EMERGING THERAPEUTIC APPROACHES FOR CYSTIC FIBROSIS

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PUBLISHED IN: Frontiers in Pharmacology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-367-8
DOI 10.3389/978-2-88963-367-8

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EMERGING THERAPEUTIC APPROACHES FOR CYSTIC FIBROSIS

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Citation: Lopes-Pacheco, M., Pedemonte, N., Kicic, A., eds. (2020). Emerging Therapeutic Approaches for Cystic Fibrosis. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-367-8

Table of Contents

frontiers in Pharmacology

EDITORIAL
published: 29 November 2019
doi: 10.3389/fphar.2019.01440

Editorial: Emerging Therapeutic Approaches for Cystic Fibrosis

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Keywords: cystic fibrosis transmembrane conductance regulator, mutations, therapeutic approach, drug development, lung, inflammation, infection, modulators

Editorial on the Research Topic

Emerging Therapeutic Approaches for Cystic Fibrosis

Cystic fibrosis (CF) is the most common life-shortening inherited disease in Caucasian populations. It affects 90,000 to 100,000 individuals worldwide and results in multi-organ dysfunction, although the respiratory disorder represents the major cause of morbidity and mortality of these patients. Understanding of cellular and molecular defects of CF-causing mutations has substantially improved since the discovery of the CF transmembrane conductance regulator (CFTR) gene in 1989 (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989), bringing perspectives for the development of novel therapies. Up to 2012, therapeutic regimens were exclusively focused on disease symptoms. These comprised of physical and inhaled therapies, and numerous daily medications to enhance airway clearance, mitigate inflammation, eradicate lung infections, and supplement absent pancreatic enzymes. Together with early diagnosis and multidisciplinary healthcare, patients' life expectancy has risen from early childhood in the 1960s to 40 to 50 years in several countries nowadays (Lopes-Pacheco, 2016).

From 2012 to date, four drugs targeting the root cause of CF have reached the market: ivacaftor (Kalydeco™), the dual combinations lumacaftor/ivacaftor (Orkambi™) and tezacaftor/ivacaftor (Symdeko™), and the triple combination elexacaftor/tezacaftor/ivacaftor (Trikafta™). In patients carrying specific CF genotypes, these pharmacotherapies have been demonstrating short- and long-term therapeutic outcomes, including improvements in lung function and body mass index, and reduction in detection of *Pseudomonas aeruginosa* and frequency of pulmonary exacerbations (Ramsey et al., 2011; Wainwright et al., 2015; Rowe et al., 2017; Taylor-Cousar et al., 2017; Keating et al., 2018). Other novel therapies targeting CFTR dysfunction, modulating alternative ion channels, and acting on diseases symptoms are under experimental and clinical investigations. Furthermore, new models have been developed to accelerate and continue expanding the pipeline of therapeutic options.

This Research Topic gathers a collection of 22 original and review articles that provide novel information regarding the "Emerging Therapeutic Approaches for Cystic Fibrosis" at basic, translational, and clinical levels. The articles have been divided in three main chapters.

The first chapter is composed by an overview of the literature with some insights on hot topics in CF research, and articles focused on the regulation of CFTR trafficking, stability, and degradation. Pranke et al. introduce the pipeline of emerging therapeutic approaches to restore CFTR function that is being investigated in both experimental and clinical studies. They describe strategies to target specific defects caused by CFTR mutations, including the CFTR modulators (read-through agents, correctors, potentiators, stabilizers, and amplifiers) and antisense oligonucleotides. They also mention the advances

OPEN ACCESS

Edited and reviewed by:
Diana Conte Camerino, University of Bari Aldo Moro, Italy

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *30 October 2019*
Accepted: *12 November 2019*
Published: *29 November 2019*

Citation:
Lopes-Pacheco M, Pedemonte N and Kicic A (2019) Editorial: Emerging Therapeutic Approaches for Cystic Fibrosis. Front. Pharmacol. 10:1440. doi: 10.3389/fphar.2019.01440

in mutation-agnostic approaches, such as cell-based and gene-based therapies, which could be applied for all individuals with CF.

CFTR folding, processing, and trafficking involve complex and hierarchical steps that take place in multiple cellular compartments and engage several networks of protein machineries. Bodas and Vij summarize some key cellular mechanisms that are impaired in CF epithelia, such as protein homeostasis (proteostasis) an autophagy. They also describe some molecules that might modulate the underlying dysfunction in these mechanisms and then control the pathogenesis in CF lung disease. In the review of Cruz et al., the authors elucidate the role of Lemur tyrosine kinase 2 (LMTK2), an incompletely characterized serine-threonine kinase that functions as a multifunctional adaptor. LMTK2 may be a potential biomarker and/or therapeutic target, as it interacts with CFTR and is involved in the regulation of several intracellular events, including protein trafficking, apoptosis, and chloride transport. In the following article, Pesce et al. demonstrate that spautin-1, an autophagy inhibitor, induces a downregulation of the expression and function of F508del-CFTR rescued either by low temperature incubation or by the corrector lumacaftor. This "anti-corrector" compound may also be interesting to investigate novel biological pathways involved in F508del-CFTR degradation.

The CFTR located at the plasma membrane must be a stable protein; otherwise, it will be removed by peripheral quality control mechanisms. Fukuda and Okiyoneda summarize key environmental stresses that may affect CFTR stability at the plasma membrane. They also describe cellular machineries that may be targeted to reduce internalization and deliver to lysosome degradation or to enhance recycling back to the plasma membrane. Loureiro et al. use network biology tools to identify novel candidate proteins involved in CFTR trafficking and membrane stability. They identify GABARAP, NOS2, and SMURF1 as novel regulators of CFTR levels at the plasma membrane. Furthermore, GABARAP knockdown demonstrate to increase the plasma membrane stability of lumacaftor-rescued F508del-CFTR.

The second chapter is dedicated to new methods in CF research and approaches that potentiate CFTR channel or circumvent its dysfunction. A review by Awatade et al. nicely summarize the latest updates in the development of primary epithelial cellular model systems, discussing conventional two-dimensional airway epithelial cell (AEC) cultures, and three dimensional airway and intestinal organoid models and evaluating the limitations and potential improvements in each system, focusing on their application in CF, such as for preclinical pharmacotherapy screening to identify responsive patients. Gianotti et al. describe different methods to expand and differentiate isolated bronchial and nasal cells into fully polarized monolayers of airway epithelium, in order to provide an optimized protocol to support physiopathology analysis and to evaluate therapeutic strategies. A quantitative evaluation by Matthes et al. finally provide evidence for patient-to-patient variation in cell culture responses to correctors that should be carefully considered when CFTR corrector drugs are compared *in vitro* for precision medicine. A group of researchers from Galapagos NV, headed by Conrath (Gees et al.), describes the discovery of two novel CFTR potentiators, named GLPG1837 and GLPG2451, able to improve channel activity on a series of Class III, IV CFTR mutants, and showing no negative interaction with pharmacological rescue of F508del-CFTR trafficking defect. These novel compounds may offer new therapeutic options for CF patients.

Among the mutation-agnostic approaches proposed to overcome the basic defect in CF, two of them are based on the exploitation of alternative targets. Balázs and Mall summarize recent evidences that identified the alternative chloride channel SLC26A9 as a disease modifier and supported its role in the pathophysiology of CF and other chronic lung diseases. Pharmacological activation of SLC26A9 may help to augment the effect of CFTR modulator therapies. Interestingly, Corvol et al. report that SLC26A9 variants, although not associated with lung function variability in untreated patients, are indeed associated with variability in ivacaftor-lung response, suggesting that pharmacogenomics, in addition to personalized medicine, will soon be part of CF patient care.

A review by Kunzelmann et al. is focused on another alternative target that could be exploited for CF therapy, the calcium-dependent chloride channel TMEM16A, which is upregulated during inflammatory lung disease. TMEM16A activation would improve hydration of the airway mucus and increase mucociliary clearance. However, recent evidence suggests that TMEM16A is essential for mucus secretion and possibly also for mucus production, and appears to maintain excessive mucus secretion during inflammatory airway disease. Thus, the still open question is whether TMEM16A needs to be activated or inhibited in CF.

Finally, the last article of this section, by Cossu et al., is focused on an innovative therapeutic approach for the treatment of CF using anionophores, small molecules that facilitate the transmembrane transport of anions. The authors characterized the anion transport mechanism of a synthetic molecule based on the structure of prodigiosine, a red pigment produced by bacteria. This prodigiosin derived ionophore induces anion transport in living cells and displays low toxicity and capacity to transport chloride and bicarbonate, thus constituting a promising starting point for the development of drug candidates for CF therapy.

The third chapter is comprised of those articles that introduce novel therapies that address the causal nature of CF and those tackle the downstream consequences, including infection and inflammation. Donnelley and Parsons initially revisit a long-held curative strategy for CF, namely gene therapy. They review progress in the field made to date, discuss the issues preventing translation into large-scale clinical trials, and outline recent technological advancements, including those made by their team that may overcome these hurdles and see the first trials conducted in the near future. Bardin et al. also reviewed the critical role microRNA (miRNA) play in regulating proteins including CFTR. Specifically, they discuss different strategies to identify dysregulated miRNA in CF and review the potential of antisense techniques that hold considerable potential as future corrective forms of therapy. Finally, Berical et al. discuss the potential of cell-based therapies for CF. In principal, such

therapies involve replacing cells that carry a mutant *CFTR* sequence with other cells that express a normal copy of the gene. The authors explore two cell-based therapies, i.e. induced pluripotent stem cells and human bronchial epithelial cell transplantation and how these may address the challenges which currently limits this approach.

