Directed evolution of adeno-associated virus to an infectious respiratory virus

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Respiratory viruses evolve to maintain infectivity levels that permit spread yet prevent host and virus extinction, resulting in surprisingly low infection rates. Respiratory viruses harnessed as gene therapy vectors have illustrated this limitation. We used directed evolution in an organotypic human airway model to generate a highly infectious adeno-associated virus. This virus mediated gene transfer more than 100-fold better than parental strains and corrected the cystic fibrosis epithelial Cl^- transport defect. Thus, under appropriate selective pressures, viruses can evolve to be more infectious than observed in nature, a finding that holds significant implications for designing vectors for gene therapy and for understanding emerging pathogens.

The complexity of evolutionary forces that drive the adaptation of pathogens to host systems has become progressively more evident as human, agriculture, and wildlife systems have come into increasingly close proximity, resulting in emerging infectious diseases. Several theories have been developed to define relevant interactions and consequences (1, 2). The classic theory behind the evolution of viral transmission is summed up in the basic reproductive number R_0: the number of secondary infections resulting from one infected host in a naïve host population. R_0 depends on a delicate balance of viral transmission and virulence: increased R_0 due to enhanced transmission or infectivity (the chance a susceptible host will be infected) is counteracted by greater virulence which reduces R_0 by limiting the infectious period and opportunity for transmission. On the extremes of this balance, it is predicted that viruses with both high infectivity and virulence will lead to extinction of both host and virus, whereas high infectivity and low or no virulence will exhaust the reservoir of naïve hosts and lead to broad host population immunity, again resulting in viral extinction. Thus, a low infectivity for pathogenic and potentially nonpathogenic viruses may impart an evolutionary advantage by permitting the existence of a persistent naïve host population, while still facilitating viral survival within the remaining population (1, 2).

At the molecular level, the infectiousness of a virus involves several key steps: cell surface binding, entry into the target cell, and replication. To achieve evolutionary success, viruses may alter or compromise each of these steps to reduce the efficiency of infection and achieve a balance between the production of viruses that spread, but do not cause, host (and hence virus) extinction. This concept is strikingly illustrated by the inefficiency, and consequent lack of therapeutic end points, of gene transfer vectors based on respiratory viruses for respiratory diseases (3–6). A better understanding of evolutionary principles governing natural viral evolution will drive more advanced methods to successfully engineer novel virus-based gene therapeutics.

Directed evolution strategies have demonstrated the power of mutagenesis and DNA shuffling methods to investigate and enhance preexisting functions of or generate novel functions in a protein without underlying mechanistic knowledge (7, 8). Recent efforts have increasingly demonstrated the impact of these methods to address novel and high impact problems in protein engineering (9–11); however, few studies have focused efforts on engineering structurally complex protein assemblies (10) or on direct clinical application (11). We hypothesized that directed evolution in an organotypic human airway model employing recombination and mutagenesis, akin to natural evolutionary mechanisms but under selective pressures not constrained by nature, could greatly enhance the respiratory infectivity of a virus.

Adeno-associated viruses (AAV) are members of the parvovirus family and share a similar size, structure, and dependence on a helper virus for replication and gene expression. Wild-type AAV is a 4.7 kb single-stranded DNA virus that contains 2 ORFs: *rep*, which encodes 4 proteins necessary for genome replication; and *cap*, which expresses 3 proteins (VP1–3) that assemble to form the viral capsid (12). Although not necessarily considered a respiratory virus, AAV is a promising candidate to explore viral evolution for multiple reasons. First, the capsid—which determines viral infectivity and tropism (13–16)—is encoded by a single gene, and the existence of multiple serotypes with distinct tropisms indicates that the capsid is highly evolvable (17). Second, AAV evolves naturally via mutagenesis and recombination similar to other viruses (18) and, in stark contrast to pathogenic viruses, is a helper-dependent virus with a remarkable safety profile in humans (19–21). Finally, the creation of a more infectious variant will have therapeutic implications for gene therapy for airway diseases and may improve our understanding of viral evolutionary selective pressures (12, 22).

