and Cy3 labeling efficiencies varied among

samples, we determined a global normalization factor for each microarray image, as described previously [37]. The adjusted Cy5/Cy3 ratio for each CpG island locus was then calculated, and data were exported into a spreadsheet for analysis. For quality control, we also conducted a self-hybridization study in which two equal portions of a control DNA sample were labeled with Cy5 and Cy3, respectively, and then cohybridized to the microarray slide.

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