In an original research article, Delpiano et al. explore novel approaches to address altered air surface liquid (ASL) pH that occurs in CF. Initially, they trialed inhibiting a H^+/K^+-ATPase, namely, ATP12A, *via* ouabain *in vitro* using human AECs grown at air-liquid interface. Although pH was found to be increased, chronic exposure of primary AEC to ouabain, was deleterious on barrier integrity and function. As an alternative approach, the authors then inhibited a related H^+/K^+-ATPase, i.e. ATP4A, with the proton pump inhibitor (PPI), esomeprazole. Encouragingly, treatment raised pH without effect on airway barrier integrity or function, illustrating the potential of PPI in addressing altered ASL pH that manifests in CF.

A second group of articles focused on some of the accompanying complications of CF particularly relating to infection and inflammation. Firstly, Ling et al. address early life triggers of inflammation in very young children with CF. In addition to reviewing the types of respiratory infections that occur, the authors focus on viral triggers of inflammation, in particular rhinovirus. In addressing the limitations with currently available therapeutics, they discuss the potential use of "omics" platforms, particularly transcriptomics to elucidate host-pathogen responses to potential identify targets for potential therapeutic intervention. In an original research article, Trend et al. address the issue of antibiotic resistant bacterial lung infections in CF and explore bacteriophages or "viruses that infected specific bacteria" as an alternative therapeutic regimen. Utilizing an *in vitro* airway model comprised of primary AECs from non-CF and CF children, the authors screen candidate phage and identify one, namely E79, to have high specificity and activity against *P. aeruginosa*. It also fails to induce any deleterious effects, including programmed cell death or inflammation in AECs. The findings provide rationale for the continued exploration of bacteriophage therapy to address the global issue of antibiotic resistant bacterial lung infections not only in CF but beyond.

Any new interventional therapy requires a translational pipeline, and Semaniakou et al. review the current animal models of CF available. They discuss the pros and cons to the various models and conclude that presently no one model mirrors the complexity of CF that manifests in humans. However, the authors advocate that these models do have utility and may complement each other to advance our knowledge of CF disease pathogenesis and progression. Finally, Schultz et al. discuss the need of an adaptive trial platform over traditional clinical trial assessment for future therapeutics in CF. They explain the concepts of the Bayesian adaptive platform, where modeling and response adaptive randomization, can facilitate multiple treatments across different management domains and document improvement in patient outcomes throughout the trial period, and outline the necessary steps needed to implement this into practice in the CF sphere.

In conclusion, this issue collates current and emerging therapeutic approaches for CF. These range from; curative strategies, those aimed at correcting defective function, and approaches that target accompanying complications, such as infection and inflammation. The impact of all these approaches if successful will significantly improve not only quality of life of individuals with CF but also extend current life expectancy. With the breath of work currently being conducted by global researchers in their respective fields of specialty, the horizon looks optimistic for several approaches to translate into clinic applications.

AUTHOR CONTRIBUTIONS

ML-P, NP, and AK wrote and reviewed the Editorial.

ACKNOWLEDGMENTS

ML-P is a recipient of the 2018 Gilead Sciences Research Scholars for Cystic Fibrosis. Work in NP lab is supported by the Italian Ministry of Health through Cinque per mille and Ricerca Corrente (Linea1), and by grants from the Fondazione per la Ricerca sulla Fibrosi Cistica (FFC no. 9/2017 with the contribution of "Delegazione FFC di Genova con Gruppo di Sostegno FFC di Savona Spotorno," "Un fiore per Valeria [Assemini–Cagliari]," "Gruppo di Sostegno FFC di Vigevano," "Delegazione FFC della Valdadige," and "Delegazione FFC di Lodi"; FFC n0. 9/2019 with the contribution of "Delegazione FFC di Genova con Gruppo di Sostegno FFC di Savona Spotorno," "Delegazione FFC di Valle Scrivia Alessandria," "Delegazione FFC di Montescaglioso," and "Delegazione FFC di Ascoli Piceno"). AK is a Rothwell Family Fellow.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 27 February 2019
doi: 10.3389/fphar.2019.00121

Emerging Therapeutic Approaches for Cystic Fibrosis. From Gene Editing to Personalized Medicine

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An improved understanding of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein structure and the consequences of *CFTR* gene mutations have allowed the development of novel therapies targeting specific defects underlying CF. Some strategies are mutation specific and have already reached clinical development; some strategies include a read-through of the specific premature termination codons (read-through therapies, nonsense mediated decay pathway inhibitors for Class I mutations); correction of CFTR folding and trafficking to the apical plasma membrane (correctors for Class II mutations); and an increase in the function of CFTR channel (potentiators therapy for Class III mutations and any mutant with a residual function located at the membrane). Other therapies that are in preclinical development are not mutation specific and include gene therapy to edit the genome and stem cell therapy to repair the airway tissue. These strategies that are directed at the basic CF defects are now revolutionizing the treatment for patients and should positively impact their survival rates.

Keywords: cystic fibrosis, CFTR, ivacaftor, CFTR modulator, gene therapy

OPEN ACCESS

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Reviewed by:
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *02 October 2018*
Accepted: *31 January 2019*
Published: *27 February 2019*

Citation:
Pranke I, Golec A, Hinzpeter A, Edelman A and Sermet-Gaudelus I (2019) Emerging Therapeutic Approaches for Cystic Fibrosis. From Gene Editing to Personalized Medicine. Front. Pharmacol. 10:121. doi: 10.3389/fphar.2019.00121

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease that affects approximately 75,000 people in North America, Europe, and Australia alone. The life expectancy of CF patients has been constantly increasing because of symptomatic therapies. As our knowledge of the CF transmembrane conductance regulator (CFTR) structure and the functional consequences of its mutations has improved, therapeutics to restore CFTR expression and function have begun to emerge. Search for mutation-specific and mutation-independent tactics have now opened the path toward a revolutionizing approach in treating CF patients.

BACKGROUND

CFTR Biology and Cystic Fibrosis

The CFTR is a transmembrane chloride (Cl^-) and bicarbonate (HCO_3^-) ion channel that is expressed in the apical membranes of the epithelial cells of multiple exocrine organs, where it regulates salt and fluid homeostasis (Linsdell, 2014). The CFTR glycoprotein has

multiple membrane-integrated subunits that form two membrane-spanning domains (MSD), two nucleotide-binding domains (NBD), and a regulatory (R) domain that acts as a phosphorylation site (Rowe et al., 2005; Riordan, 2008). MSD1 and MSD2 form the walls of the channel pore, and their conformational changes drive the channel's opening and closing (Muallem and Vergani, 2009). Phosphorylation of the R domain, which is driven by cAMP-dependent protein kinase A and C, enhances adenosine triphosphate (ATP) association to the NBD domains and hence mediates their conformational change and their dimerization in a head-to-tail configuration. This conformation defines the open state of the channel (Riordan, 2008). Inversely, the hydrolysis of ATP drives the channel to a basal closed state (Vergani et al., 2005).

CFTR protein maturation, which is characterized by complex domain folding, assembly, and double *N*-glycosylation of the MSD2, starts early in the endoplasmic reticulum (ER) during translation and continues in the Golgi apparatus. This complex process can be mismatched and retarded at multiple steps, leading to important (60–80%) degradation of even wild-type CFTR (WT-CFTR) (Varga et al., 2004) by the ER-associated ubiquitin-dependent degradation system (Jensen et al., 1995; Gelman et al., 2002) or autophagy. After reaching the plasma membrane (PM), the WT-CFTR channel is internalized by clathrin-dependent endocytosis and recycles back to the cell surface through recycling endosomes. Because the mature WT-CFTR is very stable at the PM, a pool of 10% WT-CFTR is internalized and recycled back to the PM each minute (Sharma et al., 2004; Pranke and Sermet-Gaudelus, 2014).

Almost 2,000 mutations in the *CFTR* gene have been found to cause CF; they decrease the flow of Cl^- and HCO_3^- through the epithelia of multiple organs, including the lung, pancreas, sweat glands, vas deferens, liver, and intestine. As a consequence, they interfere with their normal functioning. In the respiratory tracts, the lack of a CFTR function drives the accumulation of abnormally thick and sticky mucus that underlies chronic lung inflammation and recurrent bacterial infections, leading to progressive lung degradation. There is increasing evidence that airway inflammation and infection are frequently present before the appearance of symptoms; however, it is not clear which comes first. Studies on CF animal models suggest that CF causes congenital airway abnormalities, such as a narrowed trachea and that the airway surface liquid has a reduced pH level in CF, leading to an impaired bacteria-killing potential. The accumulation of mucus gives rise to sterile inflammation. These pathological conditions initiate a vicious circle that leads to bronchial wall inflammation and air trapping. The cumulation of neutrophils further enhances inflammation through the production of elastase and proinflammatory cytokines (Nichols and Chmiel, 2015). This ultimately leads to the formation of bronchiectasis. Lung disease is responsible for >95% of CF deaths (Davis et al., 1996; Stoltz et al., 2015). Currently, according to the CF foundation registry, the median survival age of those born in 2016 is predicted to be 47.7 years of age (Cystic Fibrosis Registry 2016). Because the morbidity and mortality of CF patients is mainly caused by lung disease, the principal focus of research in the CF domain and therapy development has been targeted at minimizing lung disease.

