ORIGINAL RESEARCH

A Novel AAV-mediated Gene Delivery System Corrects CFTR Function in Pigs

Ashley L. Cooney[1,2,3], Ian M. Thornell[2,3,4], Brajesh K. Singh[1,2,3], Viral S. Shah[2,3,4], David A. Stoltz[2,3,4], Paul B. McCray, Jr.[1,2,3], Joseph Zabner[2,3,4], and Patrick L. Sinn[1,2,3]

[1]Stead Family Department of Pediatrics, [2]Pappajohn Biomedical Institute, [3]Center for Gene Therapy, and [4]Department of Internal Medicine, University of Iowa, Iowa City, Iowa

ORCID ID: 0000-0003-2717-9138 (A.L.C.).

Abstract

Cystic fibrosis is an autosomal-recessive disease that is caused by a mutant *CFTR* (cystic fibrosis transmembrane conductance regulator) gene and is characterized by chronic bacterial lung infections and inflammation. Complementation with functional *CFTR* normalizes anion transport across the airway surface. Adeno-associated virus (AAV) is a useful vector for gene therapy because of its low immunogenicity and ability to persist for months to years. However, because its episomal expression may decrease after cell division, readministration of the AAV vector may be required. To overcome this, we designed an integrating AAV-based CFTR-expressing vector, termed *piggyBac* (PB)/AAV, carrying CFTR flanked by the terminal repeats of the *piggyBac* transposon. With codelivery of the *piggyBac* transposase, PB/AAV can integrate into the host genome. Because of the packaging constraints of AAV, careful consideration was required to ensure that the vector would package and express its CFTR cDNA cargo. In this short-term study, PB/AAV-CFTR was aerosolized to the airways of CF pigs in the absence of the transposase. Two weeks later, transepithelial Cl^- current was restored in freshly excised tracheal and bronchial tissue. Additionally, we observed an increase in tracheal airway surface liquid pH and bacterial killing in comparison with untreated CF pigs. Airway surface liquid from primary airway cells cultured from treated CF pigs exhibited increased pH correlating with decreased viscosity. Together, these results show that complementing *CFTR* in CF pigs with PB/AAV rescues the anion transport defect in a large-animal CF model. Delivery of this integrating viral vector system to airway progenitor cells could lead to persistent, life-long expression *in vivo*.

Keywords: adeno-associated virus; piggyBac transposon; CFTR; cystic fibrosis; gene therapy

Clinical Relevance

Large-animal disease models are critical tools for evaluating advances in viral vector–based delivery tools. Here, we designed a vector with the potential for lifelong expression of a therapeutic transgene and performed a short-term study that resulted in *in vivo* phenotypic correction of a cystic fibrosis pig. This study has widespread implications for long-term gene therapy–based corrections of monogenetic diseases.

Cystic fibrosis (CF) is an autosomal-recessive genetic disease that affects multiple organ systems; however, the leading cause of morbidity and mortality in patients with CF is chronic lung disease. CF is caused by a mutant *CFTR* (cystic fibrosis transmembrane conductance regulator) gene that leads to loss of a functional anion channel (1, 2). Without CFTR, viscous mucus accumulates on the airway surface and becomes a milieu for bacterial adherence (3–6). Bacterial infections and mucus accumulation lead to plugged airways and ultimately organ failure (7, 8). There are hundreds of potential

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Correspondence and requests for reprints should be addressed to Patrick L. Sinn, Ph.D., Stead Family Department of Pediatrics, 169 Newton Road, 6318 PBDB, University of Iowa, Iowa City, IA 52242. E-mail: patrick-sinn@uiowa.edu.

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disease-causing *CFTR* mutations (www.genet.sickkids.on.ca). Although small-molecule therapeutics provide relief for many people with CF (9–11), there is a need to treat all *CFTR* mutations. Ideally, a single-dose reagent would be delivered early in life to prevent the onset and progression of lung disease.

Newborn CF pigs are free from infection and inflammation at birth, but develop lung disease weeks to months after birth (12). In the absence of CFTR, the airway surface liquid pH (pH_{ASL}) is relatively acidic, and respiratory secretions exhibit a reduced bacteria-killing ability compared with that observed in non-CF pigs (13). We previously reported that complementing *CFTR* in CF pig airways using an adeno-associated virus (AAV) vector increased anion channel activity, increased pH_{ASL}, and improved bacteria-killing ability (14). In addition, AAV transduced ciliated and nonciliated cells in pig airways, and expressed CFTR at the apical surface (14). Efficient AAV-mediated gene therapy can potentially correct the CF airway defect and prevent lung infections.

