ORIGINAL ARTICLE

rAAV-CFTRΔR Rescues the Cystic Fibrosis Phenotype in Human Intestinal Organoids and Cystic Fibrosis Mice

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Abstract

Rationale: Gene therapy holds promise for a curative mutation-independent treatment applicable to all patients with cystic fibrosis (CF). The various viral vector–based clinical trials conducted in the past have demonstrated safety and tolerance of different vectors, but none have led to a clear and persistent clinical benefit. Recent clinical breakthroughs in recombinant adeno-associated viral vector (rAAV)-based gene therapy encouraged us to reexplore an rAAV approach for CF.

Objectives: We evaluated the preclinical potential of rAAV gene therapy for CF to restore chloride and fluid secretion in two complementary models: intestinal organoids derived from subjects with CF and a CF mouse model, an important milestone toward the development of a clinical rAAV candidate for CF gene therapy.

Methods: We engineered an rAAV vector containing a truncated CF transmembrane conductance regulator (CFTRΔR) combined with a short promoter (CMV173) to ensure optimal gene expression. A rescue in chloride and fluid secretion after rAAV-CFTRΔR treatment was assessed by forskolin-induced swelling in CF transmembrane conductance regulator (CFTR)-deficient organoids and by nasal potential differences in ΔF508 mice.

Measurements and Main Results: rAAV-CFTRΔR transduction of human CFTR-deficient organoids resulted in forskolin-induced swelling, indicating a restoration of CFTR function. Nasal potential differences demonstrated a clear response to low chloride and forskolin perfusion in most rAAV-CFTRΔR-treated CF mice.

Conclusions: Our study provides robust evidence that rAAV-mediated gene transfer of a truncated CFTR functionally rescues the CF phenotype across the nasal mucosa of CF mice and in patient-derived organoids. These results underscore the clinical potential of rAAV-CFTRΔR in offering a cure for all patients with CF in the future.

Keywords: gene therapy; airways; patient-derived organoid cultures; viral vectors; nasal potential difference

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At a Glance Commentary

Scientific Knowledge on the Subject: The modest successes of previous clinical trials for cystic fibrosis (CF) gene therapy raise the question of how to further improve gene transfer efficacy and how to more accurately determine functionality of CF transmembrane conductance regulator (CFTR)-encoding vectors in preclinical studies. Organoid experiments are valuable because they allow functional vector assessment in CF patient–derived material using a sensitive and quantitative swelling assay. The CF mouse model complements the organoid data because a potential rescue of the CF phenotype can be assessed *in vivo* by nasal potential differences, a widely accepted biomarker used for evaluation of response to treatment in patients with CF.

What This Study Adds to the Field: In the process of developing a clinical candidate for CF viral vector–based gene therapy, a thorough examination of preclinical efficacy in relevant cell and animal models is a prerequisite. Here we describe that a single dose of a therapeutic vector, recombinant adeno-associated viral vector–truncated CFTR, rescues the CF phenotype in intestinal organoids derived from subjects with CF and in the nasal mucosa of CF mice, highlighting its clinical potential to correct both respiratory and gastrointestinal pathology in CF. These results pave the way to assess long-term efficacy and functional restoration of CFTR activity in a large animal model recapitulating human CF pathology.

Cystic fibrosis (CF) is caused by mutations in *CFTR*, which codes for the CF transmembrane conductance regulator protein (CFTR), a chloride/bicarbonate channel regulating fluid transport across epithelia. Almost 2,000 mutations have been described (www.genet.sickkids.on.ca). The major clinical manifestations of CF are related to respiratory and gastrointestinal tract pathology. In 2012, a breakthrough in CF therapy was achieved with the U.S. Food and Drug Administration approval of the first curative drug (Kalydeco; Vertex Pharmaceutics, Cambridge, MA), a potentiator correcting the CFTR gating defect (www.ema.europa.eu). It can, however, only be applied to 4–5% of patients with CF carrying gating mutations. Very recently the U.S. Food and Drug Administration approved the first therapy, which tackles the underlying defect of the most common CF-causing mutation (ΔF508) with a combination of a CFTR potentiator (Kalydeco, ivacaftor) and a CFTR corrector (lumacaftor), a drug that partially rescues ΔF508-CFTR trafficking to the membrane (1). In a phase III clinical trial this combination therapy resulted in a significant, albeit modest (2.6–4%) improvement in lung function in patients with CF homozygous for the ΔF508 mutation (2), underscoring the need for further development of more potent therapeutic strategies.

In contrast to small molecules that act mutation-specific (3), gene therapy offers a mutation-independent treatment for all patients with CF, with the potential to cure the disease. In the early 1990s, clinical trials using adenoviral and recombinant adeno-associated viral vectors (rAAV2/2) did not improve lung function (4–6). Recent successes in gene therapy using rAAV to treat congenital blindness, hemophilia B, and lipoprotein lipase deficiency encouraged us to reexplore rAAV gene therapy for CF (reviewed in Reference 7). rAAV is derived from wild-type AAV and not associated with human pathology. It has emerged as a promising and safe vector because of its low immunogenicity, nonintegrating nature, and absence of viral genes (8). In 2012, the European Medicines Agency approved the first rAAV gene therapy product (Glybera; UniQure, Amsterdam, the Netherlands) for patients with lipoprotein lipase deficiency (www.ema.europa.eu). Unlike rAAV clinical trials in the past (9), our approach uses rAAV2/5, an airway-tropic serotype (10–12). Additionally, we incorporate a truncated CFTR (CFTRΔR) that allows insertion of a promoter to enhance gene expression. CFTRΔR has a deletion in the regulatory domain (Δ708–759) but retains channel activity (13).

We investigated the therapeutic potential of rAAV-CFTRΔR in two complementary models: intestinal organoids derived from subjects with CF and a CF mouse model. Human intestinal organoids are primary stem cell–based cultures generated from rectal biopsies (14–16). CFTR activation leads to rapid volumetric expansion of organoids (17, 18), providing a platform to study CFTR function following *CFTR* gene transfer. Here we report that viral vector-mediated gene transfer results in a rescue of the CF phenotype in human CFTR-deficient organoids.