Mutations Inducing CF

CFTR mutations are divided into six classes determined by the specific defect in CFTR protein synthesis, trafficking, function, or stability (O'Sullivan and Freedman, 2009) (**Figure 1** and **Table 1**), although many CFTR mutants present multiple defects, such as F508del-CFTR with deficient trafficking, function, and stability (Veit et al., 2016).

Class I mutations lead to severely defective protein production. They are primarily nonsense or frameshift mutations introducing a premature termination codon (PTC), leading to unstable messenger RNA (mRNA) degraded by the mRNA decay pathway (NMD) (Maquat, 2004; Popp and Maquat, 2016; Martiniano et al., 2016). If the mRNA is translated, PTC decoding results in ribosome disruption and premature translation termination, usually resulting in synthesis of a non-functional, shortened CFTR protein. Large insertions/deletions and splicing mutations resulting in the absence of proteins at the PM are also included in Class I.

Class II mutations introduce defects in CFTR processing. The main example of this class is the in-frame deletion of the 508 amino acid phenylalanine (p.Phe508del, legacy name F508del) which affects about 80% of patients. Defective folding of the newly synthesized F508del-CFTR renders NBD1 instable energetically and impairs the assembly of the interface between NBD1 and MSD1/MSD2 (Du et al., 2005; Billet et al., 2013). This misfolding impairs its stability at the ER, promoting premature degradation by the ubiquitin-dependent proteasome (Lukacs and Verkman, 2012). Consequently, little or no CFTR is trafficked to the apical PM. This mutation is also associated with the impairment of Cl^- channel gating and decreased CFTR stability at the PM because of increased degradation by a peripheral ubiquitination-related protein quality control system (Lukacs et al., 1993).

Class III mutations produce CFTR protein localizing at the PM but with defective activation, leading to a severe decrease in the ion channel's open probability. The c.1652G > A mutation, legacy name G551D (located in exon 11), affects about 2–4% of patients and is the most frequent in this class. In this case, the substitution of the amino acid glycine by aspartate occurs at a crucial point in the NBD1–NBD2 interface, inactivates ATP-dependent gating, and decreases open probability by ∼100-fold compared with WT-CFTR.

Class IV mutations induce channel dysfunction by defective ion conductance; these mutations mostly involve the MSD regions of the CFTR protein, forming the pore of the channel. The Class IV missense mutations provide a protein located in the apical membrane but with only the residual activity of a cAMP-dependent Cl^- secretion. The most common Class IV mutation is c.350G > A, legacy name R117H (located in exon 4), which affects 0.7% of patients. This substitution of arginine with histidine at position 117, which is located in the region of TMD2, reduces the channel open probability by 75%, and changes Cl^- and HCO_3^- conductance (LaRusch et al., 2014). Because Class IV mutations lead to the biosynthesis of CFTR retaining residual

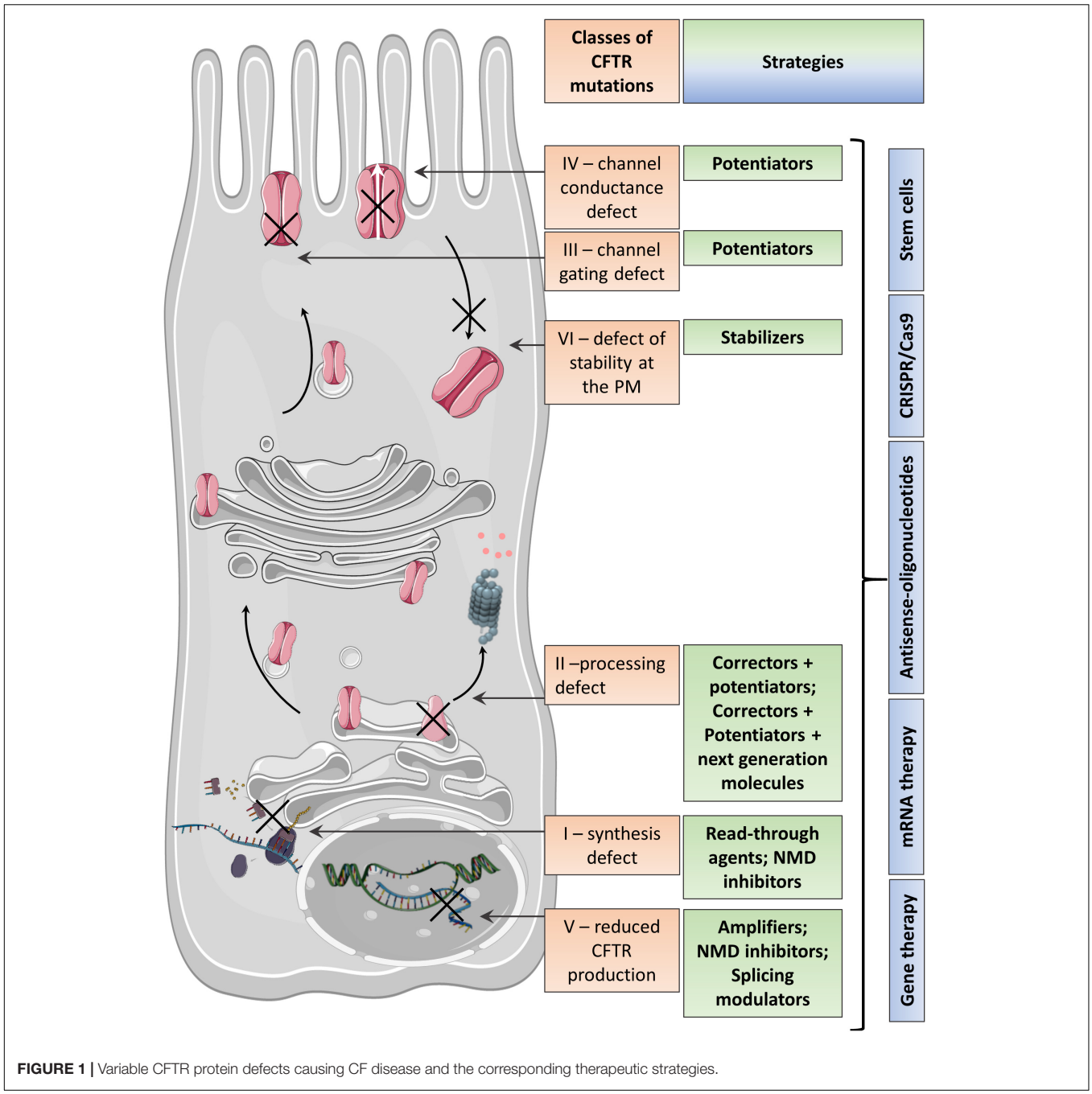

FIGURE 1 | Variable CFTR protein defects causing CF disease and the corresponding therapeutic strategies.

function and normal regulation, simple therapies to improve their activity are efficient.

Class V mutations are characterized by reduced amounts of normally functioning CFTR at the apical PM. Most of these mutations affect pre-mRNA splicing. This induces complete or partial exclusion of the exon, generating missense, silent, or nonsense mutations and, consequently, the production of defective CFTR. Class V mutations are either intronic mutations inducing the incorporation of cryptic exons or exonic mutations altering splicing enhancer motifs. The most common mutation from this class is c.3718-2477C > T, legacy name 3849+10 kb C → T (located in intron 19), and affecting about 0.58% of patients but with a higher frequency in specific populations, such as Ashkenazi Jews.

Class VI mutations result in the decreased stability of CFTR at the apical membrane as a result of increased endocytosis or decreased recycling to the PM. An example of Class VI mutations is c.120del23. This deletion of nucleotides 120 up to 142 in exon 1 eliminates the translation initiation codon at nucleotides 133–135, and the translation instead initiates at sites in exon 4 at M150/M152/M156. This produces N-truncated

proteins that are unstable and display reduced Cl^- channel activity (Ramalho et al., 2009).

Although mutations of Classes I–III provoke more severe CF disease with absent or very weak residual CFTR activity, mutations representing Classes IV–VI lead to relatively high residual function and are associated with milder forms of CF.

Different Treatment Strategies

Some strategies are specific to the CFTR mutation and aim to (i) bypass a specific PTC and restore mRNA levels (read-through therapies, NMD inhibitors therapy for Class I mutations), (ii) correct CFTR folding and trafficking to the apical PM (correctors for Class II mutations), or (iii) increase the CFTR channel function (potentiators therapy for Class III mutations and any mutant with residual function located at the PM) (**Figure 1** and **Table 1**). Other therapies in preclinical development are not mutation specific and include gene therapy to edit the genome and stem cell therapy to repair the airway tissue.

The personalization of therapy for a given patient is based on the paradigm of selecting the most effective molecule or association of molecules. The functional assays that directly or indirectly measure the CFTR activity in *in vitro* cultures of primary nasal epithelial cells (Pranke et al., 2017) and the cultures of organoids developed from intestinal epithelia (Dekkers et al., 2013, 2016) and nasal/bronchial spheroids (Brewington et al., 2018; Guimbellot et al., 2018) are promising tools to use as patient-specific biomarkers, predictive of clinical efficacy of these novel therapies.

