Engineered mutant α-ENaC subunit mRNA delivered by lipid nanoparticles reduces amiloride currents in cystic fibrosis–based cell and mice models

Anindit Mukherjee1, Kelvin D. MacDonald1,2, Jeonghwan Kim1, Michael I. Henderson3, Yulia Eygeris1, Gaurav Sahay1,4,*

Cystic fibrosis (CF) results from mutations in the chloride-conducting CF transmembrane conductance regulator (CFTR) gene. Airway dehydration and impaired mucociliary clearance in CF is proposed to result in tonic epithelial sodium channel (ENaC) activity, which drives amiloride-sensitive electrogenic sodium absorption. Decreasing sodium absorption by inhibiting ENaC can reverse airway surface liquid dehydration. Here, we inhibit endogenous heterotrimeric ENaC channels by introducing inactivating mutant ENaC α mRNA (αmutENaC). Lipid nanoparticles carrying αmutENaC were transfected in CF-based airway cells in vitro and in vivo. We observed a significant decrease in macroscopic as well as amiloride-sensitive ENaC currents and an increase in airway surface liquid height in CF airway cells. Similarly, intranasal transfection of αmutENaC mRNA decreased amiloride-sensitive nasal potential difference in CFTRKO mice. These data suggest that mRNA-based ENaC inhibition is a powerful strategy for reducing mucus dehydration and has therapeutic potential for treating CF in all patients, independent of genotype.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder that affects 70,000 patients worldwide. Mutations in the CF transmembrane conductance regulator (CFTR) gene leads to defects in CFTR protein that cause impaired Cl− ion transport at the luminal airway surface along with tonic Na+ and water reabsorption (1). The result is thick inspissated mucus that is retained in the airways, leading to permanent bacterial colonization and progressive pulmonary deterioration (2). Current strategies to restore airway hydration include CFTR protein modulator therapy that partially corrects defective membrane trafficking and function of CFTR, restoring chloride transport and mucus hydration in the airways (3). However, partial restoration of CFTR protein function by gene therapy does not appear to alter dysregulation of Na+ absorption (4). Thus, continued Na+ dysregulation and water reabsorption may account for the variability in clinical endpoints (e.g., lung function and sweat chloride) with the otherwise highly effective CFTR modulator therapy (3, 5).

In the airway, epithelial sodium channel (ENaC) performs electrogenic absorption of Na+ from the mucosal surface that results in water reabsorption (6). The drug amiloride specifically inhibits ENaC while being a poor inhibitor of other channels and transporters at lower concentrations. Thus, amiloride-sensitive sodium current is used to measure ENaC activity (7). ENaC activity is proposed to be regulated by CFTR (8). Dysregulation of ENaC activity is a common feature across many CFTR gene mutations (9). In CF, excessive water absorption due to unopposed ENaC activity results in dehydrated airway surface liquid (ASL), which, in turn, impairs ciliary motion (10). Restoring ASL height enhances mucociliary clearance and leads to improvement of pulmonary function (11, 12). One proposed approach to increase ASL height and restore mucus hydration is to reduce mucosal absorption of Na+ and water by inhibiting ENaC. Unlike CFTR modulator therapy that is only effective for specific CFTR mutations, this strategy would benefit all CF patients (3, 5). Previous and current approaches of ENaC inhibition have included drugs acting on ENaC directly, agents targeting ENaC-specific proteases, and RNA interference (RNAi) or antisense-based oligonucleotides against specific ENaC subunits (10). ENaC as a target in CF has not yet translated to a clinical therapeutic because of concerns for efficacy and duration of action as well as off-target or systemic effects of the various molecules tested so far (10).

ENaC is a heterotrimeric channel composed of three homologous α, β, and γ subunits, with the α-ENaC subunit being essential in forming a functional channel (13). Channels lacking α subunit are completely nonfunctional, whereas channels lacking β or γ subunits are hypofunctional (13). Human airways express a lesser studied ENaC δ subunit that is phylogenetically close to the ENaC α subunit (14). The α and δ subunits can be substituted for each other to express an atypical δγ channels along with the more common αβγ channels, which have differing bioelectric properties (15). The ENaC inhibitor amiloride has an IC50 (median inhibitory concentration) in the concentration range of 0.1 to 0.5 μM for human αβγ ENaC and approximately 2.5 μM for human δγ ENaC (16). Previous attempts at blocking ENaC activity with amiloride failed because of its rapid clearance from the lung (10). We hypothesized that ENaC activity can be reduced by deploying mRNA that encodes for a channel-inactivating ENaC α (αmutENaC) subunit packaged inside lipid-based nanoparticles (LNPs) for delivery. LNPs are clinically approved for small interfering RNA (siRNA) delivery and are in clinical trials for delivery of mRNA-based therapeutics and vaccines (17). We postulated that exogenous αmutENaC would compete with endogenous α and δ subunits to form inactivated channels resulting in reduced amiloride-sensitive currents in target cells. We also propose that reduction in ENaC activity would increase ASL height in CF airway cells (CFBE410-). In this study, we used an in vitro model to determine the effect of αmutENaC mRNA transfection on ENaC

1Department of Pharmaceutical Sciences, College of Pharmacy, Robertson Life Sciences Building, Oregon State University, Portland, OR 97201, USA. 2Department of Pediatrics, School of Medicine, Oregon Health & Science University, Portland, OR 97239, USA. 3Department of Cell, Developmental and Cancer Biology, Oregon Health & Science University, Portland, OR 97201, USA. 4Department of Biomedical Engineering, Robertson Life Sciences Building, Oregon Health & Science University, Portland, OR 97239, USA. *Corresponding author. Email: sahay@ohsu.edu
activity as well as its effect on regulating ASL height in polarized CFBE41o- cells. For in vivo experiments, we used intranasal transfection of \( \alpha_{\text{mut ENaC}} \) mRNA containing LNP and measured amiloride-sensitive nasal potential difference (NDP) in CFTR knockout mice. Together, our experiments show that intracellular delivery of mutant ENaC subunits can be a novel and promising strategy to inhibit sodium and water reabsorption activity in vitro and in vivo and should be pursued as a CF therapeutic.