We therefore used directed evolution of the AAV capsid to select viral variants with enhanced infection of human airway epithelium. PCR-based mutagenesis coupled with high-throughput in vitro recombination generated a diverse library of chimeric *cap* genes with components from 2 divergent serotypes that use distinct receptors, AAV2 (heparan sulfate) and AAV5 (sialic acid) (13–16). Subsequent selection of this library for enhanced infection of organotypic human airway epithelial cultures identified a single novel AAV chimera with a unique point mutation that exhibits enhanced binding to the apical surface of airway epithelia as well as improved gene transfer. Furthermore, the novel AAV virus mediates successful cystic fibrosis transmembrane conductance regulator (CFTR) cDNA-

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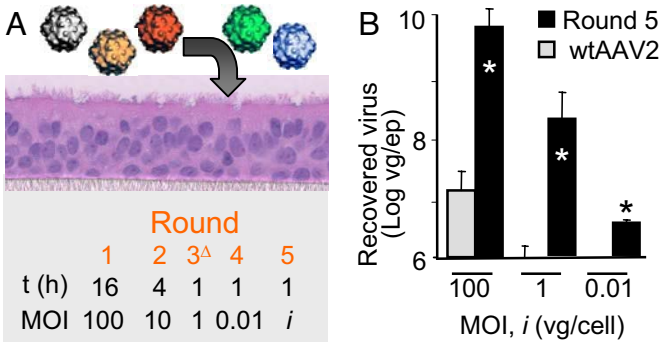

Fig. 1. Evolution of highly efficient AAV vector for pulmonary gene delivery. Directed evolution platform in organotypic polarized, well-differentiated human airway epithelia (*A*). AAV library was applied apically for decreasing times and multiplicity of infection (MOI = *i* (vg/cell)) over 5 rounds. Additional diversification (Δ) was performed after round 3. Recovered virus per epithelium (vg/ep) was approximately 550-fold greater than wtAAV2 after 5 rounds (*B*). $*P < 0.01$.

gene transfer to correct the chloride transport defect in human cystic fibrosis (CF) epithelia.

Results

Library Construction and Selection Results in a Novel AAV with Improved Airway Infection. We combined 2 divergent serotypes that use distinct receptors, AAV2 and AAV5, by subjecting the *cap* genes encoding the viral capsomeres to DNA shuffling and error-prone PCR. This strategy produced a highly diverse library of approximately 10^6 unique chimeric viral *cap* genes that were used to generate replication competent viruses, each carrying its own chimeric viral capsid sequence (10, 23). Extensive selections by apical inoculation of the AAV viral library were performed on organotypic human airway epithelial cultures from 3 different donors during each round (15 donors total)(Fig. 1*A*). AAV infection was allowed to proceed for 3 days, to allow for entry and trafficking and followed by basolateral inoculation with helper wild-type adenovirus to drive the amplification of AAV variants that successfully reached the nucleus. The stringency of selection was gradually increased during subsequent rounds by decreasing the dose and incubation period. After round 3, the successful *cap* genes were subjected to additional diversification via recombination and mutagenesis. With every round of selection, we recovered increasing numbers of viral progeny relative to wild-type AAV2. By round 5, recovery of the evolved progeny was approximately 550-fold higher than AAV2 (Fig. 1*B*). Surprisingly, sequencing 8 random clones from the selected pool revealed a single AAV variant, AAV2.5T, which is a chimera between AAV2 (aa1–128) and AAV5 (aa129–725) with one point mutation (A581T) (Fig. S1).

Improvement of Apical Airway Transduction. Apical transduction (50,000 vg/cell) by the novel AAV chimera; several corresponding variants; and serotypes 2, 5, and 9—each harboring luciferase—was quantified over a 28 day time course (Fig. 2*A*). To improve the time course of gene expression, these cultures were treated basolaterally with Hoechst 33342 at the time of apical infection. Hoechst treatment of AAV-infected airway only affects gene expression from the CMV promoter and does not affect other transduction steps. Strikingly, 21 days posttransduction, AAV2.5T outperformed all other AAV serotypes evaluated: AAV2-Luc (100-fold), AAV5-Luc (10-fold), and AAV9-Luc (20-fold). The decrease in expression at day 28 may represent cell turnover, promoter silencing, or vector genome loss. No significant difference in basolateral transduction between AAV2.5T and other wild-type serotypes was observed