AAV for CF gene therapy has been evaluated in five phase I and II CF clinical trials. Aerosolized AAV2 was tested in over 100 trial participants and determined to be safe, but no significant improvements in lung function were reported (15, 16). These studies relied on the weak inverted terminal repeat (ITR) promoter to drive expression from the 4.5-kb *CFTR* gene. However, efforts to move forward with AAV faced three limitations: a low transduction efficiency at the airway surface, a small packaging capacity, and proteasomal degradation of input virions. Directed-evolution strategies are providing new AAV capsids with enhanced tropism for airway epithelial cells, including the H22 capsid for pig airways and 2.5T for human airways (14, 17). The generation of a shortened *CFTR* cDNA by removing a portion of the R-domain (CFTRΔR) (18) and the development of a strong synthetic promoter and polyadenylation signal (19) fit within the packaging capacity of AAV. Delivery of AAV in the presence of doxorubicin increases AAV expression through proteasome inhibition (20, 21). Increasing transgene expression from an AAV vector through these modifications could help enable future AAV-based clinical trials.

AAV-mediated transgene expression persists for months in the lung (22). However, preclinical studies with AAV in the rabbit lung suggest that its transgene expression will wane and readministration may ultimately be required (23). If the goal is to express CFTR for the lifetime of an individual with CF after a single dose of a gene therapy reagent, genomic integration is likely required. AAV episomal expression only persists for the life of the cell (24), but an integrating vector transducing a progenitor cell population could lead to continuous repopulation of $CFTR^{+}$ cells at the airway surface. The nonviral *piggyBac* DNA transposon is a two-part system composed of TRs flanking a gene of interest and a transposase that catalyzes integration of the gene of interest into the host genome (25). Previously, we reported that *piggyBac* (PB)/AAV with full-length TRs conferred persistent transgene expression in immunocompetent mouse airways *in vivo* (26).

In this study, we used a *piggyBac* with shortened TRs (27) to accommodate the size constraints of AAV and to create an integrating PB/AAV vector system for gene delivery. Between the minimal TRs lies a strong synthetic promoter, shortened *CFTRΔR*, and synthetic polyA. Using the gut-corrected CF pig model (28), we delivered *CFTR* using PB/AAV pseudotyped with an H22 capsid and observed a phenotypic correction. Thus, we confirm that the addition of the minimal TRs results in a functional vector for delivery to cells *in vitro*, as well as a phenotypic correction of a large-animal CF model *in vivo*.

Methods

Constructs

Single-stranded recombinant AAV (ssAAV) carrying the *piggyBac* transposon with minimal TRs carrying an F5Tg83 promoter driving puromycin was designed *in silico* and synthesized by Genscript. Human CFTRΔR cDNA was a kind gift from Lynda Ostedgaard (18) and was cloned into the PB/AAV vector with minimal TRs with the restriction enzymes SpeI and SalI. The adenovirus expressing *piggyBac* transposase ($Ad^{transposase}$) was previously described (26, 29). In this study, we used a *piggyBac* transposase (iPB7) that includes seven mutations to make it hyperactive (30). ssAAV and scAAV vectors with F5Tg83 driving transposase expression were cloned using SpeI and KpnI restriction enzyme sites. AAV vectors were generated using the AAV2 genome and pseudotyped with the AAV5 capsid for *in vitro* studies and the AAVH22 capsid for *in vivo* pig studies. Ad and AAV vectors were produced as a fee for service at the University of Iowa Viral Vector Core (https://medicine.uiowa.edu/vectorcore/).

Colony Formation Assay

HeLa cells were transduced in a 24-well plate (5×10^4 cells/well) with PB/ssAAV-F5Tg83-$Puro^R$ with or without Ad- or AAV-delivered transposase as indicated. AAV multiplicities of infection (MOIs) ranged from 10^3 to 10^5, and Ad^{GFP} or $Ad^{transposase}$ was delivered at an MOI of 10. Twenty-four hours after transduction, the cells were trypsinized and expanded into 100-mm plates in puromycin-containing media. The media was changed three times per week. Two weeks after expansion, the cells were fixed in 2% paraformaldehyde and stained with methylene blue, and colonies were counted.

CF Pigs

All animal procedures were reviewed and approved by the University of Iowa Institutional Animal Care and Use Committee in accordance with the U.S. Department of Agriculture and National Institutes of Health guidelines. CF pigs were generated by homologous recombination in fibroblasts as previously described (31). Gut-corrected pigs were created by somatic cell nuclear transfer cloning (28). All pigs were housed at the University of Iowa animal care facility for the duration of the study. The animals were humanely killed by intravenous administration of Euthasol (90 mg/kg).