GENETIC THERAPIES

Cystic fibrosis genetic therapies rely on delivering DNA or RNA, which encodes the CFTR protein or on the restoration of the *CFTR* gene (genome editing) or the CFTR mRNA (mRNA editing).

Gene Therapy

Gene therapy implies the relocation of the proper copies of the *CFTR* gene to the epithelial cell layer in the airways with the goal of replacing the mutated gene and express functional CFTR protein. For high efficiency of this therapy, DNA coding for CFTR together with regulatory components must be adequately administered to the airways, reach the target cells, enter (transduce) the cell, and express CFTR protein. Because CF is a monogenic disease, gene therapy is particularly attractive. Despite the fact that CF is a multiorgan disease, improving respiratory manifestations will lead to a significant improvement in the patient's quality of life and may be associated with a decrease in mortality. The inhaled route is the easiest way to access the targeted abnormal zones.

Although gene therapy carries promise, it has several limitations. First, finding the appropriate plasmid DNA molecule model is important in terms of clinical potency (Pringle et al., 2009; Dhand, 2017). Second, natural barriers such as mucus, versatile immune responses, and intracellular limitations considerably impair gene transfer into the lungs (Osman et al., 2018). Finally, because the airway epithelium is constantly renewing, genetic therapies necessitate repeated administration. Therefore, the selection of the appropriate delivery method is

TABLE 1 | Classification of *CFTR* mutations.

Class of mutation	CFTR defect	CFTR function	CFTR apical expression	Examples of mutations	Potential therapy	
I	Defective production	No	No	G542X, W1282X, R553X, R1162X, E822X, 1717-1G > A, 711+1G > T, 621+1G > T	Read-through agents, NMD inhibitors	Gene therapy, antisense-oligonucleotide therapy, mRNA therapy, CRISPR/Cas9, Stem cells therapy
II	Impaired processing	No	No	F508del, N1303K, I507del, R1066C, S549R, G85E	Correctors, Correctors + Potentiators (C + P), C + P + next generation correctors, C + P + Amplificators, C + P + Stabilizers	
III	Defective regulation	No	Yes	G551D, G178R, G551S, R560T, V520F, G970R, G1244E, G1349D	Potentiators	
IV	Defective conductance	Reduced	Yes	R334W, R117H, R347P, R1070W	Potentiators	
V	Reduced amount	Reduced	Reduced	3272-26A > G, 3849+10 kb C > T, A455E, D565G	NMD inhibitors, Splicing modulators, Amplifiers	
VI	Defect of stability at the PM	Reduced	Reduced	1811+1.6 kb A > G	Stabilizers	

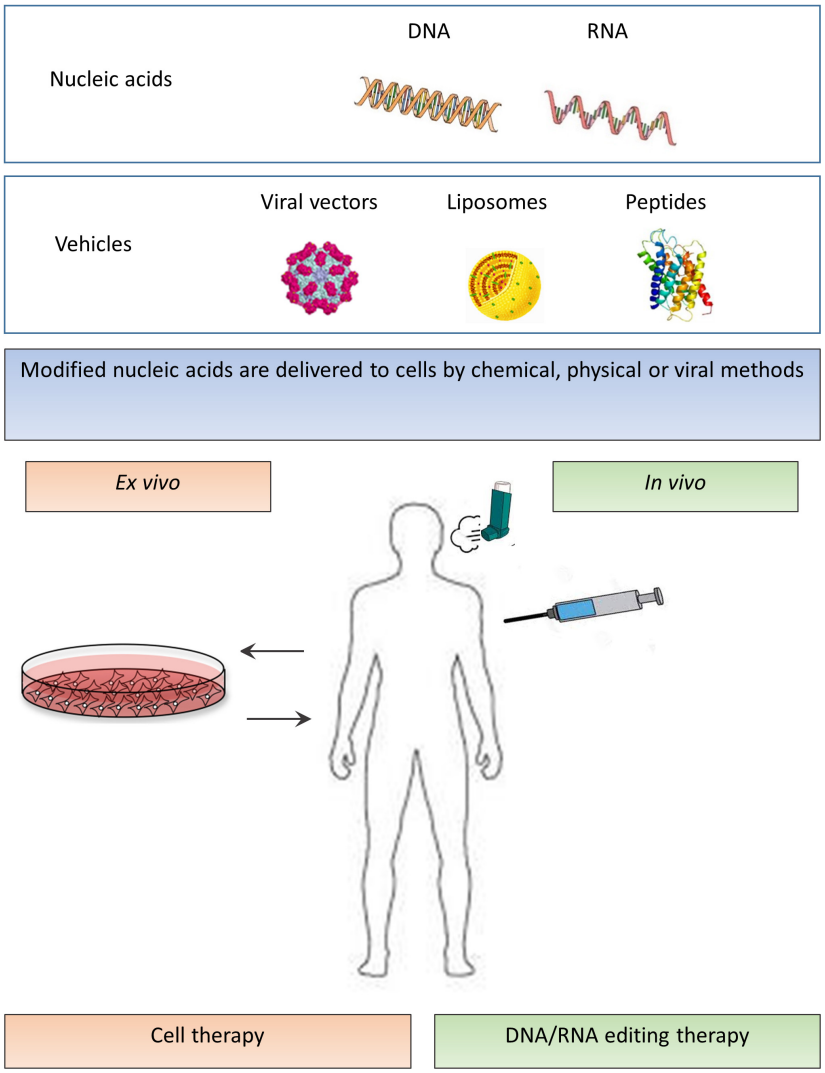

FIGURE 2 | *In vivo* and *ex vivo* strategies of mutation independent approaches. Modified nucleic acids (upper panel – nucleic acids) are delivered to cells by various methods (second upper panel – vehicles). In *ex vivo* cell therapy, stem or progenitor cells are derived from the subject, and after the required modification, the cells are transferred back into the patient (lower left part of the panel). In *in vivo* DNA/RNA editing therapy, a direct transfer of genes to the patient is performed by viral or non-viral methods (lower right part of the panel).

essential. The most commonly used agents in gene therapy for CF are viral vectors: adenoviruses, adeno-associated viruses, and lentiviruses, but also non-viral lipoplexes and peptide nanoparticles (**Figure 2**).

In 1990, for the first time, Drumm et al. (1990) proved that it is possible to deliver a healthy *CFTR* gene into the adenocarcinoma cell of a CF patient by means of retrovirus transduction. Expression of a normal *CFTR* gene was linked to a cAMP-dependent Cl^- channel regulation in CF epithelial cells.

The first ever use of viral and subsequent non-viral gene transfer factors on nasal and bronchial epithelium was seen in clinical trials in 1993 (Zabner et al., 1993; Crystal et al., 1994; Caplen et al., 1995). Many subsequent trials have demonstrated evidence of CFTR expression but have not achieved clinical efficacy.

Viral Vectors—Adenoviruses

Rosenfeld et al. (1992) showed that the adenovirus-mediated transfer of DNA coding for human CFTR to a cotton-rat model by intratracheal introduction resulted in mRNA and functional protein expression. In turn, Zabner et al. (1993) performed adenoviral gene therapy tests in humans. Although neither the CFTR mRNA nor the protein were detectable after a single nasal application of the *CFTR* gene-containing vector, the nasal potential difference showed a limited improvement of the conductivity of the Cl^- channel. Crystal et al. (1994) was the first to detect CFTR protein in the lung and nose tissue after adenoviral vector administration. Additional surveys on gene transfer by adenovirus transduction of the nasal epithelium proved that the expression of CFTR mRNA and/or CFTR protein is transient and that the Cl^- transport was not fully recovered, as

shown by the nasal potential difference (Hay et al., 1995; Knowles et al., 1995; Zabner et al., 1996; Bellon et al., 1997).

Despite preliminary promising data from preclinical models of nasal and pulmonary tissues (Katkin et al., 1995; Scaria et al., 1998) and a good tolerance at low-to-intermediate doses (Harvey et al., 2002), adenovirus-mediated gene transfer proved inefficient in CF patients (Joseph et al., 2001). This mostly occurred because of the lack of the coxsackie-adenovirus receptor, which is absent from the apical surface of most human airway epithelial cells (HAE) (Walters et al., 1999). The transduction efficiency was increased by a tight junction opener (Gregory et al., 2003) and the association of the adenovirus to the 2-(diethylamino) ethyl ether (DEAE) dextran. However, clinical use of these tight junction openers could introduce the risk of systemic invasion when considering the significant presence of bacteria in the CF lung.

Adeno-Associated Virus (AAV) Vectors

The substantial achievement in gene therapy by using rAAV for congenital blindness, hemophilia B, and lipoprotein lipase deficiency boosted scientists' interest in more carefully investigating a possible rAAV-based gene therapy approach for CF (Vidović et al., 2015). Here, rAAV is a derivate of wild-type AAV and is not related to human pathology; this vector holds promise because it is perceived to be a safe vector due to its low immunogenicity, lack of viral genes, and non-integrating character (Dismuke et al., 2014). Vidović et al. (2015) showed that the rAAV-mediated gene delivery of a shortened R domain deleted-CFTR led to the correction of the CF phenotype in CF mice nasal mucosa and in the intestinal organoids derived from CF patients. Scientists must now focus on methods to enlarge AAV tropism and to diminish its immunogenicity while improving CFTR expression and perseverance in the lungs. The efforts to improve the AAV vector efficacy in significant animal models of CF and the confirmation of the potent transduction of human epithelia for therapeutic use still remain challenging, but not unreachable (Hart and Harrison, 2017).