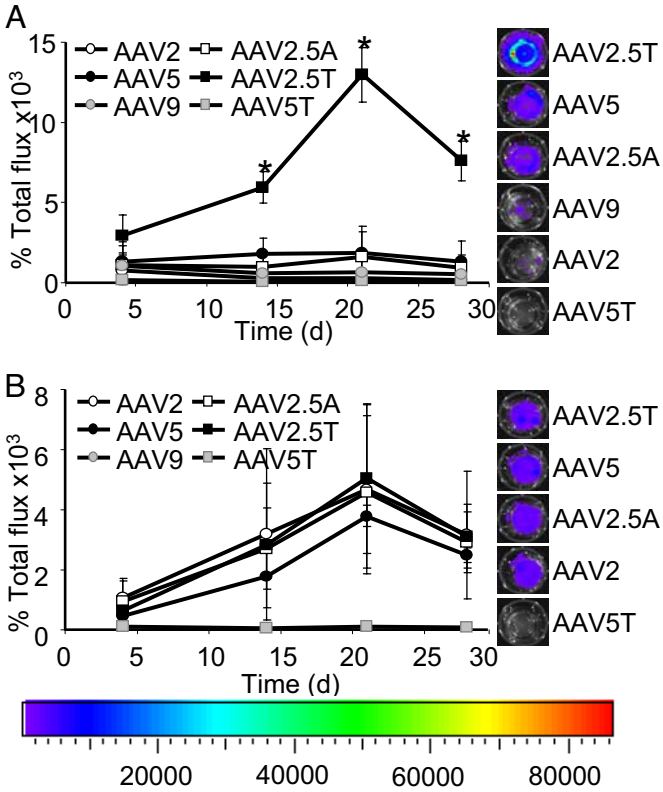

Fig. 2. AAV2.5T mediates significantly improved apical airway transduction. AAV-luciferase expression after apical (*A*) or basolateral (*B*) transduction was monitored over 28 days and peaked at day 21 posttransduction. Quantitative expression was determined after d-luciferin addition to each culture by IVIS imaging (Xenogen). $*P < 0.02$

(Fig. 2*B*), indicating that the advantage of AAV2.5T was specific for the apical surface.

Binding Is Significantly Improved and Requires Sialic Acid. To investigate the mechanism of improved transduction, apical binding was analyzed. Recombinant AAV2.5T bound to the apical surface significantly better than AAV5 (100-fold; Fig. 3*A*), and in contrast to AAV5, binding of AAV2.5T did not saturate at doses ranging from 10 to 1000 genome copies/cell. This suggested a dramatic increase in the number of viral receptors and possibly in binding affinity. Studies on cell lines with specific deficiencies in glycosylation revealed that, similar to AAV5 and in contrast to AAV2, AAV2.5T requires sialic acid (Lec2 cells) but not heparan sulfate (pgsA or pgsD cells) for efficient transduction (Fig. 3*B*). Furthermore, apical neuraminidase pretreatment of airway epithelia significantly decreases AAV2.5T binding, indicating that sialic acid binding is required for efficient transduction (Fig. 3*C*). Additional transduction studies on other cell types indicate the advantage of AAV2.5T is cell-type specific (Fig. 3*D*).

Interestingly, when the point mutant (AAV5-A581T) or chimera (AAV2.5) was studied independently, neither was better than AAV5 at binding or transduction (Fig. 2 *A* and *B*). The A581T mutation occurs in a region critical to AAV5 sialic acid binding; therefore, a mutation in this region may influence the binding affinity for sialic acid and/or the type of linkages recognized. For example, a study of a related parvovirus demonstrated that point mutations adjacent to the sialic acid binding pocket conferred recognition of additional sialic acid linkages (24). However, the AAV5-A581T virus produced low genomic titers and failed to bind or transduce airway epithelia (Fig. 2 *A*