In Vivo Viral Vector Administration

Newborn (<1 wk old) gut-corrected CF pigs were sedated with inhaled 2% isoflurane for viral delivery while oxygen levels, heart rates, and respiratory rates were monitored. Viral vector was aerosolized into the trachea by passing a Penn Century Microsprayer through a 2.0 endotracheal tube. Approximately 1×10^{12} vector genomes (vg) of $PB/AAV^{CFTR\Delta R}$ formulated with 200 μM doxorubicin (Sigma Aldrich) were delivered to each pig. Vector was delivered to the nasal turbinates by a bolus dose through a 34G Teflon catheter. Two weeks later, the pigs were analyzed for phenotypic correction.

Ussing Chamber Studies

CFTR anion channel correction was measured by Ussing chamber analysis. Freshly excised tissue or well-differentiated airway epithelial cultures were mounted in Ussing chambers, and apical and basolateral chambers were maintained under a symmetrical Ringer's solution (135 mM NaCl, 5 mM HEPES, 0.6 mM KH_2PO_4, 0.4 M K_2HPO_4, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 5 mM dextrose). Transepithelial current was measured as previously described (32). Baseline currents were measured and the following drugs were added to inhibit ion channels: amiloride (100 μM; Sigma Aldrich) to inhibit Na^+ channels, and 4,4′-dilsothiocyano-2,2′-stilbenedifulonic acid (Sigma Aldrich) to inhibit Cl^- (100 μM). After a low Cl^- gradient in the apical chamber was established, the cAMP agonists forskolin (10 μM) (Cayman Chemical) and 3-isobutyl-1-methylxanthine (IBMX, 100 μM; Sigma Aldrich) were added. After the current stabilized, GlyH-101 (GlyH) was added to block CFTR-mediated Cl^- current. Transepithelial currents (I_T) relative to baseline measurements are reported.

In Vivo Tracheal pH_{ASL} and Bacterial Killing

Pigs were anesthetized with ketamine (20 mg/kg) and xylazine (2 mg/kg), and sedation was maintained with propofol (1 mg/kg). After a tracheal window was opened, a pH-sensitive foil was placed on the tracheal surface as previously described (13, 14, 32, 33). Bacteria-killing assays were performed as previously described (13, 14, 32). Briefly, *Staphylococcus aureus* isolate SA43 was conjugated to electron microscopy grids via biotin and streptavidin interaction and placed on the airway surface of the sedated pigs for 1 minute. Live/dead bacteria were imaged by confocal microscopy after SYTO and propidium iodide staining (Invitrogen). Live/dead bacteria were quantified using ImageJ.

In Vitro pH_{ASL} Measurements

The pH_{ASL} was assayed using the ratiometric pH-sensitive dye SNARF-1 as previously described (34–36). Briefly, SNARF-1 conjugated to 70 kD dextran (Molecular Probes) was applied to the apical surface of the airway cultures and fluorescence was measured with a Zeiss LSM510 inverted confocal microscope. The SNARF-1 was excited with a 510-nm argon laser and emissions were collected at 565–597 and 619–661 through an acousto-optic tunable filter. Consistent with other studies (37–40), our empirical SNARF-1 pK_a of 7.39 was acid-shifted to the documented pK_a of ∼7.5 at room temperature. For each cell culture, images were acquired from at least five separate regions. Then each region was averaged for each culture, and each culture was averaged for each pig, which involved three replicates for each animal. Mean pig ratios were converted to pH values in GraphPad Prism by interpolating the calibration curve. Data are represented as mean values among porcine donors.

Viscosity and Dye Immobilization

As previously described (33), FITC-conjugated dextran was applied to the surface of airway cultures. Viscosity was measured by fluorescence recovery after photobleaching, specifically, the duration of time it took for the FITC-dextran to recover in the photobleached regions of the culture. In the instances where full recovery did not occur, the proportion of the net dye loss was presented as a percentage of immobilized dye.

Statistics

All statistically significant differences were calculated using one-way ANOVA in GraphPad Prism (GraphPad Software). Unless otherwise noted, all data are presented as mean ± SE. $P < 0.05$ was considered statistically significant.