Finally, we evaluated rAAV2/5-CFTRΔR gene therapy efficacy in CF mice homozygous for the ΔF508 mutation (19). We assessed a possible CFTR correction in airways by measuring *in vivo* nasal potential differences (NPDs), because the nasal epithelium of CF mice mimics transepithelial ion transport defects observed in patients with CF (20). We demonstrate that a single dose of rAAV2/5-CFTRΔR could restore Cl^- conductance. Taken together, our results underscore the therapeutic potential of rAAV-CFTRΔR as gene therapy vector for CF, opening new avenues toward a generic cure for all patients. Some of the results of these studies have been previously reported in the form of abstracts (21–23).

Methods

Viral Vector Production

Production of lentiviral vector (LV) and rAAV was performed as described previously with minor modifications (12, 24, 25). Vector production and cloning strategies are provided in the online supplement.

Generation of HeLa Cell Lines Stably Expressing CFTR Constructs

HeLa cells stably overexpressing triple flag (3F)-tagged CFTR constructs were generated by transduction with vesicular stomatitis virus glycoprotein G (VSV-G) pseudotyped LV encoding the transgene of interest under the control of the human cytomegalovirus (CMV) promoter.

Detection of CFTR Expression

Detailed protocols and antibodies used for Western blotting, immunocytochemical, and immunohistochemical analysis are available in the online supplement.

Iodide Efflux Assay

The assay was performed as described previously with minor modifications provided in the online supplement (26).

Patch Clamp

Whole-cell patch-clamp analysis was performed as described previously with minor modifications provided in the online supplement (27).

Transduction of Human Intestinal Organoids

The Ethics Committee of the University Medical Center Utrecht approved this study, and informed consent was obtained. Organoids were generated from rectal biopsies and cultured as described previously (14, 18). For viral vector transduction, cultures were trypsinized (TrypLE; Gibco BRL, Invitrogen, Merelbeke, Belgium) and seeded in 96-well plates in 4 μl Matrigel (Corning, Corning, NY) and viral vector (1:1) containing single cells and small organoid fragments. These cells were incubated at 37°C (10 min for LV; 30 min for rAAV) and immersed in medium. rAAV transduction efficiency was verified by measuring Firefly luciferase (Fluc) activity after addition of 50 mM D-luciferin using an IVIS Spectrum (Xenogen; Caliper LS, Hopkinton, MA). Quantification of forskolin-induced organoid swelling (FIS) was performed as described previously (18). A detailed protocol is available in the online supplement.

Mouse Model and Viral Vector Administration

Adult FVB/N mice homozygous for the ΔF508 mutation (*Cftr*^{tm1Eur} mice) (19) were obtained from CDTA (Cryopréservation, Distribution, Typage et Archivage animal) (Orléans, France) and housed on a fiber-free diet. A total of 50 μl of vector suspension (9×10^{10} GC/animal) rAAV2/5-CFTRΔR or control rAAV2/5-eGFP-P2A-Fluc was administered by nasal instillation to anesthetized mice. All animal procedures were approved by the local ethical committee in compliance with European Community regulations.

NPD Measurements

CFTR activity in the nasal epithelium of ΔF508 mice was assessed by NPD as described previously with minor modifications provided in the online supplement (28).

Statistical Analysis

For NPD measurements, response to vector treatment between groups was compared by Mann-Whitney test for unpaired observations. A P value less than 0.05 was considered statistically significant ($^*P < 0.05$, $^{**}P < 0.01$). GraphPad Prism 5 (GraphPad Software, San Diego, CA) software was used for statistical analysis.

Results

CFTR Truncated in the Regulatory Domain (CFTRΔR) Is a Functional and Regulatable Chloride Channel

rAAV has a limited packaging capacity and the full-length *CFTR* complementary DNA (cDNA) does not fit the small vector. As a first step in the development of a rAAV-based gene therapy for CF, we evaluated and compared functionality of two truncated versions of CFTR with that of the full-length CFTR channel. We generated HIV-based LVs that carry the 3F-tagged full-length *CFTR* cDNA (4,443 bp), CFTRΔN (3,729 bp, Δ27–264, missing the first four transmembrane segments at the N terminus) (29), or CFTRΔR (4,287 bp, lacking residues 708–759 of the regulatory R-domain) (30) (Figure 1A) and generated stable HeLa cell lines. The subcellular distribution of CFTRΔR was comparable with that of full-length CFTR as shown by immunocytochemistry (Figure 1B). By Western blot analysis, we could demonstrate that the expression level of CFTRΔR was similar to that of full-length CFTR, showing the immature protein (band B) and the glycosylated, fully mature protein (band C) (Figure 1C). In contrast, 3F-CFTRΔN showed only a single band on Western blot and located mainly to the cytoplasm (Figures 1B and 1C).

Next, we compared functionality of the respective CFTR versions in an iodide ($^{125}I^-$) efflux assay (Figure 1D). $^{125}I^-$ efflux increased in cells expressing 3F-CFTRΔR after exposure to a mixture of forskolin and genistein (indicated by an *arrow*), comparable with that of full-length 3F-CFTR-expressing cells, whereas no $^{125}I^-$ efflux was observed for 3F-CFTRΔN cells or nontransduced HeLa cells (Figure 1D, *negative*). The fact that $^{125}I^-$ efflux was only observed after activation of adenylyl cyclase activity by forskolin underscores that CFTRΔR is regulated by the cAMP/protein kinase A pathway and has no constitutive activity.

Next, we evaluated 3F-CFTRΔR and full-length 3F-CFTR protein function by whole-cell patch-clamp analysis (Figures 1E–1G). Protein expression was verified by Western blot (*see* Figure E1 in the online supplement). Currents were recorded both under basal conditions to control for constitutive channel activity and following CFTR activation with a cAMP agonist cocktail. The presence of a CFTR-specific current (ΔI_{CFTR}) was evaluated by using inh172, a CFTR inhibitor (Figures 1E–1G) (31). For 3F-CFTRΔR cells, only a very low current was detectable under basal conditions demonstrating absence of constitutive CFTRΔR activity. Interestingly, ΔI_{CFTR} generated by CFTRΔR was comparable with that of full-length CFTR (Figure 1G), consistent with the results from the $^{125}I^-$ efflux assay (Figure 1D) and in line with earlier reports (13).