**RESULTS**

**Formulated LNPs effectively transflect mRNA into CFBE41o- cells**

Our LNPs consisted of four types of lipids: (i) ionizable lipid \([O-(Z,Z,Z,Z,\text{heptatriaconta-6,9,26,29-tetraen-19-yl})-4-(N,N-\text{dimethylamino})\text{(DLin-MC3-DMA})] ;\) (ii) cholesterol; (iii) helper lipid, distearoylphosphatidylcholine; and (iv) dmyristoyl-rac-glycerol-3-methoxypropylethylene glycol-2000 (DMG-PEG2k). They were formulated by microfluidic mixing of mRNA and lipids. Further characterization for size and encapsulation by dynamic light scattering analysis showed a hydrodynamic diameter of 73 nm with a polydispersity index of less than 0.1 (Fig. 1A). LNPs had high mRNA encapsulation (>99%) and a spherical shape (Fig. 1, A and B). LNPs were used to transflect either enhanced green fluorescent protein (EGFP) or firefly luciferase (Luc) mRNA in CFBE41o- cells, as reporter genes. Fluorescent signal was detected in CFBE41o- cells treated with the LNPs encapsulating EGFP mRNA by confocal microscopy 24 hours after transfection (Fig. 1C). Moreover, a dose-dependent response was observed with LNPs used to deliver Luc mRNA transfected at 24 hours after transfection (Fig. 1D). No significant change in cell viability was observed after luciferase transfection, whereas 10% dimethyl sulfoxide (DMSO) treatment killed approximately 65% (% viability: 36.89 ± 3.13) of cells (fig. S1, A and B).

\( \alpha_{\text{mut ENaC}} \) mRNA transfection reduces amiloride-sensitive current in CFBE41o- cell

The \( \alpha \) ENaC subunit is cleaved at two sites by furin, a pro tease that resides in the trans-Golgi network, to release an imbedded inhibitory peptide that leads to activation of ENaC (18). To generate a channel-inactivating \( \alpha_{\text{mut ENaC}} \) mutant, we engineered an mRNA containing two sets of modifications. First, the conserved arginines at the degenerin site of the \( \alpha \)-ENaC subunit were mutated to alanines (Fig. 2A; also see fig. S2, A and B) (19). These mutations prevent furin from excising an 8-mer imbedded inhibitory peptide in the \( \alpha \) subunit that is critical for ENaC activation (19). The resultant channel will have a low open probability. Second, a single Ser → Ala substitution mutation was made on the conserved serine residue at the degenerated site in the second transmembrane domain of \( \alpha \) ENaC subunit (Fig. 2A). Serine residues are prone to undergo enzymatic and nonenzymatic posttranslational modifications such as acetylation, methylation, N-linked or O-linked glycosylation, pyrophosphorylation, and carboxymethylation that entail addition of a bulky residue on the amino acid side chain (20, 21). Previous studies have shown that modification or substitution of the conserved serine with a bulky amino acid residue at the degenerin site leads to a significant increase in channel open probability even after furin modification (22). To prevent such bulky posttranslational modifications of the serine that might compromise the desired effects of the engineered mutations in the extracellular domain, we conservatively substituted serine with alanine that is resistant to modifications (23). Together, the mutant \( \alpha \) subunit carrying these two sets of mutations will drive assembled ENaC to an inactivated state.

Expression of \( \alpha_{\text{mut ENaC}} \) protein was confirmed 72 hours after transfection of C-terminal V5 epitope–tagged \( \alpha_{\text{mut ENaC}} \) mRNA by LNP in CFBE41o- cell lysates by immunoblotting (Fig. 2B). Polarized CFBE41o- cells grown on polycarbonate supports were transfected with \( \alpha_{\text{mut ENaC}} \) mRNA–LNP, while the luciferase–transfected or untransfected cells served as controls. Short-circuit currents (\( I_{SC} \)) of \( \alpha_{\text{mut ENaC}} \)-transfected cells were recorded 72 hours after transfection before and after amiloride, a specific ENaC inhibitor, was added to the apical side and compared with untransfected controls (Fig. 2, C and E). In short-circuit current experiments, we noted that the overall current is significantly lower (\( P < 0.05 \)) in \( \alpha_{\text{mut ENaC}} \)-transfected (0.9 ± 0.78 \( \mu \)A) cells compared to untreated cells (6.09 ± 3.1 \( \mu \)A) (Fig. 2, C and E) at rest, whereas transfection of luciferase mRNA LNP did not alter \( I_{SC} \) compared to the control group at rest (Fig. 2, D and E). Addition of amiloride led to rapid depolarization of \( I_{SC} \) in the controls (Fig. 2, C and D). Amiloride-sensitive current likely reflects ENaC activity and is measured by calculating the difference between \( I_{SC} \) pre- and post-amiloride treatment (24). Amiloride responses of luciferase–transfected controls were similar to untreated controls (Fig. 2F). However, our data show that \( \alpha_{\text{mut ENaC}} \) transfection (0.61 ± 0.44 \( \mu \)A) led to a significant decrease (\( P < 0.05 \)) in the amiloride-sensitive current compared to controls (1.39 ± 0.66 \( \mu \)A), suggesting an inhibition of ENaC activity (Fig. 2F). The cell viability upon transfection with the mutant ENaC mRNA was not compromised (fig. S1, A and B).