Another way to tackle packaging restraints and expand AAV tropism is the use of human Bocavirus-Type-1 (HBoV1) with AAV2 genome. Like AAV, the HBoV1 is a parvovirus that exhibits a high level of tropism for both transduced human cells polarized in the air–liquid interface cultures and for the apical membrane of the human airway epithelium. HBoV1 possesses a bigger capsid, allowing for the packing capacity of 5543 nt compared with an AAV of 4679 nt. The chosen animal model for exploring therapeutic approaches with rAAV2/HBoV1 vector has been CF ferrets because these animals mimic very well the physiological aspects of CF lung disease. The study by Yan et al. (2017) confirmed that *in vitro* and *in vivo* ferret epithelium is susceptible to transduction by the rAAV2/HBoV1 vector. Moreover, the experiments showed that repetitive dosing *in vivo* was efficient in sustaining transgene expression (Yan et al., 2017).

Non-viral Vectors

The anxiety concerning unwanted immunogenic reactions, possible transgene miss-insertions, difficulty in packing a nucleic acid of an excessive size, and issues in bulk-production have shed light on non-viral vector alternatives (Foldvari et al., 2016). Advancements in liposomal vectors have demonstrated the secured and feasible delivery of bulky DNA molecules (Foldvari et al., 2016; Hart and Harrison, 2017). A randomized, double-blind, placebo-controlled Phase 2b trial carried out in the United Kingdom with a repeated nebulization of non-viral *CFTR* gene showed a modest improvement in FEV_1 (forced expiratory volume in 1 s) compared with placebo at 1 year, demonstrating the stabilization of the lung function in treated patients (Alton et al., 2015). The conclusion is speculative because the difference was mainly a consequence of decrease in the placebo group. Moreover, there was no evidence of WT-CFTR expression in respiratory cells (Mottais et al., 2017). Although disappointing, this trial demonstrated that dosing repetition is harmless, so there is a necessity to improve nucleic acid delivery to the target cell (Hart and Harrison, 2017).

To protect the DNA from extracellular factors such as mucus, bacteria, or inflammation and physical deterioration during inhalation, DNA nanoparticles can be shielded by biodegradable poly(b-amino esters) (PBAEs) polymers with a thick sheet of polyethylene glycol (Mastorakos et al., 2015). Furthermore, a cationic lipid labeled GL67A38 has shown endosomal discharge of plasmid DNA and stabilization during aerosol implementation although transfection efficiency was low (Dhand, 2017). Recent preclinical work has also demonstrated the ability of transferring circular pieces of DNA that retain transgene and regulatory elements by nanoparticles (Hart and Harrison, 2017). The use of synthetic vectors may also be considered when trying to decrease immunogenicity and improve integration (Lentz et al., 2005; Catanese et al., 2012).

CRISPR/Cas9 Approach

The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 approach is a gene-editing strategy in which the specific mutated sequence of the defective *CFTR* gene is corrected by changes introduced into the DNA (**Figure 3**). CRISPR/Cas9 technology has been developed based on the bacterial defense mechanisms against "foreign" DNA (e.g., virus) (Alapati and Morrisey, 2017). In this mechanism, "foreign" DNA incorporates multiple small pieces into a locus consisting of short palindromic repeats, called CRISPR. Upon re-exposure to the introduced DNA, the CRISPR locus is transcribed into small RNAs that lead the Cas9 endonuclease to a particular spot in the added DNA, based on the DNA–RNA sequence complementarity, which creates a double-stranded opening and protects the host bacterium. CRISPR/Cas9 technology uses a protein-RNA complex composed of an enzyme—Cas9 endonuclease bound to a guide RNA (gRNA) molecule. Engineered Cas9 (Type II bacterial endonuclease) cleaves the DNA in a sequence-specific mode defined by the gRNA component that recognizes through the complementarity the mutated sequence and creates a specific double-stranded break (**Figure 3**). The cell can then fill the excised portion with the correct gene sequence through homologous directed repair (HDR), which is the desirable plan of action but can fail occasionally as in the case of the non-homologous end joining (NHEJ), which results in insertions/deletions formation. CRISPR has been recognized as

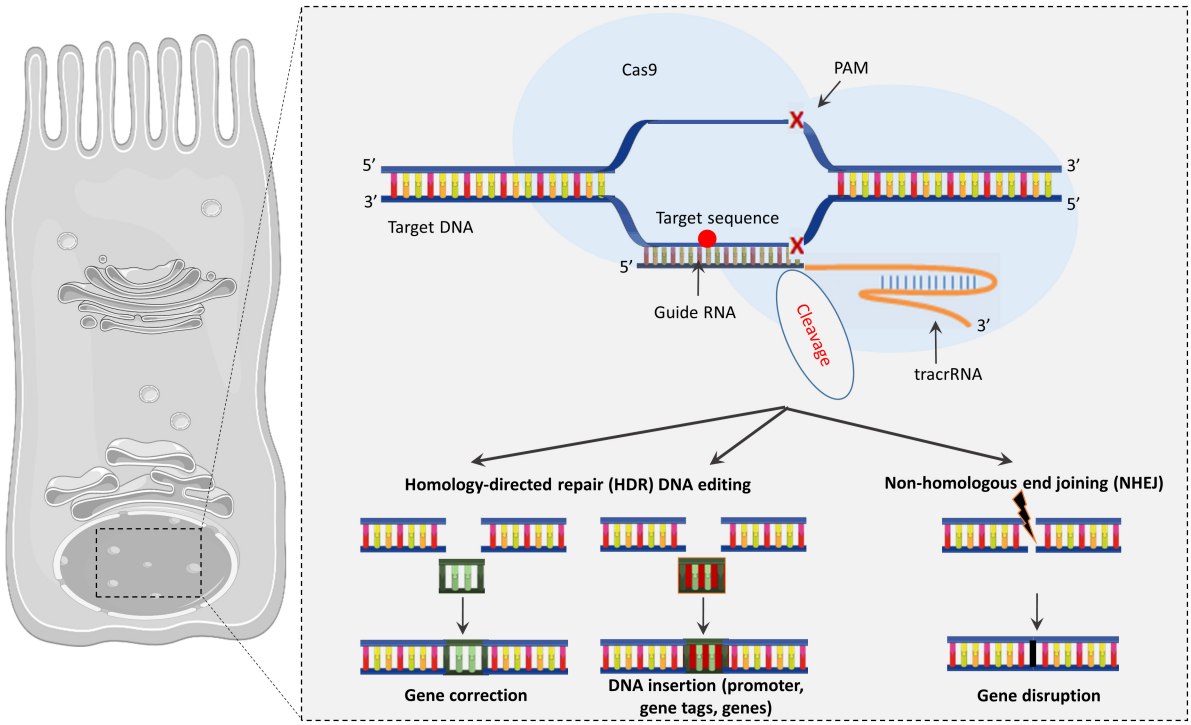

FIGURE 3 | The CRISPR/Cas9 system. Cas9 endonuclease binds to the target site using a guide RNA to precisely cut DNA allowing genome editing. CRISPR/Cas9 may drive to gene correction or DNA insertion through HDR or modify the sequence through NHEJ, which results in insertions/deletions.

the most powerful gene-editing tool when compared with zinc-finger (ZFN) and transcription activator-like effector (TALEN) endonucleases. The designed gRNA has an identical sequence as the desired site in the genome, which allows for intervention at the level of the DNA sequence with high precision, acting with molecular "scissors" to cut the DNA at the desired point and replace it with the correct sequence (White et al., 2017).

The two main benefits of this precision-made correction of the faulty gene are as follows: (i) the modified gene prevails under control of its endogenous promoter, allowing life-long expression and natural adjustment in the cell; (ii) gene repair has the ability to bypass the engagement of the external DNA, hence decreasing the chance of insertional mutagenesis.

Non-viral (lipidic or polymeric) vectors seem suitable for achieving CRISPR/Cas9 expression probable transgene integration or secondary tumor initiation (Li et al., 2015). These vectors also prevent immune responses, which has been observed in the case of viral vectors, and limit off-target activity. The optimal mode of CRISPR/Cas9 introduction into the lungs is aerosol delivery devices combined with nanoparticle suspensions. Nevertheless, inhaled therapy can become entrapped and unable to cross the dense and viscous pathological mucus layer. Despite the fact that the CRISPR approach is in its early beginnings, it presents possible significant outcomes for future CF therapy benefits.

The first studies on CRISPR/Cas9 to develop a potential therapy for CF were published in 2013. Schwank et al. (2013) tested the recovery of a functional CFTR protein in intestinal organoids obtained from CF pediatric patients carrying the F508del mutation. After lipofectamine-mediated transfection of intestinal stem cells, CRISPR/Cas9 gene editing repaired the mutation at the *CFTR* locus through the *CFTR* gene substitution approach (HDR), improving the forskolin-induced response and prompting organoid swelling. The same group suggested colonic transplantation of genetically corrected organoids as a probable perspective (Yui et al., 2012). Another successful delivery of CRISPR/Cas9 was reported by Bellec et al. (2015). They knocked-down the *CFTR* gene in HAE cells and Calu-3 cells using the CRISPR/Cas9 approach delivered with HIV-1 lentivirus. CRISPR/Cas9-directed gene modification was associated with a decline of transepithelial Cl^- secretion and a decrease in response to a CFTR inhibitor, as measured in polarized cell cultures in Ussing chambers.