CFTRΔR Restores Chloride and Fluid Secretion in Intestinal Organoids Derived from Subjects with CF

After showing functionality in a HeLa-based cell culture model, we set out to evaluate the potential of CFTRΔR to rescue the CF phenotype in human CFTR-deficient organoids (E60X/4014delATTT; referred to as CF) and to compare activity with full length 3F-CFTR. CF organoids were transduced with LVs encoding CFTRΔR, full-length CFTR, or a vector encoding a reporter gene (eGFP) to control for possible transduction-related side effects and monitored for CFTR activity using the FIS assay (Figures 2A–2C). Healthy control organoids, expressing endogenous levels of CFTR and transduced with an eGFP-control vector, were included as a reference. In LV-CFTRΔR transduced CF organoids, forskolin addition resulted in a strong increase in organoid swelling (50% increase after 120 min; compare with $t = 0$ min), in line with the rescue observed for LV-CFTR transduced CF organoids (Figure 2B). Similar results were obtained at lower vector doses (*see* Figure E2). Compared with nontransduced CF organoids, a sixfold increase in organoid swelling (i.e., area under the curve obtained from time periods measured in Figure 2B) was reached after correction with LV-CFTRΔR and a fivefold increase after correction with LV-CFTR (Figure 2C). Truncated and full-length CFTR proteins migrated at the predicted molecular weight as detected by Western blot analysis, albeit at lower expression levels than the endogenous CFTR expressed in healthy control organoids

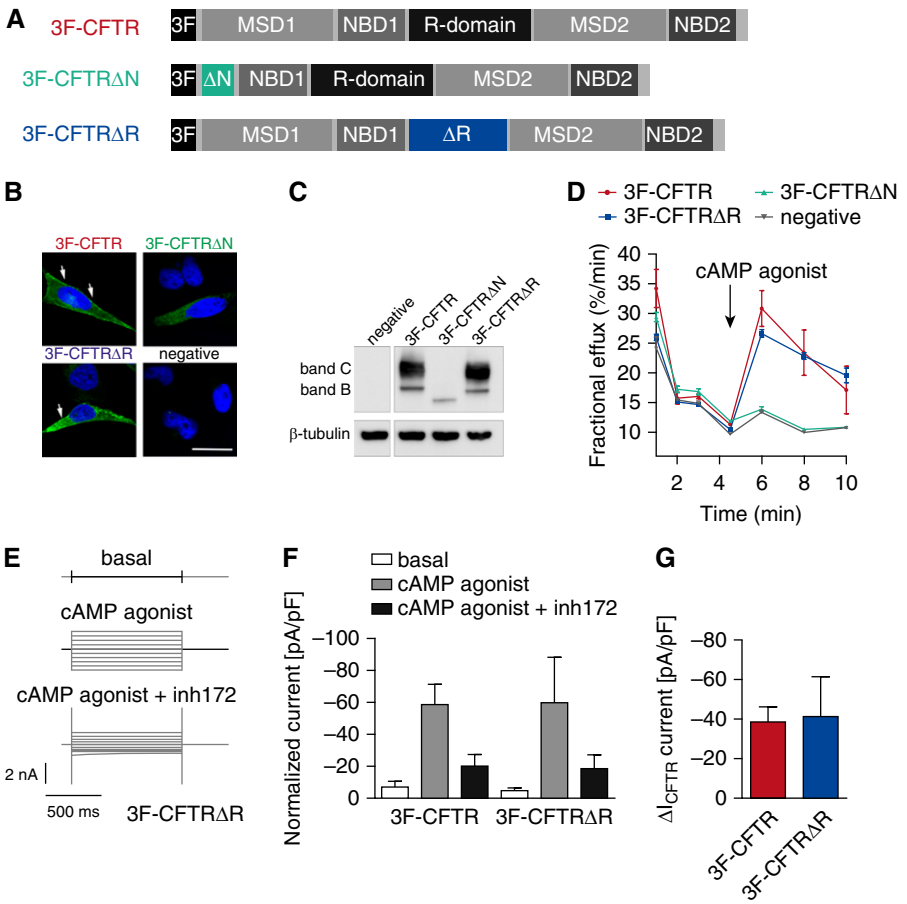

Figure 1. Functional validation of trimmed cystic fibrosis transmembrane conductance regulator (CFTR) constructs. (*A*) Overview of triple flag-tagged (3F) CFTR constructs: 3F-CFTR and two 3F-truncated CFTR constructs (CFTRΔN and CFTRΔR). (*B*) Immunocytochemistry of HeLa cells transduced with lentiviral vectors expressing indicated CFTR constructs. Anti-CFTR-Alexa-488 staining visualized CFTR-positive cells (*green*); 4′,6-diamidino-2-phenylindole–stained nuclei (*blue*). *Arrows* indicate membrane localization. *Scale bar* = 25 μm. (*C*) Western blot analysis of CFTR (anti-flag antibody) and β-tubulin (loading control) in whole-cell lysates of transduced HeLa cells. *Band B* shows immature CFTR protein and *band C* the glycosylated, fully mature protein. (*D*) Iodide ($^{125}I^-$) efflux in HeLa cells transduced with the indicated CFTR constructs. *Arrow* depicts time point of CFTR activation by forskolin and genistein. (*E*) A representative recording of whole-cell patch clamp in HeLa-3F-CFTRΔR cells. (*F*) Quantification of whole-cell currents before activation, on activation with a cAMP-analog and 3-isobutyl-1-methylxanthine (referred to as cAMP agonist), and subsequent addition of CFTR inhibitor (inh172) in monoclonal HeLa cells 3F-CFTR (n = 8) and 3F-CFTRΔR (n = 5). (*G*) Quantification of CFTR-mediated current (ΔI_{CFTR}) calculated by subtracting the current after addition of inh172 from the current obtained on activation. Measured currents were normalized to cell capacitance. All data are depicted as mean + SEM. MSD = membrane-spanning domain; N = N terminus; NBD = nucleotide-binding domain; R-domain = regulatory domain.

(Figure 2D). Altogether, these results strengthen the data obtained in the $^{125}I^-$ efflux assay and the patch-clamp analyses (Figure 1), and demonstrate that CFTRΔR rescues Cl^- secretion to similar levels as observed for the full-length protein.