In parallel to \( I_{SC} \) measures, we also recorded transepithelial resistance (TER) of the monolayers during short-circuit current experiments in \( \alpha_{\text{mut ENaC}} \) mRNA–transfected and control groups before and after addition of amiloride (table S1). As expected, amiloride treatment increased resistance across the inserts in all groups due to a decrease in electrogenic ENaC activity, thus ensuring that the epithelial tight junction integrity was preserved after treatment with either \( \alpha_{\text{mut ENaC}} \) or the luciferase mRNA–LNP (Fig. 2G). \( \alpha_{\text{mut ENaC}} \) transfection did not alter TER in polarized CFBE41o- cells (fig. S3), suggesting that one or more of the apical, basolateral, or paracellular resistances that contribute to the TER may be altered to keep the overall TER unchanged. Last, change in amiloride-sensitive resistance, measured as the difference in resistances before and after addition of amiloride, was similar in \( \alpha_{\text{mut ENaC}} \)-transfected and untransfected controls. Because other ionic pathways such as that mediated by CFTR also regulate airway epithelial resistance (25), our data may indicate alteration in such pathways that additionally influence TER in response to acute and chronic ENaC inhibition in \( \alpha_{\text{mut ENaC}} \)-transfected cells.

\( \alpha_{\text{mut ENaC}} \) transfection increases ASL height in CFBE41o- cells

The ASL lines the mucosal surface of airway cells facilitating mucociliary clearance when at the normal ciliary height. Mucus moves above the watery periciliary liquid fraction of the ASL because of ciliary movement (26). In CF, dehydration of the periciliary liquid reduces ASL height, impairing mucociliary clearance (27). We used dextran beads conjugated with rhodamine (red) to label the ASL and calcine (green), a membrane-permeable live cell marker to demarcate the cell (Fig. 3A). Using confocal microscopy, representative
three-dimensional (3D)–rendered images from the ASL to the basolateral surface of the cells were generated (Fig. 3A). The vertical height of labeled dextran from the cellular surface was considered as measure of ASL height. Confocal z-section images on live cells were obtained from six relatively fixed points on Transwell inserts. For measuring ASL height, average vertical height of rhodamine-dextran was computed from three randomly selected points in a single image representing one of the six fixed points. This measurement was repeated on images of all six points on the insert for each experimental group for obtaining average ASL height on each membrane. We hypothesized that reduction of ENaC activity will decrease mucosal Na⁺ and water hyperabsorption and result in an increase in the ASL height. CFBE-WT cells that stably overexpress wild-type human CFTR (WT) were used as a positive control. Expectedly, the ASL height of CFBE-WT cells (39.05 ± 1.40 μm) was greater (P < 0.01) than that of the untransfected CFBE41o- cells (24.51 ± 2.83 μm) (Fig. 3B). αmutENaC
mRNA–LNP transfection in CFBE41o- cells led to a significant increase ($P < 0.001$) in ASL height (35.16 ± 4.06 μm) compared to untransfected controls (23.03 ± 2.73 μm) (Fig. 3C), suggesting that LNP transfection of αmut ENaC led to increased ASL height in CF patient–derived cell lines.

**αmutENaC transfection reduced amiloride-sensitive NPD in CFTR KO mice**

Having observed αmut ENaC-mediated inhibition of endogenous ENaC activity in vitro, we proceeded to assess these effects in vivo using mouse models. We used intranasal instillation to transfet murine nasal epithelium with a luciferase reporter gene and imaged luciferase activity. Five hours after administration of LNP-delivered luciferase mRNA, a strong bioluminescence was observed in the nasal cavity indicating effective gene delivery in the nasal epithelium (Fig. 4A). A previous study from our group has shown that Luc mRNA transfection does not alter electrical properties of nasal epithelia in mouse (28).

**In vivo efficacy of αmut ENaC mRNA was evaluated by measurement of NPD.** NPD measurement is a well-established procedure...
performed clinically on patients for a definitive diagnosis of CF, based on net transepithelial ion transport activities of CFTR and ENaC (29). Nasal respiratory epithelium can be used as a substitute for measurements of electrophysiological activities of the lower airways, because they share common bioelectric properties (29).