Two other groups showed gene correction in CF-specific iPSCs (induced pluripotent stem cells) with the use of gene editing (Firth et al., 2015; Crane et al., 2015). Firth et al. (2015) used CRISPR technology and a piggyBac-based donor to obtain footprint-free gene correction at the *CFTR* locus in patient-derived iPSCs. The recovery of expression and function of CFTR, as measured by patch-clamp, in lung epithelial cells that were differentiated from edited iPSCs was demonstrated (Firth et al., 2015). Crane et al. (2015) used ZFN technology, which is less efficient than CRISPR, to correct the *CFTR* gene. When the repaired CF-iPSCs were differentiated into lung epithelial cells, a mature CFTR glycoprotein was expressed, which in turn recovered CFTR Cl^- channel activity (Crane et al., 2015).

Sanz et al. (2017) investigated the application of a CRISPR/Cas9-based NHEJ method to edit a small number of CF-causing mutations: c.1679 + 1634A > G, c.3140-26A > G, and c.3718-2477C > T CF, which create alternative splice sites that produce pseudo-exons or extend existing exons. The group demonstrated that CRISPR Cas9/gRNA pairs are useful for successful excision via a NHEJ pathway. NHEJ-mediated excision took place in ≥25% of transfected cells, a degree of editing that is 10-fold higher than their previous study of homology directed repair gene editing, with Cas9/gRNA in the same locus in the same cells (Hollywood et al., 2016).

Antisense-Oligonucleotide-Mediated Therapy

Antisense oligonucleotides (ASOs) are single-stranded synthetic RNA-like molecules that can selectively change gene expression by means of various techniques regulated by their chemistry and antisense oligomer design. Because these mechanisms of action are based on the complementary base pairing to the target sequences, oligonucleotides are highly specific compounds. Antisense oligonucleotides are being chemically modified to improve cellular uptake and intracellular stability and to decrease cell toxicity. From a pharmacological point of view, they are attractive forms of medication because they are resistant to nucleases and have good pharmacokinetic properties.

Zamecnik et al. (2004) constructed a modified oligodeoxyribonucleotide to replace the three missing bases caused by F508del mutation in the CFTR mRNA. This treatment *in vitro* showed improved CFTR function (Zamecnik et al., 2004). The company ProQR employs single-stranded antisense RNA-based oligonucleotides that encompass the lacking bases and behave as guide sequences to restore the targeted abnormal mRNA in cells carrying the F508del mutation. Phase II clinical trials with this modified RNA oligonucleotide QR-010 (Eluforsen), which is dedicated to F508del mutation, showed increased CFTR function in patients' nasal mucosa, good drug tolerance, and improved quality of life.

Antisense oligonucleotides can repair abnormal mRNA or alternatively target an RNA transcript for degradation through RNaseH activation. Recently, Crosby et al. (2017) tested ENaC-specific antisense oligonucleotides delivered by inhalation in mouse models for the prevention and reversal of lung symptoms in CF. Aerosol-delivered ENaC ASOs down-regulated ENaC and mucus marker expression, ameliorated goblet cell metaplasia, inflammation status, and airway hyper-responsiveness (Crosby et al., 2017).

Most of the efforts in antisense oligonucleotides research focus on splicing alteration to skip an exon enclosing a nonsense or frameshift mutation or alternatively recover the reading frame, expecting here that the end isoform will retain improved function compared with the mutated protein. ASOs have also been shown to modulate splicing in cells with the CFTR splicing mutation c.2657+5G > A, which causes exon 16 to be omitted along the splicing (Igreja et al., 2016). Single-stranded DNA oligonucleotides of 19 nucleotides having 2′-*O*-methyl modified ribose and a phosphorothioate backbone were modeled to hybridize to pre-mRNA and correct aberrant splicing in HEK293 cells expressing the c.2657+5G > A mutant *CFTR* minigene (Igreja et al., 2016). A similar correction was achieved for 3849 + 10 kb C → T, a mutation identified in 5% of Ashkenazi Jewish patients, which constitutes a novel donor site in intron 19, causing an 84 base-pair pseudo-exon to be incorporated into the mRNA, and generating a downstream PTC (Friedman et al., 1999).

mRNA-Mediated Therapy

Whereas conventional gene therapy targets the nucleus, in mRNA therapy, a right nucleotide sequence coding for CFTR is targeted at the cell cytoplasm. In both cases, a normal protein is produced, though the concepts based on mRNA delivery are more convincing because they are not required to overcome the nuclear membrane barrier. Apart from this, chemically changed mRNA displays various benefits over other nucleic acids. The most valuable characteristics are a transient protein expression, decreased immunogenicity, superior translation efficacy, enhanced stability, and pharmaceutical safety because modified mRNA is not incorporated into the host genome (Kuhn et al., 2012). The mRNA can be delivered to the cell by using liposomal or polymeric non-viral vector formulations that are administered via several routes, for example, intraperitoneally, intravenously, or intratracheally.

The transfection of chemically modified WT-CFTR mRNA in CFBE41o-cells homozygous of the F508del mutation re-established cAMP-induced CFTR currents (measured in Ussing chambers) similar to WT cells as a result of the mRNA-driven replacement of functional channels (Bangel-Ruland et al., 2013). Immunofluorescence and biochemical approaches have confirmed the expression of apically located WT-CFTR after optimized WT-CFTR mRNA transfection. Primary cultured human nasal epithelial cells were characterized by nearly a twofold improvement in the cAMP-stimulated CFTR current after mRNA transfection.

Robinson et al. (2018) used a clinically relevant lipid-based nanoparticle (LNP) for the packaging and transport of large chemically modified CFTR mRNA (cmCFTR) to bronchial epithelial cells derived from patients. The experiments showed an increase in membrane-localized CFTR and rescue of its primary function as a Cl^- channel. Nasal application of LNP-cmCFTR recovered CFTR-mediated Cl^- secretion to conductive airway epithelia in CFTR knockout mice for at least 14 days. The CFTR activity peaked on the 3rd day post-transfection, retrieving up to 55% of the net Cl^- efflux that is distinctive for the healthy mice (Robinson et al., 2018).

STEM CELL THERAPY

The biggest obstacle to overcome in regenerative medicine is to determine the relevant cells that will be capable of repairing a defect. The desired cell should be non-immunogenic, patient specific, easy to make proliferate, and easy to modify (Guo et al., 2017). Because endogenous progenitor cells are difficult to

recognize and insufficient in their quantity and activity, focus has been directed on exogenous cell sources (McQualter et al., 2010; Rock and Königshoff, 2012). The discovery of iPSCs inspired a discussion about whether iPS cells are a copy of embryonic stem cells (ESCs). Yamanaka's group first obtained the iPS cells by reprogramming mouse fibroblasts and next confirmed the results with the use of human fibroblasts (Takahashi et al., 2007). Nowadays, the existing therapeutic probability of ESCs is even more alluring when combined with the CIRSPR/Cas9 approach (Wagner et al., 2016). Indeed, repairing a CFTR mutation has been demonstrated in human iPSC cells by means of CRISPR/Cas9. Skin fibroblasts derived from CF individuals were reprogrammed toward iPSCs, transfected with the CFTR/Cas9 gRNA vector, corrected to their WT phenotype, and further entirely differentiated into cells of proximal and distal airways (Crane et al., 2015; Pollard and Pollard, 2018). This suggests that theoretically, stem lung cells restored to their WT phenotype could be regrafted to lung niches to further redifferentiate them into respiratory cells.

Moreover, the CRISPR/Cas9 method showed the total recovery of CFTR protein function in organoids—intestinal stem cells placed in culture—derived from children with CF (Schwank et al., 2013). The possibility to engraft cultured colon organoids was tested in a mouse by using effective transplantation into a superficially damaged mouse colon. The integration of grafted cells into the mouse colon resulted in a coating with epithelium in the deprived area of the damaged colon (Yui et al., 2012; Firth et al., 2015). However, this technology is time- and labor-intensive and challenging in terms of obtaining entirely differentiated lung-specific cell subsets (Wagner et al., 2016).

Presumably, ESCs/iPSCs assays will become a potent method to better forecast patients' clinical responses to CFTR modulators. The creation of an array of iPS cell lineages possessing the characteristics of different CF mutations will provide a powerful tool for selecting the potential drug to repair functional deficiencies (Schmidt et al., 2016; Simsek et al., 2016; Conese et al., 2018). Importantly, safety concerns about ESC- and iPSC-based cell grafts, including the transmission of possible genetic abnormalities and tumor risk, may hamper potential clinical therapeutic purpose. A clear answer is needed to define if the mutations were already pre-existing in the cells of origin or if they were introduced during the reprogramming process. Future preclinical risk assessments need to better establish tumor and disease risk related to the therapeutic use of iPSC derivatives (Martin, 2017).