Optimization of Trimmed Expression Cassettes to Accommodate CFTRΔR

Because the rAAV genome size is limited to 5 kb, this excludes the addition of the full-length *CFTR* cDNA in combination with regulatory elements. We opted for a truncated CFTR (CFTRΔR), which allows insertion of an additional promoter to enhance gene expression levels. We evaluated a shortened version of the CMV promoter (comprising 173 bp of the full-length CMV, referred to as CMV173) and a minimal polyadenylation signal (49 bp, SPA) described by Ostedgaard and coworkers (30) and compared reporter gene expression levels with our standard rAAV vector carrying a full-length CMV and a BGHpA signal (rAAV-CMV173-Fluc and rAAV-CMV-Fluc, respectively) (Figure 3A). The latter expression cassette, in combination with CFTRΔR, would exceed the rAAV packaging capacity. Fluc activity was compared following transduction of HEK293T cells with the respective rAAV vectors (Figure 3B). Even though rAAV-CMV173-Fluc showed a 35-fold lower Fluc signal than the control vector (rAAV-CMV-Fluc), the signal was still 3-log higher than background (Figure 3B). Similar data were obtained when evaluating rAAV-CMV173-Fluc and rAAV-CMV-Fluc transduction in primary intestinal organoids (Figure 3C), demonstrating that a shorter expression cassette results in lower transgene expression levels, but opens the possibility to incorporate a larger cDNA, such as *CFTRΔR*.

rAAV-Mediated CFTRΔR Gene Transfer Rescues CFTR Activity in Human Intestinal Organoids

After successful validation of rAAV-CMV173-Fluc, we replaced the reporter genes with CFTRΔR to generate a therapeutic rAAV vector, referred to as rAAV-CFTRΔR with a genome size of 4,964 bp, which is just below the theoretical rAAV packaging limit of 5 kb (Figure 3D). rAAV-CMV173-Fluc was included as control (further referred to as rAAV control). Next, rAAV-CFTRΔR was functionally validated in human CFTR-deficient organoids (E60X/4014delATTT; referred to as CF) (Figures 3E–3I). Even though the episomal nature of rAAV does not allow stable transduction of rapidly proliferating organoids, rAAV-CFTRΔR resulted in FIS of transduced organoids at $t = 120$ min (Figure 3G) compared with organoids transduced with rAAV control (Figure 3F), which corresponds to an average surface area increase of 13% (Figure 3H), being fourfold higher than organoids treated with rAAV control (Figure 3I). Taken together, these data demonstrate that the engineered therapeutic rAAV-CFTRΔR vector is functional and that the ion transport-induced organoid swelling is CFTR-dependent. Although the expression cassette is at the limit of the rAAV packaging capacity, data imply that intact genomes are incorporated into vector

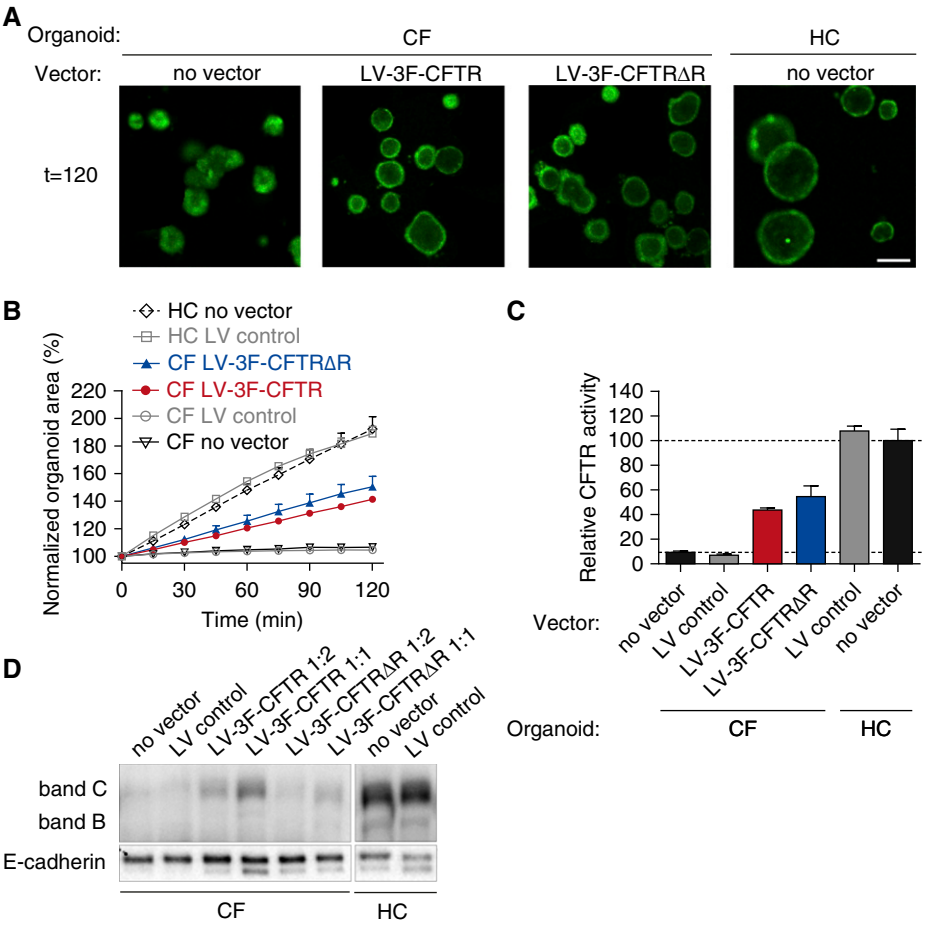

Figure 2. Truncated cystic fibrosis transmembrane conductance regulator (CFTRΔR) restores chloride and fluid secretion in human intestinal organoids. (*A*) Confocal images of calcein green–labeled and forskolin-stimulated (120 min) organoids from a cystic fibrosis (CF) or healthy control (HC) subject after transduction with a lentiviral vector (LV) encoding triple flag-tagged (3F) cystic fibrosis transmembrane conductance regulator (CFTR) or 3F-CFTRΔR. Untransduced CF or HC organoids are taken along as a reference. In the CF panels, cell debris and unviable structures have been removed from the image following standard operating procedures. *Scale bar* = 100 μm. (*B*) Forskolin-induced swelling of transduced organoids is expressed as the total organoid surface area increase relative to $t = 0$ (normalized area) averaged from four independent wells. (*C*) Forskolin-induced swelling bars represent organoid swelling expressed as the area under the curve calculated from the time periods shown in *B* relative to the average HC response (100%). The *dashed lines* correspond to the relative area under the curve for nontransduced CF (9.3%) and nontransduced HC organoids (100%), respectively. (*D*) Western blot analysis of CFTR and E-cadherin (loading control) in whole-cell lysates of organoids transduced with an LV expressing indicated CFTR or control constructs. All results are representative of at least three independent experiments and are presented as mean + SEM. CF organoids = E60X/4015ATTTdel.