NPD measurements were performed on WT control mice (Fig. 4B) as a baseline, by sequential perfusion with the following buffers (table S2): (i) Ringer’s: modified Ringer’s buffer to measure the net potential difference arising from the baseline activity of all channels in the nasal airway epithelium; (ii) Amil: modified Ringer’s buffer containing amiloride, which targets ENaC activity; (iii) 0Cl−/Iso: modified zero [Cl−] Ringer’s buffer containing isoproterenol, which raises adenosine 3′,5′-monophosphate (cAMP) in the cells, leading to CFTR-mediated Cl− secretion along with amiloride for continuing ENaC inhibition; (iv) ATP (adenosine 5′-triphosphate): modified zero [Cl−] Ringer’s buffer containing ATP for stimulating Cl− efflux by activation of calcium-activated chloride channels and isoproterenol and amiloride (28). We found that the baseline potential difference in WT mice was −1.58 ± 1.23 mV, n = 4 (table S3). Expectedly, WT mice showed a typically small amiloride response (amiloride-sensitive NPD: 1.40 ± 0.58 mV, n = 4) (table S3), whereas the magnitude of CFTR-mediated isoproterenol response was 22.11 ± 4.95 mV (n = 4) (table S3), suggesting a subdued ENaC and normal CFTR activity that is expected in WT mice (30). Additional calcium-activated chloride channel–mediated hyperpolarization response induced by ATP served as an internal control, indicating both the intactness of the nasal epithelium and correct positioning of the nasal bridge (Fig. 4B).
Next, CFTRKO mice were used to perform electrophysiological studies for measuring the effect of $\alpha_{\text{mut}}$ENaC mRNA–LNP transfection in the nasal respiratory epithelia. Baseline NPD recording was performed in CFTRKO mice on day 0, before any treatment, following the same in vivo perfusion protocol used for WT mice (Fig. 4C). After a rest period, the same animals were transfected with $\alpha_{\text{mut}}$ENaC mRNA–LNP in the nasal epithelia on days 7 and 8; thus, each animal was its own control. NPD recording assessing the effect of LNP...
transfection was performed after 72 hours on day 11. After 2 weeks of rest, NPD recording was repeated on the same animal to confirm extinction of the effect of LNP transfection on day 27 (Fig. 4C). NPD recording before transfection shows a hyperpolarized baseline (−14.41 ± 1.97 mV) followed by a characteristically large depolarization after perfusion with amiloride-containing Ringer’s buffer (amiloride-sensitive NPD: 7.42 ± 1.92 mV) (Fig. 4, D and E) (31). As expected, the CFTRKO mouse exhibited no isoproterenol response. Large hyperpolarizing response was observed after ATP treatment, indicating intact epithelia in these animal models (Fig. 4D, gray line). After gene transfection of αmutENaC mRNA, we found that the amiloride-mediated depolarization (amiloride-sensitive NPD: 3.57 ± 1.92 mV) was blunted (P < 0.005) in animals (Fig. 4, D and E). Amiloride-mediated depolarization regained perhaps due to clearance of the exogenous mutant mRNA after 2 weeks (Fig. 4D, blue line). The overall potential difference before amiloride treatment in the transected (−12.86 ± 2.74 mV) and untransfected (−14.41 ± 1.97 mV) groups remained similar (Fig. 4E). Addition of amiloride in the untransfected group led to a significant decrease (P < 0.01) in overall potential difference (−Amil versus +Amil: −14.41 ± 1.97 mV versus −6.99 ± 1.88 mV), whereas in the αmutENaC-transfected animals, the amiloride response did not change significantly (−Amil versus +Amil: −12.86 ± 2.74 mV versus −9.29 ± 1.66 mV) (Fig. 4E). The amiloride-sensitive potential difference was greater in untransfected animals compared to animals treated with mutant mRNA, signifying that ENaC activity was inhibited (Fig. 4F). The third NPD recording performed after 2 weeks showed a restoration of the amiloride-sensitive potential difference to the levels of untransfected animals, suggesting that the mutant protein was cleared (Fig. 4F).

DISCUSSION

In this study, we have shown that engineered ENaC α mRNA mutants decreased amiloride-sensitive currents as a measure of ENaC activity. Unopposed or excessive ENaC channel activity is a longstanding proposed mechanism in CF pathophysiology (Fig. 5, A and B) (32). ENaC mutations in the α and β ENaC subunits that result in increased channel activity cause a CF-like phenotype (33, 34). Lung-specific β ENaC subunit–overexpressing mice develop thick inspissated mucus and lung disease similar to CF (35). In contrast, patients with an ENaC-inactivating mutation showed increased ASL height and greater mucociliary clearance (36). Heightened amiloride responses in CF patients and patient-derived cell lines suggest enhanced ENaC activity (1, 37) such that the channel function is either increased or unopposed. The exact mechanism of ENaC regulatory activity in the context of a functional decrease in CFTR activity is unclear. However, it is conceivable that in CF, a decrease in either the expression or functional CFTR activity alters inhibitory influences on ENaC. ENaC is activated by specific proteases (38) and conversely inhibited by extracellular Cl− (39). In CF, mucosal [Cl−] is decreased (37). Moreover, contraction of ASL volume leads to increased concentration of extracellular proteases that cleave ENaC and result in its activation (38). Previous studies have shown that inhibiting ENaC-activating proteases results in an increase in ASL height in cultured cells (38). Thus, ENaC–mediated sodium absorption and attendant mucus dehydation is an independent therapeutic target in the treatment of CF.