READ-THROUGH THERAPIES AND NMD INHIBITORS

Read-through agents (**Table 2**) are directed at in-frame nonsense mutations. Some PTCs are more "permissive" than others to NMD, leading to residual levels of mRNA. This residual level of mRNA is the target for "read-through" agents, whose goal is to reduce the ribosomal ability to proofread and enable ribosomes to skip the PTC, leading to the formation of functional protein. Several factors are challenging when it comes to read-through treatment efficacy: (i) the level of drug-induced read-through, (ii) the amount of target transcripts, and (iii) the activity of the recoded protein (Pranke et al., 2018).

Aminoglycosides were the first read-through agents tested for CF disease (Howard et al., 1996). Aminoglycoside antibiotics are amino sugars that interact with the ribosome at the A-site and imitate a conformational change in the ribosomal RNA that normally is induced by codon–anticodon pairing; therefore, this promotes near-cognate tRNA incorporation and increases the number of PTC misreading, allowing translation to continue to the correct termination codon. Gentamicin and geneticin present read-through potential (Wilschanski et al., 2000; Wilschanski et al., 2003; Kandasamy et al., 2012). Gentamicin was found to display beneficial effects in patients with at least one Class I mutation, as assessed by an improvement in nasal potential difference after topical nasal application (Wilschanski et al., 2003) and intravenous administration (Sermet-Gaudelus et al., 2007). These small-scale clinical trials provided a proof-of-concept for read-through efficiency. However, gentamicin and geneticin cannot be used in clinics because of serious renal toxicity and ototoxicity. Several studies have been performed to develop chemically modified aminoglycosides to provide higher activity and less toxicity (Nudelman et al., 2009; Rowe et al., 2011; Xue et al., 2014). NB124 (ELX-02, ELOXX Pharmaceuticals) is a derivative of aminoglycoside that is modified to provide a higher level of read-through activity than gentamicin (Xue et al., 2014). It has been shown that NB124 restores CFTR function to roughly 7% of WT levels. Its read-through potency has been shown in respiratory cell lines for the most prevalent PTCs—G542X, R553X, R1162X, and W1282X—and in primary human bronchial epithelial (HBE) cells from patients carrying the G542X mutation. Moreover, tests of ototoxicity in the tissue-based model showed that this compound is also less cytotoxic than gentamicin. Lower levels of toxicity and a higher level of PTC suppression by NB124 are achieved through a strong preference for cytosolic versus mitochondrial ribosomes. A Phase II clinical trial will begin in 2019 in Belgium.

High-throughput screening (HTS) identified PTC124 (Ataluren®, Translarna™ PTC Therapeutics) (Welch et al., 2007). The systemic administration of PTC124 in CF mice expressing a human CFTR-G542X transgene induced CFTR function rescue. This small molecule was tested in a Phase II clinical study (Kerem et al., 2008; Sermet-Gaudelus et al., 2010) with contradictory results. A first Phase III trial did not deliver any significant changes in FEV_1 although there was a positive trend favoring Ataluren®(−2.5% change) over the placebo (−5.5% change; $p = 0.124$) (Wilschanski et al., 2011). New clinical placebo-controlled Phase III trials excluding inhaled tobramycin, which could antagonize the effect that PTC124 has on the ribosome, demonstrated no evidence in improving FEV_1, nor any benefit in bronchial exacerbations. The clinical development of the compound is now stopped in the field of CF (Kerem et al., 2014).

Other factors may also influence the read-through efficiency, for example the PTC identity, the neighboring mRNA sequence, the NMD efficiency controlling the level of mRNA, the geometry of the tRNA-mRNA complex in the presence of the drug at

the ribosome-decoding center, and the function of the protein, which is neoformed.

Therefore, new tracks of research for PTC-associated mutations are now being explored, including (i) NMD inhibitors, (ii) the association with CFTR modulators to improve CFTR expression/function of the neoformed protein, and (iii) factors that force mismatched base pairs to adopt a Watson–Crick geometry.

ReCode Therapeutics has been developed as an innovative therapeutic approach that utilizes suppressor transfer RNA (tRNAs) with the goal of correcting CF-causing nonsense mutations. RCT101 is a therapeutic nucleic acid actively studied in preclinical models. This modified tRNA is transported to cells by patented nanoparticles to correctly "recode" the translating protein. The unpublished data from experiments on human HBEs from patients with genotypes G542X/G542X and F508del/G542X showed that RCT101 significantly increased CFTR-dependent Cl^- secretion as a single active agent or in combination with VX-809 and VX-770 (Hagemeijer et al., 2018).

CFTR MODULATOR THERAPIES

A CFTR modulator is a pharmaceutical agent that targets a specific defect in the CFTR protein that is caused by mutation in the *CFTR* gene. This modulator does not correct mutations in the gene but rather targets the errors that occur post-transcriptionally, either during protein folding, trafficking up to the PM, or CFTR functioning.

The CFTR modulators are classified into four main groups: potentiators, correctors, amplifiers, and stabilizers (**Table 2**). They are different in their mechanisms of action, which can be determined by the type of protein defect they target (Sloane and Rowe, 2010).

Potentiators

Therapeutic agents that improve the channel-open probability and potentiate mutated CFTR gating are called potentiators. A large number of proof-of-concept studies have been published to demonstrate that ATP analogs and small-molecule agents have good potentiation activity. Chemically modified analogs of ATP have demonstrated a significant increase in the open probability of CFTR, with P-ATP [N^6-(2-phenylethyl)-ATP] having the highest affinity and efficacy (Zhou et al., 2005; Bompadre et al., 2008). It has been demonstrated that P-ATP increases the open probability of G551D-CFTR and F508del-CFTR (Miki et al., 2010). Another ATP analog 2'-dATP (2′-deoxy-ATP) also enhances the gating of WT-CFTR and G551D-CFTR (Aleksandrov et al., 2002; Cai et al., 2006). Remarkably, both modifications in ATP have synergic effects in the potentiating gating of G551D- and F508del-CFTR (Miki et al., 2010). Nevertheless, issues with bioavailability and potential unspecific binding to other proteins involved in multiple physiological functions limit the utility of ATP analogs in clinics.

The agents that increase the intracellular concentration of cAMP and hence amplify PKA activity and the phosphorylation level of the R domain might also improve the activity of defective CFTR. First, Drumm et al. (1991) found that IBMX (3-isobutyl-1-methylxanthine), a compound that increases intracellular cAMP by inhibiting phosphodiesterase, increases PKA activity and makes F508del-CFTR more responsive to activation. Second, an isoflavone derivative genistein is a tyrosine kinase inhibitor that

TABLE 2 | Strategies of treatment for personalized CF medication and compounds tested pre-clinically and clinically.

Therapy		Compounds
Read-through agents	Pre-clinical	Geneticin, RCT101
	Clinical	Gentamicin ↓, NB124 (ongoing), PTC124 ↓
Potentiators	Pre-clinical	ATP analogs (P-ATP, 2′-dATP, P-dATP), IBMX, PG-01, VRT-532, dihydropyridine blockers of L-type calcium channels, CO-068, CB subunit of crotoxin, P5, G01, A01, A02, H01, H02, H03
	Clinical	Genistein ↓, curcumin ↓, VX-770 ↑, PTI-808 (ongoing in triple combination), GLPG1837 (ongoing)
Correctors	Pre-clinical	Curcumin, HDAC inhibitors (SAHA), Corr-4a, VRT-325, glafenine, RDR1, 407882, FDL169,
	Clinical	4PBA ↓, miglustat ↓, sildenafil ↓, VX-809 (Orkambi®) ↑, VX-661 (Symdeko®) ↑, VX-440, VX-152, VX-659, VX-445 (ongoing in triple combinations), cavosonstat (ongoing), GLPG2222, GLPG2851, GLPG2737, GLPG3221 (ongoing in triple combinations), PTI-801 (ongoing in triple combination)
Amplifiers	Clinical	PTI-428 (ongoing in triple combination)
Stabilizers	Pre-clinical	HGF (hepatocytes growth factor), VIP (vasoactive intestinal peptide)
	Clinical	Cavosonstat

↑-indicates succesfull clinical outcome and the FDA approval, ↓-indicates unsuccesfull clinical attempt.

can also activate CFTR in intact cells (Illek et al., 1995, 1996). Illek et al., (1995 1996) proposed that this effect was mediated by increasing intracellular cAMP; however, other independent studies have demonstrated that genistein directly targets and binds to the CFTR protein (French et al., 1997; Hwang et al., 1997; Weinreich et al., 1997). Genistein entered the clinical study as a duo therapy with phenylbutyrate; however, the results were not satisfying. Other flavonoids (apigenin, kaempferol, and quercetin) have been found to enhance the currents *in vitro* and *in vivo* by increasing the CFTR channel's open probability (Illek and Fischer, 1998). Naturally existing curcumin also extends the channel opening duration of WT-CFTR (Berger et al., 2005), F508del-CFTR (Berger et al., 2005), and G551D-CFTR (Wang et al., 2007) although data from clinical investigations have not been conclusive. Similarly, it was proposed that sildenafil, a phosphodiesterase (PDE5) inhibitor, activates the guanylate cyclase and increases the intracellular cGMP and hence CFTR activity (Lubamba et al., 2008).