particles and allow efficient second strand DNA synthesis and transgene expression.

rAAV2/5-CFTRΔR Corrects the CF Phenotype in a ΔF508 Mouse Model

In a next step, we applied rAAV intranasally to ΔF508 mice (rAAV2/5-CFTRΔR and rAAV2/5-eGFP-P2A-Fluc, respectively) and assessed transgene expression by immunohistochemistry in nasal tissue (*see* Figure E3). CFTRΔR expression was detected in the respiratory epithelium in a nonhomogeneous, patchy manner. In positively stained epithelial cells, CFTRΔR was detected as a characteristic apical signal (*see* Figure E3A, *black arrow*) in rAAV2/5-CFTRΔR-treated animals, whereas the signal was much lower in animals treated with rAAV control (*see* Figure E3B, *orange arrow*). This signal likely presents the detection of endogenous ΔF508-CFTR situated both in the cytoplasm and to a limited extent in the plasma membrane, as described by others for this mouse strain (32). However, a small part of the apical signal could be aspecific because low-level apical staining was occasionally observed in nasal tissue of CFTR knock-out mice (*see* Figure E3C).

In a final step, we evaluated functionality of our gene therapeutic approach in a ΔF508 mouse model (19). The experimental protocol is detailed in Figure 4. One week before treatment, NPD measurements were performed to assess residual activity of endogenous ΔF508-CFTR (Figure 4; *see* Figure E4, *left recordings*). We applied rAAV2/5-CFTRΔR (n = 8) or rAAV2/5-eGFP-P2A-fLuc, referred to as rAAV control, (n = 5) intranasally to CF mice (Figure 3D). Two to 4 weeks after vector administration, we assessed a phenotypic rescue by measuring changes in nasal transepithelial ion transport using NPD (Figure 4; *see* Figure E4, *right recordings*). NPD recordings after rAAV2/5-CFTRΔR treatment demonstrate a clear change in potential difference in response to low Cl^- solution and to some extent after forskolin addition, which was partially inhibited by inh172 (Figure 4 demonstrates a NPD recording of a rAAV2/5-CFTRΔR-treated animal; recordings of all animals are shown in Figure E4). Hyperpolarization after low Cl^- and forskolin perfusion was taken as a main indicator to demonstrate a response to CFTRΔR gene transfer (33).

In the rAAV2/5-CFTRΔR-treated group, five out of eight mice responded to low Cl^- (Figure 5A). The highest change in potential difference value of this group measured 1 week before vector administration was taken as the cut-off value for positivity to score the effect of gene transfer. Mice that responded to treatment (five of eight) showed a significant hyperpolarization during low Cl^- perfusion of −5.8 mV (median; range, −6.9 to −3.4; n = 5) compared with the group treated with rAAV2/5 control ($P = 0.008$), indicating Cl^- channel activity (Figure 5C). For comparison, reference values obtained in wild-type mice after low Cl^- perfusion were −5 mV (median; range, −15.6 to −1.2; n = 28) (28). Additionally, a cumulative response to low Cl^- and