Early approaches that used inhaled ENaC-specific inhibitors such as benzamil and amiloride were ineffective because of rapid clearance from the lung (10). More complete ENaC inhibition by candidate compounds—GS-9411, P-552-02, and compound A—that were purported to have better pharmacokinetics and potency exhibited detrimental side effects (40, 41). Chronic and complete inhibition of ENaC and ENaC-dependent renal K+ excretion by these compounds resulted in hyperkalemia (40, 41). Thus, to reduce but not completely attenuate ENaC activity, several groups have attempted targeted gene knockdown of the ENaC subunits (10, 42). Prevailing antisense oligonucleotides and RNAi–driven, knockdown-based approach reduced ENaC activity both in vitro and in vivo by predominantly silencing of the α ENaC subunit. Both approaches led to an increase in ASL height in cultured cells (42). Although currently a promising inhalable siRNA formulation...
known as ARO-ENaC is being tested in a phase 1/2 clinical trial (43), past attempts have not succeeded in yielding an RNAi-based therapeutic for CF (10). Moreover, these approaches are inherently prone to off-target silencing of unintended genes (44). ENaC is part of the larger degenerin family of proteins that include acid-sensing ion channels (45). These channels share large homologies with ENaC and play an important role in lung physiology (46) and can be knocked down (7). However, mRNAs can be engineered to incorporate chemical modifications and regulatory elements to minimize immunogenicity and off-target protein expression, respectively (47). Last, indirect anti-ENaC approaches such as inhibition of SPLUNC1 facilitated ENaC degradation, which led to decreased channel activity (48). Although effective in vitro, clinical trials of SPLUNC1 inhibitors were not successful, leaving ENaC inhibition an unmet target in CF therapeutics.

In this study, we used mRNA as an ion channel inhibitor (Fig. 5C). Our mRNA therapeutics have the advantage of offering versatility in regulating and fine-tuning both αβγ and δβγ channel activities. The δβγ channels have been shown to be expressed widely in human airway epithelial cells (49). These channels have significantly higher open probability and sodium conductance compared to the typical αβγ channels (50). We proposed that our engineered αmutENaC might compete with both endogenous α and δ subunits to form inactivated αmutβγ channels to effectively reduce overall ENaC activity. Future studies using exogenously transfected WT and mutant ENaC subunits that carry different epitope tags will be used to conclusively show mutant αmutENaC integration into WT αβγ and δβγ ENaC channels expressed at the surface of cultured cells. Appropriately designed mutant subunits of ENaC will also be able to regulate the degree of ENaC inhibition. For example, mutating the furin cleavage site of the γ ENaC subunit has been shown to have a lesser inhibitory impact on channel activity when compared to mutations in furin cleavage sites in the α ENaC subunit (18). Other mutations such as those in the cysteine-rich domains or the thumb domain of β ENaC subunit reduce channel cell surface expression and channel half-life (51). Such mutations can be taken advantage of for targeting different aspects of channel biogenesis and function, thereby finely regulating ENaC activity by modifying channel expression, gating, or response of the channel to modulators such as proteases (7, 52, 53). Furthermore, because mutant α ENaC subunit alone cannot form functional channels, transfection of αmutENaC mRNA will likely not alter ion transport profile in cells that do not express endogenous ENaC. Thus, even without using targeted delivery mechanisms, our strategy will effectively alter ion transport specifically in ENaC-expressing cells and thus minimize off-target effect on ion transport profiles in nontarget cells (54).

On the basis of the previously discussed studies, we engineered α ENaC mRNA that lacks furin sites in the extracellular domain, while the conserved serine residue in the degenerin site was mutated to alanine (55). The furin site mutations inhibit ENaC activity, while the precautionary mutation at degenerin site helps maintain ENaC in an inhibited state. Our mutant αENaC mRNA used in this study was effective in reducing amiloride-sensitive current (by 85.22%) in CFBE41o- (untreated 6.09 ± 3.1 versus αmutENaC-transfected 0.9 ± 0.78 cells; Fig. 2, B and D), which is in comparable level to that achieved by RNAi-based methods (10, 42).

Improvement in ASL height is a precursor to improved mucociliary clearance in the airways. ASL height is maintained by the coordinated action of CFTR, ENaC, and aquaporins in the airway cells (56, 57). Transcellular water transport is driven largely by the movement of Na+ and Cl− ions across the epithelia (58). We tested whether αmutENaC-mediated suppression results in increasing the height of ASL. In our study, the immortalized cells had been grown to confluence before treatment that resembles the state of the airway in vivo. Our data show that ENaC inhibition led to an increase in ASL height in CFBE41o-cells by 34%. This is similar to a recent in vitro ASL change using siRNA ENaC targeting (42). In that study, Tagalakis et al. demonstrated that the change in ASL was enough to increase ciliary beat frequency. Our future studies will focus on determining whether delivery of CFTR or αmutENaC mRNA alone or in combination can increase ciliary beat frequency in polarized cultured primary CF patient–derived lung or nasal epithelial cells.

We have previously shown that LNP-delivered CFTR mRNA restores Cl− secretion in vitro and in vivo (28). As suggested by previous reports, we observed that amiloride-sensitive potential difference is elevated in CFTRKO mice, suggesting an increase in ENaC activity (30, 35). In our experiments, intranasal delivery of αmutENaC mRNA–LNP over two consecutive days resulted in a decrease in amiloride response, which is regained after 2 weeks in CFTRKO mice. A key challenge to be addressed in future studies will be to overcome the characteristic thick mucus barrier of CF airways for efficient delivery of mRNA via LNPs for which we will use a β-ENaC–overexpressing mouse model that replicates inspissated mucus phenotype observed in CF patients (35). In this model, LNPs can be delivered by aerosol and deposition to the lung studied. Last, mucus barriers will be applied ex vivo to epithelial monolayers to demonstrate LNP penetration. Nanoparticles coated with non–mucus-adhesive polymers such as PEG have been shown to readily penetrate CF sputum. Thus, sputum–penetrating drug- and gene-carrier nanoparticles can be developed for CF. We have not examined whether ENaC inhibition by αmutENaC transfection alters chloride conductance in cells. However, previous studies have shown that correction of CFTR chloride efflux by gene therapy does not significantly alter dysregulated sodium absorption in human and murine studies (4, 28, 59). Amiloride-sensitive NPD did not alter significantly after CFTR transfection in two studies (28, 59). Therefore, complementary treatment with αmutENaC and CFTR mRNA could lead to optimized ion transport and ASL height regulation. Lipid nanoparticles can be used to deliver these mRNA in combination (60). Similarly, ENaC inhibition in combination with partial correction of chloride transport achieved by the current triple combination CFTR modulator therapy may prove to be beneficial for nonresponders as well as patients who respond modestly to the drug regimen (3, 5). Through these studies, we have shown that engineered mRNA can be used to inactivate the ENaC channel selectively in vitro and in vivo. Our results showcase the promise of mRNA therapeutics as a potential universal treatment of CF regardless of underlying CFTR mutation.