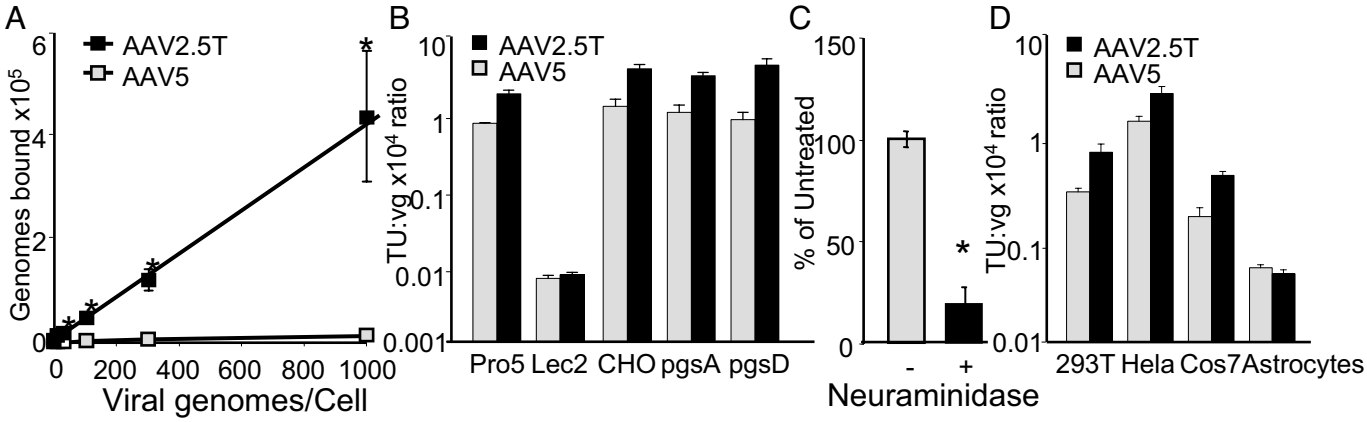

Fig. 3. Enhanced binding and cell specificity of evolved AAV2.5T. In contrast to the saturation of AAV5 binding to the apical surface of human airway epithelia, AAV2.5T binding does not saturate at doses between 300 and 1,000 viral genomes per cell (vg/cell) (*A*). AAV2.5T mirrors parental AAV5 sensitivity for sialic acid and does not efficiently transduce sialic acid deficient Lec2 cells whereas transduction is not altered on heparan sulfate deficient mutants pgsA or pgsD (*B*). TU, transducing unit. AAV2.5T binding to the apical surface of human airway epithelia is significantly reduced by pretreatment with neuraminidase (*C*) ($P < 0.001$). In contrast to airway epithelia, similar transduction was observed between AAV2.5T and AAV5 in several cell lines and primary human astrocytes (*D*).

and *B*). Likewise, AAV2.5 offered no advantage over AAV5 in airway epithelia, even though aa1–128 from AAV2 may potentially alter intracellular trafficking (25). Collectively, our data suggest that the recombination event rescues a structurally deleterious yet functionally advantageous mutation (A581T). Furthermore, the data demonstrate that under artificial pressures that select for enhanced infectivity and do not require a balance between transmission and virulence within a population, AAV can evolve to be significantly more infectious than naturally occurring serotypes.

CFTR Expression and Phenotype Correction. We next investigated potential therapeutic applications of AAV2.5T by analyzing whether it could efficiently express CFTR and correct the CF chloride transport defect. CF airway epithelia were transduced with AAV2.5T encoding a shortened CFTR expression cassette (CFTRΔR, 50,000 vg/cell), then analyzed in Ussing chambers 30 days posttransduction, as previously described (26, 27). Normal epithelia demonstrated chloride transport (Fig. 4*A*), as shown by an increase in current, after treatment with IBMX/Forskolin (ΔIsc_{cAMP} 12 ± 2 $\mu A.cm^{-2}$), that is blocked with GlyH-101, a CFTR blocker (ΔIsc_{GlyH} 10 ± 2 $\mu A.cm^{-2}$). CFTR is barely detectable by immunocytochemistry in normal epithelia (Fig. 4*B*). CF epithelia did not transport chloride (Fig. 4*C*), as shown by the lack of change in current, after treatment with IBMX/Forskolin (ΔIsc_{cAMP} 0 ± 0 $\mu A.cm^{-2}$) or with GlyH-101 (ΔIsc_{GlyH} 0 ± 0 $\mu A.cm^{-2}$). CFTR was also undetectable by immunocytochemistry (Fig. 4*D*). In contrast, AAV2.5T-CFTRΔR restored CFTR chloride current to normal levels (Fig. 4*E*, ΔIsc_{cAMP} 12 ± 4 $\mu A.cm^{-2}$, ΔIsc_{GlyH} 18 ± 9 $\mu A.cm^{-2}$), and CFTR protein strikingly localized at the apical membrane of the epithelial cells (Fig. 4*F*). This is in contrast to AAV2, which does not correct this model, and AAV5, which requires a substantially higher multiplicity of infection (MOI) (26). Furthermore, a dose-response with AAV2.5T-CFTRΔR in CF epithelia showed, surprisingly, that as little as 10 vg/cell was sufficient for chloride transport correction, and a dose of 100-1000 vg/cell was equivalent to adenovirus carrying the wild-type CFTR gene (Fig. 4*G* dotted line, 200 pfu/cell).

Discussion

These data demonstrate that under the appropriate selective pressure, viruses, in particular AAV, can evolve significantly greater infectivity within the airway epithelium than generally observed for naturally occurring variants. This result supports the theory that a highly infectious phenotype may be evolutionarily deleterious, and, therefore, naturally evolved viruses likely fail to possess optimal phenotypes for highly efficient respiratory gene delivery vectors (3, 22). Our experimental system with artificial selective pressures thus enabled the evolution of a novel viral variant with a specific phenotype (i.e., enhanced infectivity) by removing natural viral evolutionary pressures and constraints, such as host survival and long transmission times.