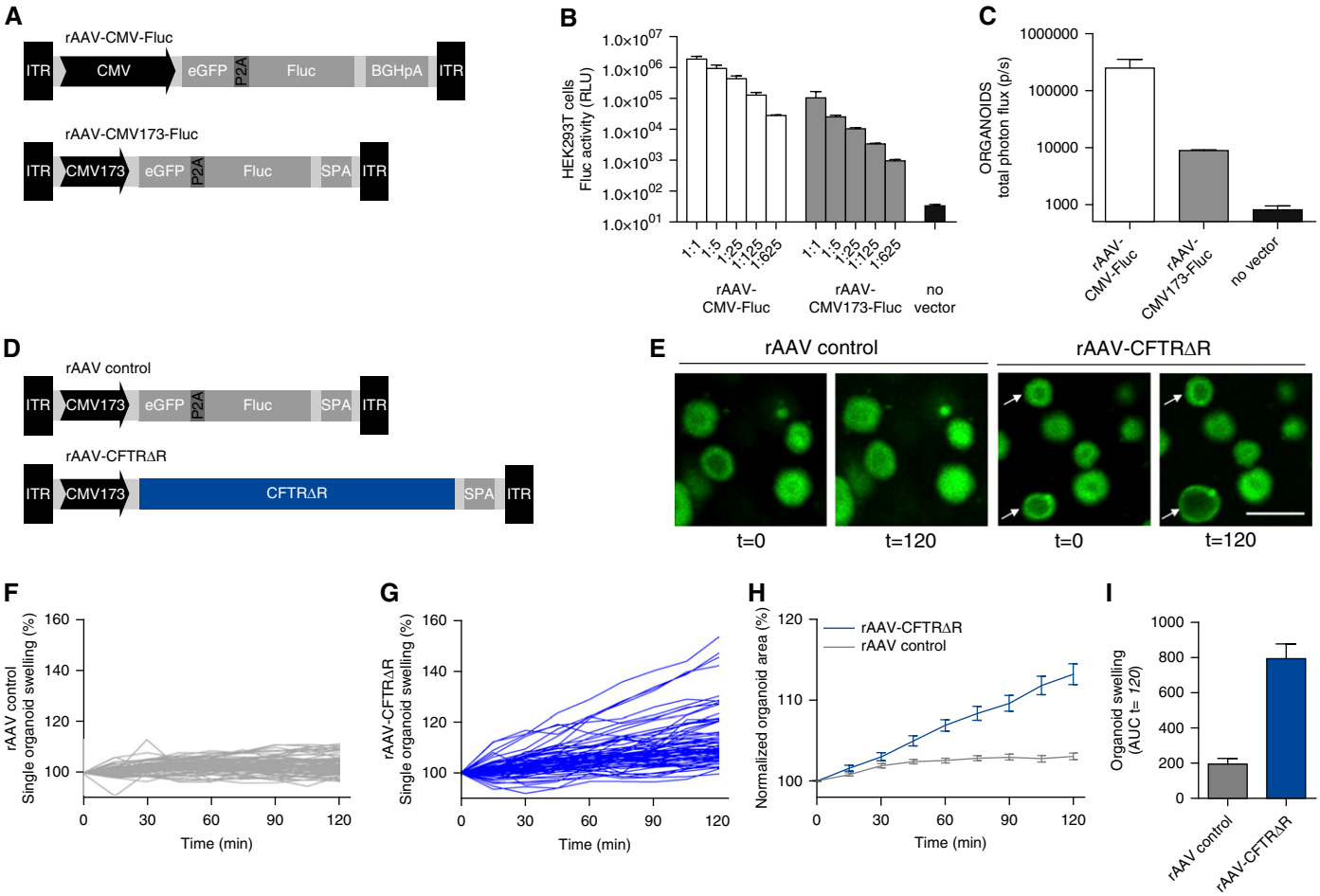

Figure 3. Recombinant adeno-associated viral vector (rAAV)-truncated cystic fibrosis transmembrane conductance regulator (CFTRΔR) gene transfer rescues cystic fibrosis (CF) phenotype in CF organoids. (*A*) Comparison of following rAAV expression cassettes: rAAV-CMV-Fluc contains a cytomegalovirus (CMV) promoter (CMV, 583 bp), bovine growth hormone polyadenylation sequence (BGHpA, 215 bp), and reporter gene construct eGFP-P2A-Fluc. rAAV genome size (inverted terminal repeats [ITRs] included) 4.4 kb. rAAV-CMV173-Fluc contains a shortened CMV (CMV173, 173 bp), minimal polyadenylation sequence (49 bp, SPA), and eGFP-P2A-Fluc (3.2 kb). (*B*) HEK293T were transduced with the respective rAAV2/5 vectors (1:1 dose: 8.0×10^9 GC/well) and scored for Fluc activity in lysates (relative light units [RLU] normalized to total cell number). (*C*) Healthy control organoids were transduced with rAAV2/9 (4.0×10^9 GC/well) and analyzed by bioluminescence imaging (photons/second [p/s]). (*B* and *C*) Background signal was determined in untransduced cells. (*D*) rAAV2/9 expression cassettes for forskolin-induced swelling (FIS) after transduction of CF organoids: rAAV-CMV173-Fluc ('rAAV control') and rAAV-CFTRΔR (5 kb), 3.0×10^8 GC/well. (*E*) Calcein green–labeled transduced organoids visualized before ($t = 0$ min) and after ($t = 120$ min) forskolin stimulation. *Arrows* indicate swelling. *Scale bar* = 100 μm. (*F* and *G*) Surface area increase relative to $t = 0$ after stimulation (normalized area) of individual organoids after transduction with (*F*) rAAV control or (*G*) rAAV-CFTRΔR. (*H*) FIS averaged from *F* and *G*. (*I*) FIS of transduced organoids expressed as area under the curve (AUC) calculated from time periods in *H* (baseline = 100%, $t = 120$ min). CF organoids = E60X/4015ATTTdel. (*B*, *C*, and *I*) Mean + SEM; (*H*) mean ± SEM. eGFP = enhanced green fluorescent protein.

forskolin perfusion was observed in five out of seven mice strengthening the previous observation (Figures 5B and 5D). Interestingly, a strong response to forskolin perfusion (−6 mV) without initial hyperpolarization to low Cl^- was observed in one animal (*see* Figure E4A, *animal 7*). In the rAAV2/5-CFTRΔR-treated group, Cl^- secretion could be inhibited to a larger extent compared with the control group (Figure 5E) ($P = 0.03$), underscoring the contribution of CFTR to the overall transepithelial ion transport. In summary, the ion transport defect in the nasal mucosa was restored by rAAV2/5-mediated CFTRΔR transfer in most treated ΔF508 mice.

Discussion

This study provides a robust proof-of-principle that rAAV-mediated gene transfer of a truncated CFTR leads to functional rescue of CF. We demonstrated a significant improvement in CFTR channel activity in two complementary CF models: first, in human intestinal organoids; and second, in the ΔF508 mouse model, which allows *in vivo* assessment of CFTR correction by NPD, routinely used in patients. Since the discovery of *CFTR* in 1989 (34), the initial high expectations on gene therapy for CF were not met because of lack of persistent clinical benefit. A very recent clinical study by Alton and coworkers (35) demonstrated

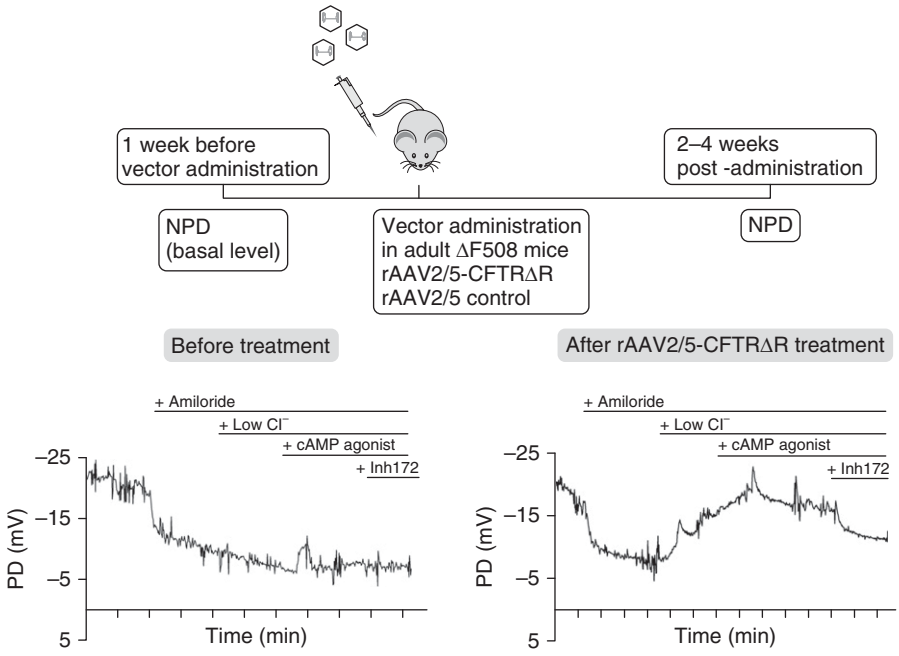

Figure 4. Recombinant adeno-associated viral vector (rAAV) 2/5–truncated cystic fibrosis transmembrane conductance regulator (CFTRΔR) rescues ion transport defect in ΔF508 mouse nasal mucosa. (*Top*) Overview of the experimental set-up. Basal NPD was recorded 1 week before intranasal administration of rAAV2/5-CFTRΔR or a rAAV2/5 control vector in adult mice homozygous for the ΔF508 mutation. NPD was measured 2–4 weeks postadministration. *Bottom* shows an example of an NPD recording before (*left*) and after rAAV2/5-CFTRΔR treatment (*right*) for the same animal. Results are represented as the best responsive nostril. Each interval on the *x-axis* depicts a 1-minute time frame. NPD = nasal potential difference.