MATERIALS AND METHODS
Materials
Engineered mRNAs were produced at TriLink BioTechnologies Inc. (San Diego, CA). 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesterol (catalog number C8667) was purchased from Sigma-Aldrich Corp. (St. Louis, MO), DMG-PEG3K was obtained from NOF America, and DLin-MC3-DMA was custom-synthesized by Biofine Inc. (Vancouver, BC, Canada).
LNP formulation and characterization
Our LNPs are composed of DLin-MC3-DMA, cholesterol, DSPC, and DMG-PEG2k, along with the engineered mRNA and are assembled using microfluidic mixing as described previously (28). Briefly, all lipids were dissolved in pure ethanol at 50:38.5:10:1.5 molar ratios, and mRNA was diluted in 50 mM citrate buffer solution. The amount of ENaC mRNA ranged from 150 to 200 μg per batch. The N/P ratio between mRNA and DLin-MC3-DMA was 5:30 in all LNPs. Lipid solution and mRNA solution were mixed by microfluidics using NanoAssemblr Benchtop (Precision NanoSystems Inc., Vancouver, BC, Canada) at a 1:3 ratio, followed by dialysis against sterile phosphate-buffered saline (PBS) overnight using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Waltham, MA) with 10,000-Da molecular weight cutoff (MWCO). The resulting LNP solutions were concentrated using Amicon Ultra centrifugal filter units with 10,000-Da MWCO (Millipore). Hydrodynamic size and polydispersity index of LNPs were characterized by dynamic light scattering using Zetasizer Nano ZSP (Malvern Instruments, Malvern, Worcestershire, UK). mRNA encapsulation was assayed using a Quant-iT RiboGreen RNA Assay kit (Thermo Fisher Scientific, Waltham, MA) and a multimode microplate reader (Tecan Trading AG, Männedorf, Switzerland).

Cell culture
CFBE41o- and hCFTR-expressing CFBE-WT cell cultures, gifts from J. P. Clancy and D. Gruenert, were maintained in vented T75 tissue culture flasks (Corning Inc., Corning, NY) using minimum essential medium culture medium (Corning Inc., Corning, NY), supplemented with 10% fetal bovine serum and penicillin/streptomycin/glutamine (Corning Inc., Corning, NY) in humidified 5% CO2 incubator. For electrophysiological studies, 200,000 cells were seeded onto collagen I–coated 12-mm Snapwells/Transwells (Corning Inc., Corning, NY). Cultures were maintained with medium change every other day. Transepithelial electrical resistance (TEER) measurements were performed with Millicell ERS-2 (EMD Millipore, Burlington, MA) 30 min after medium change for checking tight junction formation. Cells were transfected when TEER values stabilized to >200 ohm-cm2 (ERS-2) after 5 to 6 days of polarization (TEER of empty inserts approximately 80 to 85 ohm-cm2) (61).

In vitro transfection assay
For luciferase transfection assay, CFBE41o- parental cells were seeded on a black 96-well plate at 4 × 103 cells per well and allowed to attach overnight. Cells were transfected with the LNP encapsulating luciferase mRNA in various doses and incubated for 24 hours. Cell viability and luciferase expression were assayed using the ONE-Glo + Tox Luciferase Reporter and Cell Viability Assay Kit (Promega Corp., Madison, WI).

For cell viability assay, CFBE41o- parental cells were seeded and cultured in a 24-well plate. When confluent, the cells were transfected with the LNP encapsulating luciferase mRNA in various doses or 10% DMSO and incubated for 72 hours. Cell viability after the LNP treatment was assayed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI).

Immunoblotting
CFBE41o- cells were solubilized using a NE-PER kit (Thermo Fisher Scientific) following the manufacturer’s protocol. Reduced and de-natured cell extracts were electrophoresed in 4 to 12% Bolt Bis-Tris Plus (Thermo Fisher Scientific) gels in MES (Thermo Fisher Scientific) running buffer followed by dry transfer to polyvinylidene difluoride membrane. The membranes were probed with anti-V5 [Rabbit (Rb), 1:1000] (Cell Signaling Technology) and β-actin (Thermo Fisher Scientific) [Mouse (Ms), 1:10,000] primary antibodies.

Cryo–electron microscopy
Cryo–electron microscopy (Cryo-EM) acquisition was performed at 300 kV using Titan Krios with a Falcon III camera and Direct Electron Detector (DED). The sample (3 μl) was dispensed on a plasma-cleaned grid (Quantifoil R 1.2/1.3, 300 mesh, Ted Pella, Redding, CA) in the Vitrobot chamber at ~95% relative humidity and allowed to rest for 5 s. Then, the grid was blotted for 3 s with filter paper and plunged into liquid ethane cooled by liquid nitrogen. The frozen grids were then checked for visible defects and assembled into cassettes. The collected images were then processed and analyzed using ImageJ.