We combined AAV2(the best characterized serotype)with AAV5, which binds a distinct receptor, infects airway epithelia, and contains only 57% primary protein sequence homology to AAV2 (13–16). The stringency of selection was increased each round (decreased inoculation MOI and time) to drive the selection of viral variants with enhanced infectivity from the apical side of airway epithelia. Clones were isolated from the lowest MOI for which virus could subsequently be recovered by PCR, which surprisingly resulted in the enrichment of a single variant after 5 rounds of selection. The high stringency of our protocol may have prevented the identification of additional variants, as observed in a previous study (28). This novel solution is a chimera between the VP1 specific region of AAV2 and the VP2/VP3 region of AAV5, with a single mutation within loop 4 of the exposed VP3 region of AAV5 (A581T).

The AAV capsid contains approximately 3–6 VP1 molecules. The VP1 specific region of AAV2 and some other parvoviruses contains a phospholipase A_2 (PLA_2) domain that has been shown to be crucial for viral infection due to its role in virion endosomal escape (25, 28). No such activity has yet been ascribed to the AAV5 VP1 region, which possesses only 67.7% protein sequence homology to the AAV2 VP1 region and differs in key PLA_2 consensus residues. Interestingly, evidence suggests that amino acid differences within this region among other parvoviruses confers differences in PLA_2 activity of 2 to 3 orders of magnitude , along with altered specificities for various phospholipids (25, 28). Furthermore, Takeuchi *et al.* have recently discussed the VP1-VP2 boundary as a recombination hot spot (29). Two recent attempts to produce AAV vectors in insect cells have shown that swapping the AAV2 VP1 region with the corresponding regions of AAV5 and AAV8 can alter the viral tropism (30, 31). These studies along with our data highlight modularity of this critical capsid domain. Potentially, enhancement of the endosome escape process for AAV may avoid high levels of lysosomal or proteasomal degradation of the virus and thus increase viral transduction as seen in previous airway