that liposome-mediated airway delivery of *CFTR* cDNA stabilized lung function (measured by FEV_1) in treated patients with CF, whereas a further decline was observed in the placebo group, showing a first proof-of-concept of nonviral gene therapy to prevent further loss of lung function, a clinical hallmark of CF airway disease. Although the benefit achieved by this form of nonviral gene therapy is modest and at this stage too premature to become part of clinical care for patients with CF, these results fuel further research on CF gene therapy. Liposomes are safe nonviral delivery vehicles that can transfer a large genetic cargo and allow repeated vector administration. However, transport from the cytoplasm to the nucleus presents a major rate-limiting step (reviewed in Reference 36), in contrast to viral vectors (37).

A first improvement compared with previous unsuccessful rAAV-based clinical trials for CF is the selection of the serotype. We opted for rAAV2/5, which efficiently transduces airway epithelia in mice and humans (10–12, 38), in contrast to rAAV2/2 used in the past (5). It is now known that receptors for that serotype are low to absent at the apical side of the airway epithelium, which could at least in part explain the limited success of rAAV2/2 (39). A second improvement is the use of an optimized expression cassette, which incorporates a truncated CFTR (CFTRΔR) to allow addition of an external promoter (CMV173), which significantly enhanced gene expression. As a result, expression of CFTRΔR in nasal tissue of rAAV2/5-treated ΔF508 mice was detected in our study. In contrast, previous rAAV2/2-based clinical trials for CF incorporated the full-length *CFTR* cDNA into the rAAV expression cassette. Packaging size restriction necessitated the use of the inverted terminal repeat promoter, the short but weak promoter present in the rAAV genome. The inability to detect CFTR expression in biopsies and the clinical failure in those trials may thus be caused by poor transgene expression (5, 6).

The use of a minigene for rAAV-based gene therapy, which has a packaging limit of approximately 5 kb, is a strategy that has already been successfully applied in Duchenne muscular dystrophy (40). In our study, CFTRΔN did not show any intrinsic channel activity (Figure 1D). However, previous studies demonstrated that this truncated form rescues the endogenous ΔF508-CFTR mutant by transcomplementation (29, 41), which can serve as an alternative gene therapy approach. However, although CFTRΔR was developed for a rAAV-based gene therapeutic approach, functionality *in vivo* has only been reported using adenoviral-mediated gene transfer (13). In that perspective, our aim was to evaluate the therapeutic potential of rAAV-CFTRΔR as a novel treatment option for both CF airway and intestinal disease. Although stronger than the AAV inverted terminal repeat, the CMV173 promoter is weaker than the full-length CMV promoter. This moderate activity may be an advantage for CF gene therapy because strong viral promoters result in marked overexpression, with some CFTR channels even being present in the basolateral membrane, thereby reducing transepithelial Cl^- transport at the apical membrane (42). So we believe that the selection of the promoter contributed to our successful results.

Humans are naturally infected with AAV virus. Hence, preexisting immunity poses a major challenge for systemic rAAV-based gene delivery (43–45). However, when rAAV is administered locally to for example the airways, the effect of preexisting neutralizing antibodies might be less pronounced. Different approaches are currently evaluated preclinically to reduce the effect of neutralizing antibodies, such as prior transduction with empty rAAV particles to deplete the serum or bronchoalveolar lavage fluid of rAAV-specific neutralizing antibodies (46) or capsid engineering to generate serologically distinct AAV serotypes (47). Each model used for preclinical validation of gene therapeutic approaches using rAAV requires prior selection of the optimal rAAV serotype for efficient transduction. As such, the rAAV2/5 preference in mouse does not necessarily carry through to humans (48). However, assuming that rAAV2/5 would be translated into the clinic, 40% of the human population is estimated to be seropositive and 3.2% positive specifically for anti-AAV5 serum neutralizing factors (49–51). This prevalence is among the lowest of all natural AAV serotypes evaluated (49).