Fluorescent microscopy
For viable cell imaging, CFBE41o- parental cells were seeded and cultured in a 24-well plate. When confluent, the cells were treated with the ΔmutENaC-LNP mRNA in various doses or 10% DMSO and incubated for 72 hours. Live or dead cells were stained using the LIVE/DEAD Cell Imaging Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol, and the center areas of each well were automatically scanned using an EVOS FL Auto fluorescent microscope (Invitrogen, Carlsbad, CA).

Animals
All animal studies were conducted at the Oregon Health & Science University and approved by the Institutional Animal Care and Use Committee (IP00001707). Female BALB/c mice were sedated using isoflurane, and the LNP encapsulating FLuc mRNA was pipetted onto a single nostril with spontaneous inhalation (0.25 mg/kg). After 5 hours, 200 μl of Δ-luciferin substrate (150 mg/kg; Thermo Fisher Scientific, Waltham, MA) was intraperitoneally injected to the mice 10 min before bioluminescent imaging. Image acquisition and analysis were performed using IVIS Lumina XRMS and the manufacturer’s software (PerkinElmer, MA). The CFTR−/−tm1Unc Tg(FABPCFTR)1 Jaw/J double-transgenic CFTRKO mice were obtained from The Jackson Laboratory (JAX 002364) and maintained as described previously (62). For LNP administration, mice were anesthetized with an intraperitoneal injection of a mixture of ketamine and xylazine (100 μg/10 μg per kilogram body weight). LNPs were administered on two consecutive days to a single nostril (2 μl per application, 10 applications over 20 min, 0.5 mg/kg per day) (28).

Short-circuit current recording
Confluent CFBE41o- monolayers were transfected with 750 ng of ΔmutENaC or luciferase mRNA LNP on day 6 (TEER values stabilized to >200 ohm-cm2, ERS-2, seeding day considered as day 0). IscSC recording was performed on untransfected and transfected cells 72 hours after transfection. Snapwell inserts were mounted in EasyMount Ussing Chamber (P2302, Physiological Instruments, San Diego, CA) equipped with a heat block for temperature control. The apical and basolateral hemichambers contained 4 ml of Krebs buffer solution (135 mM NaCl, 1.6 mM MgCl2, 6H2O, 2.4 mM K2HPO4, 0.4 mM KH2PO4, and 2.25 mM CaCl2, 2H2O). The chamber temperature was maintained at 37°C. The hemichambers were continuously bubbled with air, which maintained the pH at 7.4. The apical and basolateral hemichambers...
were connected to Ag/AgCl electrodes via 3 M KCl agar bridges for voltage sensing and current passage. A VCC MC6 (multichannel voltage/current clamp) (Physiological Instruments, San Diego, CA) was used to generate current to maintain voltage at 0 mV. Data generated were recorded by Acquire and Analyze 2.3 software (Physiological Instruments, San Diego, CA). After an equilibration period to achieve a stable \( I_{SC} \) (\(~10\) to 15 min), the amiloride-sensitive component of the \( I_{SC} \) was then determined by adding 100 \( \mu M \) amiloride to the apical hemichamber.

Confocal microscopy and measurement of ASL height

For EGFP mRNA transfection, CFBE41o- parental cells were seeded on the collagen-coated three-well chambered slide (iBidi USA Inc., Fitchburg, WI). When cells became a complete monolayer, LNP encapsulating EGFP mRNA was added to the cells at a dose of 500 ng of mRNA per chamber. After incubating the specimen for 24 hours, cells were fixed in 4% paraformaldehyde, and nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA). Fixed specimens were mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific, Waltham, MA) and imaged under an LSM 880 confocal microscope (Carl Zeiss AG).

CFBE41o- and WT-CFBE41o- cells were polarized on (TEER >200 ohm-cm\(^{-2}\) ERS-2), Transwell (Corning) inserts for 6 days. The CFBE41o- cells were transfected with 750 ng of \( \text{tm} \text{uNaC-LNP} \) on day 6 polarization or were left untreated as controls. ASL depth (i.e., depth of both the mucus and periciliary liquid layers) was measured 72 hours after transfection (day 9 of polarization) using a confocal microscope. Untransfected WT-CFBE41o- (400,000) cells were seeded on collagen-coated Transwell and also imaged at day 9 of polarization. The ASL in each Transwell was washed with PBS and then labeled with 50 l of PBS containing rhodamine B isothiocyanate-dextran (2 mg/ml) (10 kDa; Sigma-Aldrich, Dorset, UK) by apical application 1 hour before the experiment. The cells were stained using 5 \( \mu M \) calcine AM (Thermo Fisher Scientific, Northumberland, UK) dissolved in culture medium for at least 60 min and introduced to the basolateral compartment of the insert. Perfluorocarbon (200 \( \mu l \), FC-770; Sigma-Aldrich) was added to the apical compartment of the insert to prevent ASL evaporation. Fluorescent images of the epithelial layer and ASL height were obtained using an LSM 880 confocal microscope (LRBB 478)–Zeiss Laser-Scanning Confocal Fast Airy (Water Objective C-Apochromat 40×/1.2 W Corr LSF880 confocal microscope (Carl Zeiss AG).