Results

PB/AAV Transposition *In Vitro*

Our goal in these studies was to design an AAV vector expressing human CFTR that is flanked by the *piggyBac* TRs to allow for transposase-dependent genomic integration. We previously reported that AAV successfully delivered a *piggyBac* transposon expressing a reporter gene and integrated into the host cell genome in the presence of transposase (26). In those studies, the *piggyBac* transposon contained full-length TRs (5′ 308 bp and 3′ 237 bp = 545 bp total) and the cytomegalovirus promoter (520 bp). The packaging capacity of recombinant AAV2, including the prerequisite ITRs, is ∼4.9 kb (41). The full-length *piggyBac* TRs, cytomegalovirus promoter, and *CFTR* cDNA far exceed the AAV packaging capacity. To address the size constraints, we engineered an AAV2-based packaging construct with minimal *piggyBac* TRs (99 bp total) (27), a minimal F5Tg83 promoter (183 bp) (19), and a short synthetic polyadenylation signal (54 bp)

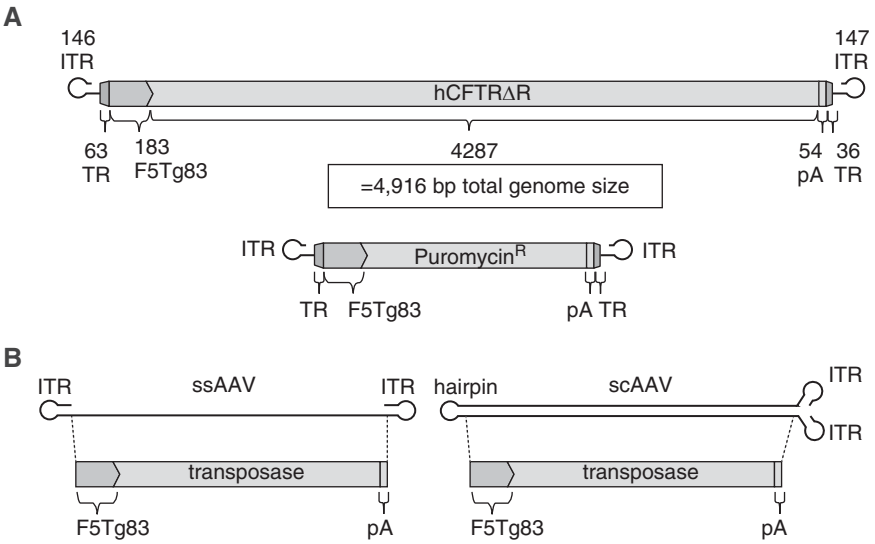

Figure 1. Schematics of hybrid vectors. (*A*) A PB/adeno-associated virus (AAV) transposon was delivered by a single-stranded AAV (ssAAV). The transposon terminal repeats (TRs) flank the F5Tg83 promoter, driving expression of human CFTRΔR or puromycin resistance, followed by a shortened synthetic polyadenylation signal (pA) (19). (*B*) The *piggyBac* transposase driven by the F5Tg83 promoter followed by the short polyA is represented schematically in either the ssAAV or self-complementary AAV (scAAV) vector. bp = base pairs; hCFTR = human cystic fibrosis transmembrance conductance regulator; ITR = inverted terminal repeat; PB = *piggyBac*.

(19) (Figure 1A). The vectors described in this study use the same promoter and polyA signal as previously described (14).

Using this packaging construct to express puromycin resistance (Figure 1A, bottom), we generated AAV with the H22 capsid, termed PB/AAV^{puro}. We first asked whether the minimal *piggyBac* TRs would confer functional transposition from the AAV genome into the cellular genome. We performed a colony formation assay as an indirect measure of transposition activity as previously described (26). Briefly, HeLa cells were cotransduced with PB/AAV^{puro} and adenovirus (Ad) expressing GFP (Ad^{GFP}) or Ad^{transposase}. Transduced cells were maintained in puromycin-containing media for 2 weeks. The cells were then fixed, stained with methylene blue, and counted. We observed colony formation in a dose-dependent manner only in the presence of the transposase (Figure 2A). These results confirm that minimal TRs and a short synthetic promoter will confer persistent drug resistance in a transposase-dependent manner. Without the transposase, the number of puromycin-resistant colonies ranged from 10 to 66, which is likely the result of low-level random integration of AAV genomes (42, 43).