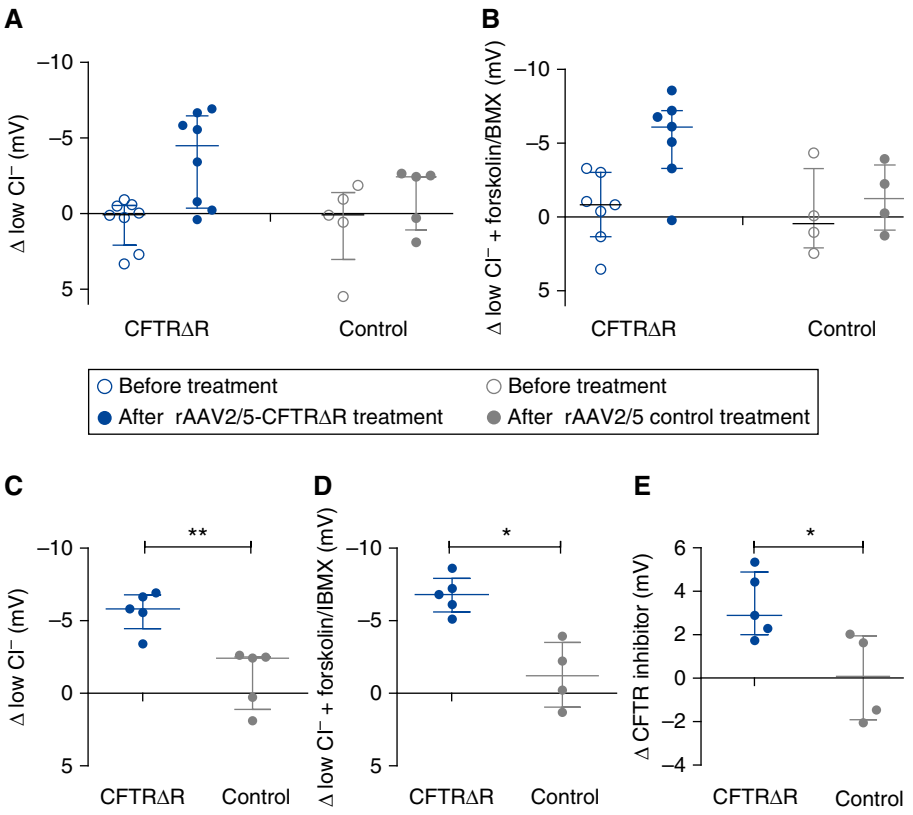

Figure 5. Nasal potential difference measurements demonstrate rescue after recombinant adeno-associated viral vector (rAAV) 2/5–truncated cystic fibrosis transmembrane conductance regulator (CFTRΔR) gene transfer. Adult mice homozygous for the ΔF508 mutation were treated intranasally with rAAV2/5-CFTRΔR (n = 8) or rAAV2/5 control vector (n = 5). At 2–4 weeks after administration, changes in transepithelial ion transport across nasal mucosa were assessed by NPD. (*A*) Response to low chloride perfusion. (*B*) Cumulative response to low chloride and forskolin/3-isobutyl-1-methylxanthine (IBMX) perfusion. (*A* and *B*) Basal values, recorded 1 week before vector administration, are presented at the left side of both graphs for each treatment group (*open circles*). (*C*) Comparison of the response to low chloride in animals that responded to rAAV2/5-CFTRΔR gene transfer compared with the group treated with rAAV2/5 control vector. (*D*) Comparison of the cumulative response to low chloride and forskolin/IBMX perfusion after vector treatment in animals that responded to rAAV2/5-CFTRΔR gene transfer compared with the group treated with rAAV2/5 control vector. The cut-off for positivity in animals that responded to rAAV2/5-CFTRΔR gene transfer was determined as the highest value obtained in basal NPD measurements 1 week before treatment (*open circles*) for the respective group. (*E*) Effect of perfusion with CFTR inhibitor (inh172) evaluated after CFTR activation with low chloride and forskolin/IBMX perfusion and plotted as the difference in potential difference (ΔPD) between activation and inhibition. Measurements of individual animals were plotted as single values, and the median ΔPD plus interquartile range per group is depicted. The response to vector treatment between the two groups was compared using a Mann-Whitney test for unpaired observations (*$P < 0.05$; **$P < 0.01$). NPD = nasal potential difference.

Compared with other AAV serotypes, AAV5 seroconversion occurs later in life (around 15–20 yr of age) (reviewed in Reference 52). This implies that a broader time window is available for rAAV2/5 gene therapy treatment in patients, situated between the decrease in maternal anti-AAV5 antibodies found in newborns during the first few months after birth and the occurrence of AAV5 infection (reviewed in Reference 52).

Although rAAV is a mainly nonintegrating vector, it can provide sustained gene expression in different low-proliferative organs including the mouse lung (11, 38) and the liver, where systemic rAAV2/8 administration resulted in a reduction of the bleeding phenotype in hemophilia B patients for more than 3 years (44, 45). Estimating the longevity of gene expression depends on the specific cell types transduced. For instance, ciliated airway epithelial cells, a primary target for CF gene therapy, are terminally differentiated and their half-life is estimated to be approximately 6 to even 17 months (53). Nevertheless, although the airway epithelium is a slowly proliferating tissue, repeated vector doses will most likely be necessary to ensure lifelong correction for specific diseases, such as CF. To that end, further exploration of approaches to circumvent an immune response to the AAV capsid is essential, such as serotype switching with a serologically distinct rAAV serotype (54), transient immunosuppression (55), vector administration to an immature immune system (12, 56), or prolonging the time period between vector administrations (11, 57). The safety of repeated rAAV2/2-CFTR doses has already been demonstrated in a clinical setting (6).

Apart from rAAV-based gene therapy for CF, other viral vectors are currently being investigated to target the airways, such as LV (for a review, *see* Reference 58). Although prolonged gene expression can be obtained, it is yet to be elucidated if sustained LV-mediated gene expression results from transduced long-living terminally differentiated epithelial cells or from vector integration into stem cells (37, 59, 60). An integrating vector is in theory capable of transducing airway stem cells. Many of these cells reside in specific anatomic niches (61); however, basal cells, one of the main stem cells described for adult airways, do not (62). They are located at the basal lamina of the pseudostratified respiratory epithelium and are thus not in contact with the lumen of the conducting airways. Therefore, the *in vivo* potential of transducing these different stem or progenitor populations via local airway delivery will have to be investigated for the specific viral vector system used.

Organoid experiments are valuable because they allow functional vector assessment in CF patient-derived material using a sensitive and quantitative FIS assay. The rescue we obtained in human CFTR-deficient organoids not only provides evidence that rAAV-CFTRΔR can restore chloride and fluid transport, but at the same time highlights that the gastrointestinal CF phenotype can be corrected by gene therapy. The CF mouse model complements the organoid data because here a potential rescue of the CF

phenotype can be assessed *in vivo* by NPD, a widely accepted biomarker used for evaluation of response to treatment in patients with CF (28, 63, 64). Our results show a clear response to low Cl^- and forskolin perfusion in most treated mice, underscoring the therapeutic potential of rAAV2/5-CFTRΔR. Importantly, the level of rescue after rAAVA2/5-CFTRΔR gene transfer was comparable with the level of rescue after CFTR corrector treatment as measured by NPD (65). Variation in treatment response could be explained by differences in transduction efficiency between animals. This could affect NPD outcome depending on where exactly the measuring electrode was placed on the nasal epithelium.

In conclusion, this study provides a robust proof-of-principle that rAAV-mediated gene transfer of a truncated CFTR leads to functional rescue of the CF phenotype in two complementary models: gastrointestinal pathology in human intestinal organoids, and *in vivo* assessment of CFTR correction in the nasal mucosa of CF mice. A next step is to investigate the therapeutic effect of rAAV-CFTRΔR in a larger animal model for CF, such as the CF rat, ferret, or pig, because these models recapitulate human CF pathology more faithfully and allow a better assessment of the level of CFTR rescue required to ameliorate CF (66–69). If successful, gene therapy would drastically improve life expectancy and life quality of patients with CF by offering a definite cure for CF in a mutation-independent manner. ■

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