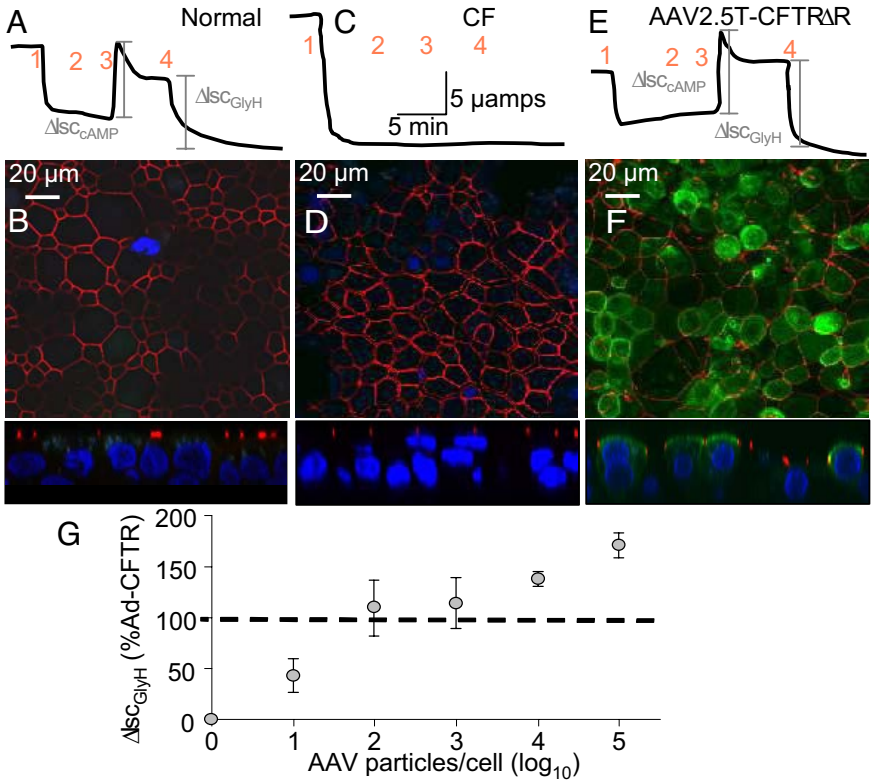

Fig. 4. AAV2.5T-CFTRΔR-mediated correction of CF eplithelia. Normal epithelia exhibit cAMP-regulated chloride transport that is sensitive to the CFTR blocker, GlyH-101 (*A*) and CFTR (green) is barely detectable by immunocytochemistry (*B*) (representative X-Y and X-Z plane shown). Tight junction protein ZO-1 (red), nuclei (blue). In contrast, cystic fibrosis (CF) epithelia lack cAMP-regulated chloride transport (*C*), and CFTR is not detectable by immunocytochemistry (*D*). AAV2.5T-CFTRΔR (MOI 50,000) corrected cAMP-regulated chloride current (*E*) and CFTR (green) is detectable by immunocytochemistry and appropriately localized to the apical membrane (*F*). Dose-response related correction of CF epithelia by AAV2.5T-CFTRΔR relative to adenovirus-CFTR correction ($n = 3–6$ CF epithelia from 2 different donors +/- SEM) (*G*). Representative Ussing chamber tracing: (1) Inhibition of Na^+ current with amiloride (10^{-4} M) hyperpolarized apical membrane voltage and increased the driving force for Cl^- secretion; (2) Inhibition of non-CFTR Cl^- channels with DIDS (10^{-4} M); (3) CFTR activity stimulation (ΔIsc_{cAMP}) by cAMP levels elevated via forskolin (10^{-5} M) and IBMX (10^{-4} M); (4) Reduction of transepithelial Cl^- current (ΔIsc_{GlyH}) with the CFTR-specific blocker GlyH-101 (10^{-4} M). ISC, short-circuit current. 60× oil immersion confocal microscopy.

studies that used proteasomal inhibitors to augment AAV transduction (32).

The A581T mutation is at the mouth of a region we have recently identified as the sialic acid-binding pocket of AAV5 (Fig. 5). Many other viruses, including influenza, polyomaviruses, coronaviruses and paramyxoviruses, have been shown to use sialic acid as a receptor (24, 33, 34), and alterations of only a few amino acids have been shown to significantly alter sialic acid binding of some viruses. For example, the type of sialic acid linkage is critical for the species specificity of influenza A. Avian influenza binds α2, 3-linked sialic acid while human viruses prefer α2, 6-linked sialic acid, and mutations within the influenza hemagglutinin protein can shift this preference between α2, 3- to α2, 6-linked sialic acid (35). Mutation at a critical receptor-binding site could thus significantly alter tissue specificity, especially for human respiratory tissues that possess a remarkably diverse array of glycan structures (36). In our case, modifying a small hydrophobic residue to a large polar group could alter the binding affinity and specificity by modulating viral capsid interactions with the terminal sialic acid moieties, adjacent sugar residues, or the underlying glycoprotein. In contrast to AAV5, AAV2.5T does not readily saturate its binding to airway epithelia, suggesting it accesses abundant receptor sites on the apical surface (Fig. 3*A*). Interestingly, the improvement in transduction appears to be airway-specific since transduction is not significantly improved on other cell types (Fig. 3 *B* and *D*). Future studies will address the underlying basis for the airway-specificity, which may be a function of airway polarization or glycosylation profile.

It is exceedingly difficult to predict which capsid regions should be reengineered to form novel viral-cell interactions that

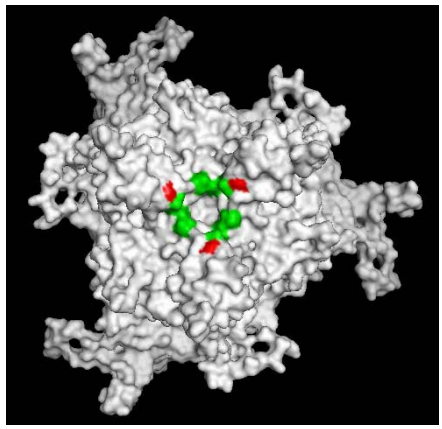

Fig. 5. Molecular model of the alanine to threonine mutation on the AAV5 capsid surface. Three-dimensional models of the AAV5 VP3 subunit were generated using Swiss Model with the coordinates of AAV2 (Protein Databank accession no. 1LP3) supplied as a template and images were rendered in Pymol and Rasmol. The A581T mutation (shown in red) occurs at the mouth of the predicted AAV5 sialic acid binding pocket (shown in green).

would be optimal to transduce particular tissues, despite advances in understanding AAV structure and function (37–40). However, the search for AAV variants with novel properties through a variety of algorithms—such as isolating naturally occurring AAV, site-directed mutants, or (most recently) laboratory-evolved variants—has yielded various chimeric AAV viruses with distinct transduction profiles (17, 41–44). Family shuffling of the AAV capsids and subsequent selection in nonhuman models have established the power and potential of this method to generate novel capsids tailored to specific applications (23, 41, 42). Here we demonstrate the human therapeutic potential of a customized AAV vector.