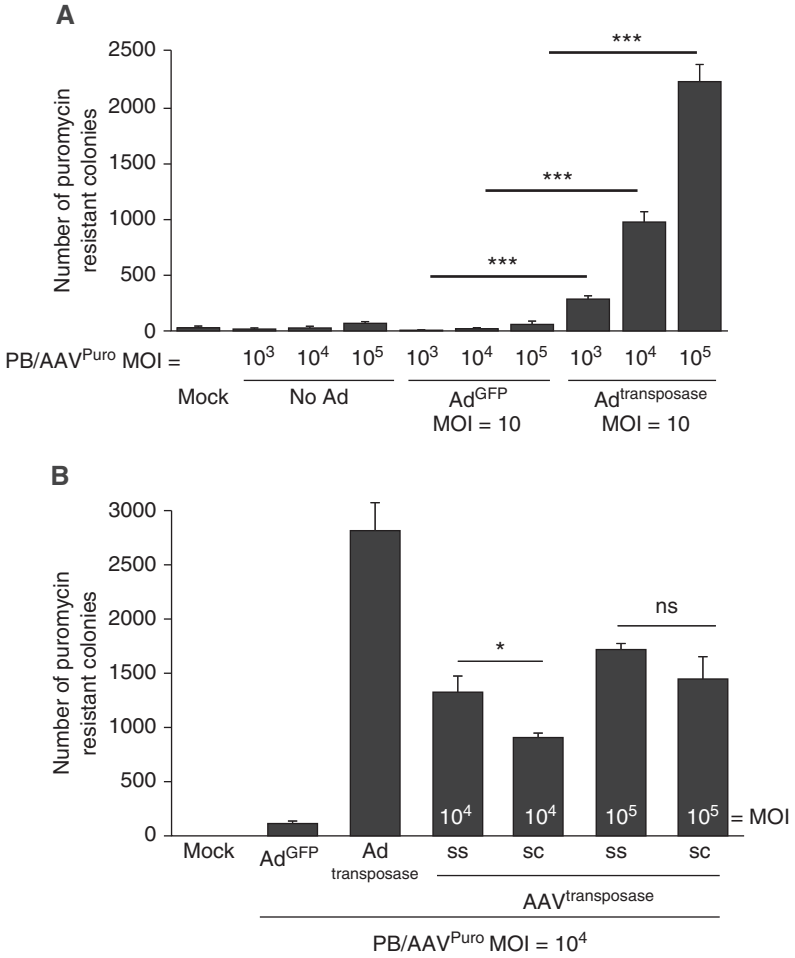

Figure 2. PB/AAV confers puromycin-resistant colonies only in the presence of the transposase. (*A*) HeLa cells were left untransduced or transduced with PB/AAV^{puro} at a multiplicity of infection (MOI) of 10^3, 10^4, or 10^5 in the absence of adenovirus (Ad), with Ad^{GFP} (MOI = 10), or with Ad^{transposase} (MOI = 10) for 4 hours. Twenty-four hours after transduction, cells were expanded from a 24-well plate into 100-mm tissue culture dishes and grown in puromycin-containing media for 2 weeks. HeLa cells were then fixed and stained with methylene blue, and colonies were counted. (*B*) ssAAV^{transposase} and scAAV^{transposase} were compared for their ability to confer puromycin-resistant colonies. HeLa cells were transduced as in *A* with the indicated viral vectors. $^*P < 0.05$ and $^{***}P < 0.005$; $n = 4$. ns = not significant.

Next, we compared the transposition activity of AAV^{transposase} with that of Ad^{transposase} (26). We evaluated both a self-complementary AAV (scAAV) and a single-stranded AAV (ssAAV) for transposase delivery (Figure 1B). We hypothesized that scAAV would increase transposition activity over ssAAV because scAAV bypasses second-strand synthesis and expresses earlier than a transgene delivered by ssAAV. Unexpectedly, the transposase delivered by ssAAV led to equal or greater transposition activity as compared with scAAV (Figure 2B). We conclude that the ssAAV^{transposase} confers dose-dependent transposase-mediated integration of the PB/AAV^{puro} transposon into the genome.

Phenotypic Correction in CF Pigs by PB/AAV$^{CFTR\Delta R}$

As shown in Figure 1A (top), we generated an integrating CFTR expression cassette to fit within the AAV packaging constraints. The minimal TRs of the *piggyBac* transposon flank an F5Tg83 promoter (19) driving CFTRΔR followed by a short polyA. In a short-term proof-of-principle study to validate the PB/AAV transposon *in vivo*, we aerosolized 1×10^{12} vg of PB/AAV$^{CFTR\Delta R}$ (without transposase) formulated with 250 μM doxorubicin into the trachea and lungs of newborn gut-corrected CF pigs. In addition, a bolus dose was delivered to the nasal epithelium of the same animals. Two weeks after delivery, we used a variety of metrics to measure the effects of CFTR complementation. Freshly excised tracheal and bronchial tissues were mounted in Ussing chambers to assess their bioelectric properties. The CF pigs that received PB/AAV$^{CFTR\Delta R}$ showed significantly greater changes in transepithelial current than the untreated CF pigs in response to the cAMP agonists forskolin and IBMX and the CFTR inhibitor GlyH (Figure 3A). We confirmed this in tracheal and bronchial tissue to ensure that various regions of the lung were transduced. To determine whether complementing CFTR in airway cells modified the tracheal pH_{ASL}, we used a pH optode to measure the pH_{ASL} of the tracheal surface. Tracheal pH_{ASL} levels in CF pigs treated with PB/AAV$^{CFTR\Delta R}$ were similar to those observed in non-CF pigs (Figure 3B). Furthermore, we observed a bacteria-killing ability in treated animals that