Measurement of NPD

Experimental setup

NPD was measured using a modification of the previously described methods (28). Briefly, \( CFTR \) null or WT mice were anesthetized with an intraperitoneal injection of a ketamine and xylazine mixture (100 \( \mu g/10 \mu g \) per gram body weight). Anesthetized animals were intubated orally beginning with direct visualization of the vocal folds using an otoscope with a 2-mm speculum (model no. 20200; Welch Allyn, Skaneateles Falls, NY). A flexible guide wire was advanced through the vocal folds (catalog number RA-04020, Arrow International Inc., Reading, PA), and a 20-gauge intravenous catheter was passed over the wire (BD Medical, Franklin, NJ). Mice were placed head down on a 15° incline. Body temperature was monitored rectally (TH-5; Physitemp, Clifton, NJ) and maintained with a heating pad and heat lamp as needed. A high-impedance voltmeter (World Precision Instruments, Sarasota, FL) was connected by silver-chloride pellet electrodes to an exploring nasal bridge and a reference subcutaneous bridge. The voltmeter offset was adjusted to zero. The nasal bridge, a single polyethylene tube (PE10, 0.28-mm inner diameter; Clay-Adams, BD, Sparks, MD), was pulled to approximately one-half its original diameter and cut at an acute angle to maximize surface area. The resulting orifice was ~0.5 mm in diameter. The tubing was marked at 3 and 5 mm from the tip. To ensure placement of the tubing in the nasal respiratory epithelia, the tubing was inserted into the naris to 3 mm and, after steady state, was advanced to the point of maximum voltage but never beyond 5 mm. The subcutaneous bridge was a 25-gauge butterfly-type (Abbott, Chicago, IL) needle containing Ringer’s solution inserted subcutaneously in the right abdominal wall. Each solution was warmed to 37°C and perfused to the naris at 7.5 nl/min using a perfusion pump.

Experimental protocol

Buffers were perfused sequentially through the nasal cavity for measuring baseline potential difference (PD), amiloride-sensitive PD, isoproterenol-sensitive PD, and ATP-sensitive PD. The buffer compositions are listed in table S2.

Experimental timeline

Sequential PDs were measured in untransfected animals by placing the nasal bridge in one nostril for establishing baseline NPD profile for an individual mouse. The animals were allowed to rest for 10 to 14 days, following which two consecutive days of nasal instillation of mRNA-LNP (2 \( \mu l \) per application, 10 applications over 20 min, 0.5 mg/kg per day) was performed. Care was taken to instill mRNA-LNP on the nostril that has not been used for the placement of the nasal bridge for baseline recording, on both days. NPD on the transfected nostril was performed 72 hours after the second day of nasal instillation, followed by a 2-week recovery period. The NPD was then performed on the same nostril to confirm the extinction of the effect of exposed mRNA and return of the NPD profile to baseline values.

Statistics

All experiments were performed at least in triplicate, and significance was determined by Student’s \( t \) test using GraphPad Prism for all analyses.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/47/eabc5911/DC1

View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES

1. C. Jiang, W. E. Finkbeiner, J. H. Widdicombe, P. B. McCray Jr., S. S. Miller, Altered fluid transport across airway epithelium in cystic fibrosis. Science 262, 424–427 (1993).
2. R. K. Rowntree, A. Harris, The phenotypic consequences of \( CFTR \) mutations. Ann. Hum. Genet. 67 (Pt 5), 471–485 (2003).
3. H. G. M. Heijerman, E. F. Mckone, D. G. Downey, E. Van Braeckel, S. M. Rowe, E. Tullis, M. A. Mall, J. J. Welte, B. W. Ramsey, C. M. McKe, G. Marigowda, J. S. Moskowitz, D. Waltz, P. R. Sosnay, C. Simard, N. Athluvalia, F. Xuan, Y. Zhang, J. L. Taylor-Cousar, K. S. McCoy, K. McCoy, S. Donaldson, S. Walker, J. Chmiel, R. Rubenstein, D. K. Froh, J. Neuringer, M. Jain, K. Moffett, J. L. Taylor-Cousar, B. Barnett, G. Mueller, P. Flume, F. Livingston, N. Mehdi, C. Teneback, J. Welter, R. Jain, D. Kissner, K. Patel, F. J. Calimano, Mukherjee et al., Sci. Adv. 2020; 6 : eabc5911 18 November 2020
11 of 12
ACKNOWLEDGMENT: We thank T. Haley and A. Paolo for technical support. We would also like to thank the OHSU confocal imaging facility. EM was performed at the Multiscale Microscopy Core (MMC) with technical support from the Oregon Health & Science University (OHSU).

Funding: This project was supported through funding from the National Heart Lung and Blood Institute (1R01HL146736-01 to G.S.) and the Cystic Fibrosis Foundation (SAHAY18G0 to G.S.).

Author contributions: G.S. directed the project. A.M. and G.S. wrote the manuscript. A.M. and K.D.M. conceived and designed studies. J.K. formulated and characterized LNPs with mRNA. S.R.D., P.K.J., M.E.H., Y.E., and G.S. performed experiments and analysis. A.M. and K.D.M. performed Ussing chamber and NPD studies. A.M. did cell culturing and microscopy studies. Y.E. performed EM studies. All authors contributed in data analysis arising from experiments that they were involved in. A.M. and G.S. wrote the manuscript.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 5 May 2020
Accepted 5 October 2020
Published 18 November 2020
10.1126/sciadv.abc5911

Citation: Mukherjee, K. D. MacDonald, J. Kim, M. I. Henderson, Y. Eygeris, G. Sahay, Engineered mutant α-ENaC subunit mRNA delivered by lipid nanoparticles reduces amiloride currents in cystic fibrosis–based cell and mice models. Sci. Adv. 6, eabc5911 (2020).