CF is a complex and lethal genetic disease, and the original hypothesis that the associated pulmonary disease could be efficiently treated with co-opted respiratory viruses containing the CFTR gene has thus far proven false. A wealth of information has been gained by these experiments, leading us to a deeper appreciation of host-pathogen interactions. Although never tested directly, it is estimated that targeting as few as 6% of epithelial cells could correct CF pulmonary disease (45), although evidence suggests that correction of specific structures such as the submucosal glands may also be required (46). Early clinical trials with adenovirus yielded short-term localized expression of CFTR, but a strong immune response to the adenoviral vectors was observed (47–49). In contrast, recent clinical trials with AAV2 have demonstrated the safety of this virus (19, 20); however, these trials were not successful at correcting the CF pulmonary phenotype, likely due to the low airway gene delivery efficiency of AAV2. Furthermore, more recently used serotypes, such as AAV5 and AAV9, possess only slightly higher gene delivery efficiencies on human airway epithelia compared to AAV2 ($<$ 10-fold) (50–52). Whether poor transduction efficiency is the sole limitation for in vivo gene therapy has yet to be tested, and future work will determine the relative importance of mucus, mucociliary clearance, and humoral- and cell-mediated responses as barriers to in vivo gene delivery by a highly infectious AAV vector. Novel AAV vectors evolved specifically to transduce a high percentage of human airway epithelia (Fig. 4) should provide a new tool to investigate these considerations and for the treatment of CF and other pulmonary diseases.

In summary, we speculate that in contrast to the low infectivity (R_0) for most respiratory viruses found in nature, viruses can evolve under artificial selective pressure to be significantly more infectious. This finding has strong relevance for the engineering of novel virus-based gene therapeutics. Directed evolution coupled with a clinically-relevant and human-specific selection platform effectively generated a highly efficient AAV vector capable of correcting the CF Cl^- transport defect to levels comparable to non-CF epithelia even at very low doses. This general approach thus enables the development of "designer" gene delivery vectors under clinically desirable selective pressures and, when coupled with the appropriate disease model, will continue to yield exciting new candidates for human gene therapy.

Materials and Methods

Materials. Alexa-488 and -568 conjugated goat anti-mouse or anti-rabbit Abs were from Molecular Probes. ZO-1 Ab was from Invitrogen (61–7300). CFTR monoclonal Abs M3A7 and MM13–4 were from Millipore.

Primary Human Airway Epithelia. Primary human airway epithelia from CF and non-CF individuals were isolated from trachea and bronchi of donor lungs. Cells were seeded onto collagen coated, semipermeable membranes (Millipore) and grown at the air-liquid interface as previously described (53, 54). Approximately 2 weeks after seeding, cultures were well-differentiated and attained a measurable transepithelial resistance.

Library Construction. For the initial plasmid library, the AAV2 *cap* gene was amplified via PCR using 5′-CATGGGAAAGGTGCCAGACG-3′ and 5′-CGCAGAGACCAAAGTTCAACTGA-3′ as forward and reverse primers, respectively and AAV5 *cap* gene was amplified via PCR using 5′-CATGGGAAAGGTGCCAGACG-3′ and 5′- AAGCGGCCGCAATGGGTTAAAGGGG -3′ as forward and reverse primers, respectively. DNA shuffling was performed as previously described (7, 8), and chimeric *cap* genes were cloned into pSub2 for rcAAV production (10). For subsequent evolution rounds, error-prone PCR was performed as previously described (10).

Cell Lines and Viral Production. Unless otherwise mentioned, cell lines were obtained from the ATCC and cultured at 37 °C and 5% CO_2. HEK293T, HeLa, CHO K1, CHO pgsA, and CHO pgsD were cultured in Iscove's modified Dulbecco's medium (IMDM) (Mediatech). Pro-5, and Lec2 were cultured in minimum essential medium, alpha modification (αMEM) (Sigma–Aldrich). AAV293 cells (Stratagene) and Cos7 were cultured in DMEM. Primary astrocytes were cultured according to the manufacturer's instructions (ScienCell). All media were supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

The rcAAV library and rAAV vectors were packaged and purified via iodixanol gradient centrifugation as previously described (10, 23). Viral vectors were harvested and titered via quantitative PCR to obtain DNase-resistant genomic titers or flow cytometry to obtain transduction titers as previously described (10, 23).