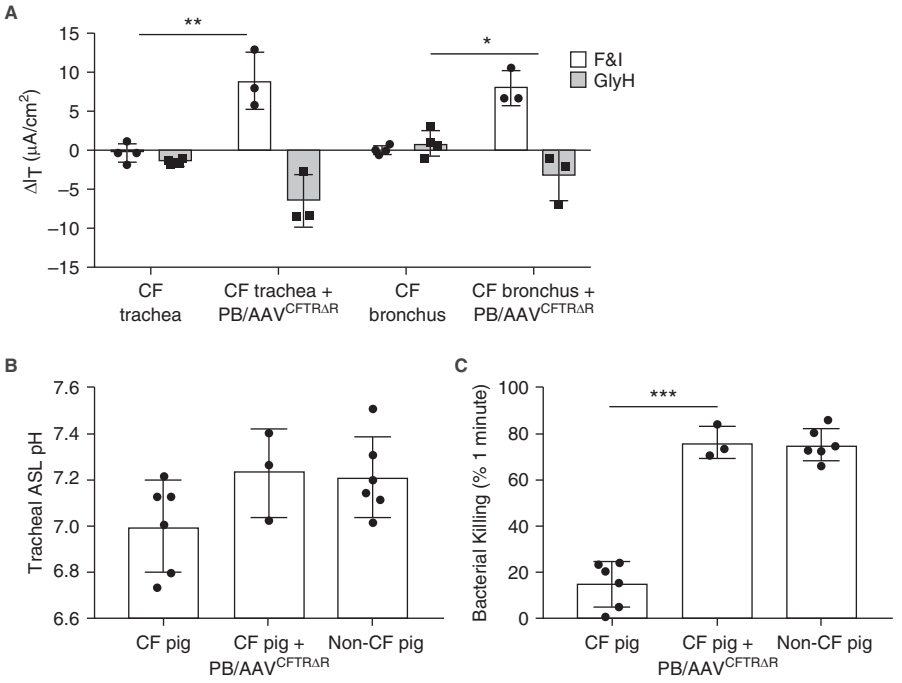

Figure 3. PB/AAV$^{CFTR\Delta R}$ corrects the anion channel defect *in vivo*. Three newborn cystic fibrosis (CF) pigs received ~1×10^{12} vg of H22 pseudotyped PB/AAV$^{CFTR\Delta R}$ via intratracheal aerosolization and nasal instillation. The AAV transposase was not included in this experiment. Two weeks after delivery, the pigs were assayed for phenotypic correction. (*A*) Freshly excised tracheal and bronchial tissues from untreated CF pigs or CF pigs treated with PB/AAV$^{CFTR\Delta R}$ were mounted in Ussing chambers to measure their bioelectric properties. Changes in transepithelial current were measured in response to forskolin and 3-isobutyl-1-methylxanthine (F&I) and GlyH-101 (GlyH). (*B*) Tracheal airway surface liquid (ASL) pH was obtained after the animal was sedated and a tracheal window was opened. A pH-sensitive foil was placed on the tracheal surface and pH was measured with a pH optode. (*C*) Bacterial killing was determined by placing a *Staphylococcus aureus*–coated electron microscopy grid on the tracheal surface for 1 minute. The grids were stained with a live/dead stain, imaged by confocal microscopy, and quantified using ImageJ. $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.005$; $n = 3$.

mirrored non-CF levels (Figure 3C). Using a previously described protocol (44), real-time qRT-PCR was used to amplify *CFTR* from bronchial tissue from both transduced pigs and naive controls. *CFTR* RNA levels in transduced animals were 2,880-fold higher than untransduced background levels. Based on the multiple metrics, we conclude that complementing CFTR by a PB/AAV vector corrects phenotypic defects in CF pigs.

pH_{ASL} and Viscosity in Primary Cells Cultured from Treated CF Pigs

We next examined pH_{ASL} and viscosity in primary cultures of airway cells derived from untreated or PB/AAV$^{CFTR\Delta R}$-treated CF pigs. These cells were cultured at an air–liquid interface and tested after differentiation (⩾2 wk). Consistent with the *in vivo* measurements, pH_{ASL} in the cultured airway cells from treated pigs was increased compared with that in untreated controls (Figure 4A). In addition to decreased pH_{ASL}, previous studies demonstrated that another consequence of reduced CFTR-dependent anion transport in CF is viscous mucus (45). We next assayed the ASL viscosity in the cultured airway cells from treated pigs by applying FITC-conjugated dextran to the apical surface of primary cultures, and quantified the fluorescence recovery after photobleaching (45). Here, we observed a reduced mucus viscosity in epithelia cultured from CF pigs that received PB/AAV$^{CFTR\Delta R}$ relative to cultures from untreated CF pigs (Figure 4B). In some cases, the fluorescent dye did not fully recover after photobleaching of CF cultures. As an additional measure of mucus viscosity, we quantified immobilization of the fluorescent dye FITC-dextran. After photobleaching, the fluorescent dye diffusion was measured over time and FITC expression after photobleaching stabilized within 30