CFTR transgene in AAV2.5T contains a deleted portion of the R domain (CFTRΔR, 708−759), a shortened CMV immediate/early (173*CMVie*) enhancer/promoter, and minimal poly(A) signal as described by Ostedgaard *et al.* (26, 27). Ad-CFTR contained the full length CMV promoter and wild-type CFTR.

Wild-type adenovirus serotype 5 (Ad5) and Ad-CFTR were produced by the University of Iowa Gene Transfer Vector Core.

in Vitro Selection and Characterization. To select the viral library, AAV was diluted in EMEM and added to the apical side of primary human airway epithelia from 3 donors in a final volume of 25 μl or 50 μl at 50,000 viral genomes per cell or at the indicated dose and incubated at 37 °C for 1, 4, or 16 h as indicated. Unbound virus was removed by washing 2 times with 250 μl of PBS. After 3 days, AAV was amplified by infection with Ad5, as described (10). Briefly, the epithelia were inverted and 25 μl of Ad5 at 10 pfu/cell was allowed to adsorb from the basolateral side. Epithelia were then placed in fresh media. Three days later, AAV viral genomic DNA was recovered by PCR amplification and cloned into pSub2 for additional rcAAV production and selection or pXX2 NotI for rAAV production (23). One round of evolution consisted of *cap* recombination and mutagenesis followed by 3 selection steps of infection of human airway epithelia. A second round of recombination and mutagenesis was performed followed by another 2 rounds of selection for a total of 5 rounds of selection.

To determine the relative transduction efficiencies of various clones in cell lines, 1–2.5 $\times$ 10^5 cells were infected with rAAV vectors carrying cDNA encoding green fluorescent protein at a genomic MOI of 10^3 or 5 $\times$ 10^4. Forty-eight hours postinfection, the percentage of GFP+ cells was quantified by flow cytometry.

For human airway epithelia, apical inoculation was performed at 37 °C for 4 h and basolateral was performed by inverting the millicell to allow adsorption, as described above. For apical inoculations only, Hoechst 33342 (5 μM in USG medium, Invitrogen) was applied basolaterally at the time of infection and incubated for 4 h at 37 °C, after which the basolateral media was aspirated and replaced with fresh USG media containing no Hoechst 33342.

Human airway epithelia infected with eGFP carrying virus were visualized by confocal microscopy. Luciferase gene expression was determined by adding 50 μl of 150 μg/ml D-Luciferin Potassium Salt (P/N 122769, Xenogen Corp.) dissolved in EMEM to the apical surface of human airway epithelia. Epithelia were then imaged using the IVIS Imaging System 200 and quantitated using Living Image 2.50.1 software.

Neuraminidase Treatment and Binding Assays. Human airway epithelia were pretreated apically with 10 milliunits of neuraminidase (*Vibrio cholerae*, Sigma–Aldrich) for 2 h at 37 °C. Cells were then put on ice, neuraminidase removed, and the apical surface washed 3 times with cold EMEM. For all binding assays, epithelia were incubated on ice for 10 min before virus adsorption. Virus was diluted in EMEM and applied to the apical side at the indicated doses for 30 min on ice. Unbound virus was removed by washing 2 times with 250 μl of ice cold EMEM. DNA was isolated with the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Quantitative PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) with primers designed to the CMV promoter (5′-

AAATCAACGGGACTTTCCAA-3′ and 5′-GGTTCACTAAACGAGCTC-3′) and compared to known DNA standards to determine the amount of viral DNA.

Ussing Studies. The short-circuit current (I_{sc}) was measured, using a Cl^- gradient in modified Ussing chambers (Jim's Instruments) as previously described (26). Epithelia were treated with forskolin (10^{-5} M) and IBMX (10^{-4} M) for 18–24 h before study in Ussing chambers to minimize basal CFTR current. Current was measured.

Immunostaining. Human airway epithelia were washed once with PBS, fixed with ice cold methanol containing 1% paraformaldehyde for 20 min at −20 °C, and blocked with 2% BSA in SuperBlock (Pierce). Cells were incubated with primary Ab, washed extensively, and incubated with goat anti-mouse or -rabbit secondary Ab. After washing, epithelia were coverslipped with Vectashield mounting media (Vector Laboratories, Inc.). Images were acquired with an Olympus Fluoview FV1000 Laser Scanning Confocal Microscope using a 60X oil immersion lens and analysis was performed with Olympus Fluoview Viewer software version 1.4a and Image J.

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