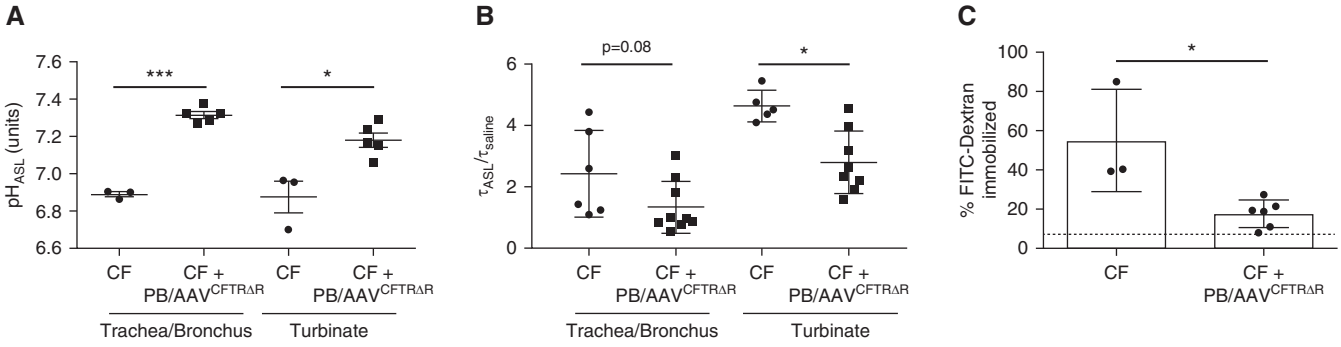

Figure 4. Primary airway epithelial cells cultured from PB/AAV$^{CFTR\Delta R}$-treated pigs exhibit increased ASL pH and decreased viscosity compared with those cultured from untreated CF pigs. Trachea/bronchus and turbinate primary airway epithelial cells were cultured from untreated CF pigs or CF pigs treated with PB/AAV$^{CFTR\Delta R}$. (*A*) Airway epithelial cultures were treated with SNARF-1 and ASL pH measurements were obtained by confocal microscopy. (*B*) Viscosity was measured by coating primary cells with FITC-conjugated dextran and measuring the tau of ASL and saline by fluorescence recovery after photobleaching. (*C*) The recovery of FITC-dextran fluorescent dye after photobleaching was quantified. Increased mucus viscosity in CF cultures prevents dye from refilling the photobleached area, and therefore the dye is immobilized in the mucus. The dashed line indicates saline background. $^{*}P < 0.05$ and $^{***}P < 0.005$; $n = 3$.

minutes. In CF cultures, the viscous mucus was partially immobilized after it was photobleached, whereas CF+AAV cultures of trachea/bronchus and turbinate recovered to near saline control levels (Figure 4C). Together, these data indicate that the PB/AAV$^{CFTR\Delta R}$ vector can rescue several CF phenotypic defects of epithelial cells cultured from trachea/bronchus and turbinates, including *1*) Cl^- current activity, *2*) pH_{ASL}, and *3*) ASL viscosity.

Discussion

A major limitation for using AAV as a CF gene therapy vector is its small carrying capacity. To overcome this limitation, we carefully considered the necessity of each nucleotide and designed an integrating PB/AAV vector carrying minimal TRs of *piggyBac*, a shortened CFTR, and a short promoter and polyadenylation signal. To validate the minimal *piggyBac* TRs in the context of a viral vector, we quantified transposition *in vitro*. In the absence of the transposase, PB/AAV behaved like a standard AAV vector; however, delivery of the transposase by a separate AAV vector led to transposase-mediated integration in a dose-dependent manner.

Using gut-corrected CF pigs, we aerosolized AAVH22 expressing CFTRΔR and rescued several phenotypic defects, similar to what was done in our previous studies (14, 19). However, the transposase was not included in this proof-of-principle experiment because the goal was to validate CFTR expression from the novel vector design, not to achieve genomic integration. We observed phenotypic correction by measuring changes in the transepithelial current, pH_{ASL}, and bacteria-killing ability. In addition, we assayed for pH_{ASL} and viscosity changes in primary airway epithelia harvested from CF pigs after PB/AAV$^{CFTR\Delta R}$ delivery. In this report, we *1*) validate that minimal PB TRs functionally confer integration from a nonintegrating viral vector *in vitro*, and *2*) demonstrate that PB/AAV$^{CFTR\Delta R}$ can correct the phenotypic defect of a CF pig model *in vivo*.

In our previously described persistence study in mice, PB/AAV-expressing luciferase lasted for 6 months (the duration of the experiment) in the presence of the transposase (26). Polidocanol treatment was used to accelerate cell turnover at a 3-month time point by denuding surface epithelial cells. Luciferase expression after the polidocanol treatment drastically declined in the absence of the transposase, but stabilized in the mice that received the transposase (26). These data suggest that a population of progenitor cells were transduced *in vivo*. These previous studies were performed in Balb/c immunocompetent mice. To our knowledge, no studies to date have specifically described the immunogenicity of the *piggyBac* transposase. Like any foreign protein, there is a potential for an immune response. However, considering that the *piggyBac* transposase is naturally endemic in moths, humans are unlikely to have preexisting immunity. Optimally, a gene therapy reagent would be delivered as a single dose, avoiding the need for repeated administration.

For our *in vitro* studies, we commonly use a standard MOI of 10,000. This requires a volume of 0.5 μl per well of a 24-well plate (1×10^{12} vg/ml transducing 50,000 cells/well). In our pig experiments *in vivo*, we delivered 1 ml of vector with a titer of 1×10^{12} vg/ml. The total number of airway epithelial cells in an 88-kg human adult has been estimated to be 1.05×10^{10} with 18× more alveolar cells than bronchial epithelial cells (46). If we extrapolate 2.4×10^{8} total airway epithelial cells for a 2-kg pig, we are delivering an MOI of 4,166 *in vivo*. However, this MOI assumes a homogeneous delivery pattern. Based in part on the 15-μm particle size delivered by the microsprayer, we suspect that most vector deposition occurs in the conducting airways. Deposition will gradually decrease as the alveoli are reached. Based on our estimates, we suspect that the achievable MOI *in vivo* is comparable to that obtained in our *in vitro* studies; however, local MOIs will vary greatly.

AAV is a promising gene therapy vector for multiple diseases, most notably neuromuscular and retinal diseases. For example, AAV vectors have been assessed in neuromuscular clinical trials involving spinal muscular atrophy type 1 (SMN); intramuscular delivery of limb-girdle muscular dystrophy, type 2D (LGMD2D) (47); and Becker muscular dystrophy (BMD) (48, 49). Encouragingly, LGMD2D and BMD delivered by AAV demonstrated persistent correction of muscle function for at least 6 months in trial participants. The U.S. Food and Drug Administration recently approved a gene therapy treatment for Leber congenital amaurosis 2 (LCA2) that uses AAV to deliver *RPE65*, with remarkable improvements in eyesight. *MERTK* (Mer proto-oncogene tyrosine kinase) and *REP1* (Rab-escort protein 1) are also in clinical trials for AAV-mediated gene replacement. These studies reported a safe vector delivery and gene correction, which led to improved vision in the participants (reviewed in Reference 50). AAV is a good choice of vector for these models because muscle cells have low turnover (51) and retinal cells are relatively immunoprivileged (52) and highly permissive to AAV (53, 54). It may be many years before the duration of expression from these studies is known.

We evaluated transposase delivery using Ad, ssAAV, and scAAV vectors. Although viral vectors are an efficient way to deliver the transposase, other delivery options may be considered. The transposase is required for the initial transposition into the host genome, but transient transposase expression may be preferred. Transposase delivery by mRNA is a promising way to improve transposition efficiency and the quality of integration (55, 56). Another way to deliver the transposase is to incorporate the transposase protein into integrase-deficient lentiviral particles (57). Short-term transposase expression could prevent the undesired effects of persistent transposase expression. Overall, there are several options for transient delivery of the transposase that confer persistent expression from an integrated transposon.

We provide evidence for the retention of dose-dependent transposition activity using minimal transposon TRs. We also show functional correction of CF defects in CF pigs after aerosolized delivery of a shortened CFTR by a PB/AAV vector as measured by the Cl^- current, tracheal pH_{ASL}, and bacterial killing. Future studies will compare the persistence of PB/AAV in the presence and absence of the transposase. We speculate that after airway epithelial cell turnover, a population of cells containing the integrated transposon will repopulate the airway epithelium and persistently express the gene of interest. The PB/AAV transposon system is a

promising vector system to efficiently and persistently complement CFTR defects in airway cells. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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