First cell-based fluorescence HTSs by Verkman's laboratory (Yang et al., 2003; Pedemonte et al., 2005b) and Vertex Pharmaceuticals (Van Goor et al., 2006), applying a Fischer rat thyroid (FRT) cell line stably expressing F508del-CFTR and an YFP sensitive to iodide, led to the discovery of several classes of small-molecule potentiators (Yang et al., 2003). Screening realized by Pedemonte et al. (2005b) showed the ability of the phenylglycine molecule PG-01 to restore the opening of F508del-CFTR almost to the level of WT-CFTR. Van Goor et al. (2006) identified VRT-532 (pyrazole). As shown by Pasyk et al. (2009) and Wellhauser et al. (2009), VRT-532 potentiates the gating of G551D- or F508del-CFTR through direct interaction with CFTR and the restoration of its defective ATPase activity (Pyle et al., 2011).

In 2009, Vertex Pharmaceuticals discovered VX-770 (Ivacaftor), which potentiates CFTR activity. First, they showed that VX-770 increases the activity of F508del- and G551D-CFTR using the patch-clamp technique and Cl^- secretion measures in bronchial epithelial cell cultures sampled from patients carrying these mutations (Goor et al., 2009). VX-770 prolongs the opening duration of WT-CFTR (Hwang and Sheppard, 2009), acting independently of ATP hydrolysis and NBD domain dimerization, because VX-770 efficiently potentiates G551D-CFTR (Van Goor et al., 2009; Eckford et al., 2012; Jih and Hwang, 2013), E1371S-CFTR (Jih and Hwang, 2013), and WT-CFTR in the absence of ATP (Eckford et al., 2012; Jih and Hwang, 2013). Eckford et al. (2012) suggested that VX-770 interacts directly with CFTR and induces an unconventional mode of gating. Although VX-770 increased the residual forskolin-stimulated channel activity in HBE cell cultures from some F508del-homozygous patients (Van Goor et al., 2009), a Phase II investigation of F508del-homozygous patients showed no improvement in FEV_1 (Flume et al., 2012).

A Phase II and Phase III study in patients carrying the G551D mutation showed that ivacaftor efficiently improved predicted FEV_1 in as early as 15 days, reaching a 10.6% increase in FEV_1 at 24 weeks of treatment ($p < 0.001$). Ivacaftor decreased sweat chloride concentration by 48 mmol/l compared with the placebo ($p < 0.001$), reduced the frequency of pulmonary exacerbations by 55% ($p = 0.001$), and increased the weight of patients to 2.7 kg ($p < 0.001$) (Ramsey et al., 2011). Phase II trials also showed significant improvements of the channel function in the nasal and sweat gland epithelia (within-subject) (Accurso et al., 2010). Children with "silent lung disease," which is characterized by normal initial FEV_1, also demonstrated a significant improvement in FEV_1 and lung clearance index. These results demonstrated that correcting CFTR at the molecular level can translate into outstanding clinical improvements. VX-770 (trade name Kalydeco®) became the first CFTR modulator approved for use in clinics and was initially approved in the United States (beginning of 2012), then in Europe and Canada (end of 2012), Australia, and New Zealand (2013).

Further development of ivacaftor demonstrated its clinical benefit in eight additional Class III gating mutations, including S549N and G551S, confirming improvement in lung function, BMI, sweat chloride, and CFQ-R; in addition, this method did not have safety concerns (De Boeck et al., 2014). Ivacaftor proved effective in a preschool population in open-label studies, highlighting an increase in fecal elastase and potential reversal in early pancreatic insufficiency status. An important concern was abnormalities of liver function tests in this population. Finally, ivacaftor demonstrated substantial activity in the non-gating mutation R117H, with amelioration in sweat chloride and CFQ-R scores in all age groups, whereas respiratory improvement was significant only in adults, perhaps because of the disease being more established in these patients (Yu et al., 2012; Carter et al., 2015).

Given these outstanding clinical benefits, the Food and Drug Administration (FDA) approved ivacaftor for marketing authorization based on *in vitro* assays for a number of mutations with residual function. Nowadays, ivacaftor is approved by the FDA for patients aged 1 and older who have one of the following gating mutations: G178R, S549N, S549R, G551D, G551S, G1244E, S1251N, S1255P, or G1349D; one of the following residual function mutations: A455E, E193K, R117C, A1067T, F1052V, R347H, D110E, D110H, F1074L, R352Q, G1069R, R1070Q, D579G, K1060T, R1070W, D1152H, L206W, S945L, D1270N, P67L, S977F, E56K, or R74W; one of the following splice mutations: 711+3A→G, 3272-26A→G, E831X, 2789+5G→A, or 3849+10 kb C→T; or the conduction mutation R117H.

Since the approval of ivacaftor, many other potentiators have been found. A number of these are still in preclinical development. A screen of the approved drugs performed by Galietta's laboratory picked out dihydropyridine blockers of L-type calcium channels to have the potentiation activity of F508del-CFTR (Budriesi et al., 2011); however, their clinical usefulness is unclear because of side effects (e.g., off-target cardiac effect). In turn, Faure et al. (2016) showed that a CB subunit of crotoxin from *Crotalus durissus terrificus* interacts with the NBD1 domain of both WT- and F508del-CFTR and increases their Cl^- channel currents. To identify the potentiators that act synergistically with correctors, Verkman's laboratory screened the analogs of previously found P5 potentiators and unrelated synthetic small molecules. They found 12 of the most active compounds, including a thiophene (G01), a 2-thioxo-4-amino-thiazoles (A01 and A02), and pyrazole-pyrrole isoxazoles

(H01, H02, and H03), with a higher potentiating efficacy in FRT cells than VX-770.

An investigational CFTR potentiator proposed by Proteostasis Therapeutics—PTI-808—was found to enhance the function of F508del-CFTR (2018 ECFS Conference, New Frontiers in Basic Science of Cystic Fibrosis) and is currently in Phase I clinical trials together with PTI-801 and PTI-428 as a combination therapy.

Interestingly, dihydro-5H-thieno[2,3-c]pyran-2-yl)-1H-pyrazole-3-carboxamide) GLPG1837, a more recent potentiator developed by Galapagos, exhibits a higher efficacy than VX-770 for G551D-CFTR (Van der Plas et al., 2018). Similar to VX-770, GLPG1837's underlying mechanism is independent of NBD domain dimerization and ATP hydrolysis. By applying GLPG1837 with VX-770 together, Yeh et al. (2017) provided evidence that these two molecules probably act in competition for the same site of action, whereas GLPG1837 and the ATP analog P-dATP work synergistically through two different sites. Two Phase II clinical studies are now conducting enrollment to test the GLPG1837 compound in patients with a S1251N mutation and G551D mutation.

Correctors

The CFTR correctors are small molecules that improve the trafficking of mutated CFTR (Class II mutations, e.g., F508del) from ER to the apical PM and increase CFTR cell surface expression. These correctors improve defective CFTR folding and cellular processing by direct binding (called pharmacological chaperones) or modulate protein homeostasis and the quality control system of the cell to modify the recognition and processing of misfolded CFTR (called proteostasis regulators). Because F508del-CFTR presents multiple defects, the development of correctors is a greater challenge than the development of potentiators (Thibodeau et al., 2010). Indeed, correction of F508del-CFTR requires the following: (i) rescue to native folding by the restoration of NBD1 energetics and interface instability; (ii) evasion of the protein from ER quality control; (iii) enhancement in the apical cell membrane localization; and (iv) improvement in CFTR-dependent Cl^- secretion. Hence, a strategy to combine the correctors with potentiators and even an amplifier or stabilizer into a "combination therapy" was tested *in vitro* and in clinical trials.

The initial studies focused on the regulation of proteostasis for F508del-CFTR. Early studies have shown that some drugs approved for other diseases have the corrector activity for F508del-CFTR. The compound 4-phenylbutryate (Buphenyl, 4PBA), a chemical chaperone that stabilizes the folding of proteins, has been found to increase F508del-CFTR PM expression in cell culture models (Rubenstein and Zeitlin, 2000). However, this clinical trial failed to confirm the correction activity, as measured by nasal potential difference (Zeitlin et al., 2002). Curcumin (Egan et al., 2004; Lipecka et al., 2006) blocks calcium from entering into the ER and thus may interfere with the calcium-dependent chaperones that are involved in the degradation of the CFTR. Initial tests in patients failed to confirm any efficacy. Miglustat (n-butyldeoxynojyrimicin) is an alpha-glucosidase inhibitor that may interfere with F508del-CFTR misfolding quality control (Noël et al., 2008; Robert et al., 2008; Norez et al., 2009). Although Miglustat efficiently corrected the functional cell surface expression of F508del-CFTR in cell culture models and mice, a small clinical study did not confirm the corrector activity. Wang et al. (2006) demonstrated that increased PM localization of F508del-CFTR can be achieved by the down-regulation of Aha1 (an Hsp90 cochaperone), whereas Hutt et al. (2010) proposed that the inhibition of histone deacetylase HDAC7 activity with HDAC inhibitors (Suberoylanilide Hydroxamic Acid, SAHA) facilitates F508del-CFTR folding and stability and corrects F508del-CFTR. However, a later study by Bergougnoux et al. (2017) had contradictory conclusions, with experiments in human nasal epithelial (HNE) cells showing that SAHA decreased CFTR transcript and protein levels.

A second strategy was based on the HTSs of small molecules. The first corrector identified with this methodology was bithiazole Corr-4a (bisaminomethylbithiazole, C4) (Pedemonte et al., 2005a), whose later analogs have improved potency (Yu et al., 2008). Corr-4a stabilizes both ER- and PM-localized F508del-CFTR. This improves the domain assembly (Loo et al., 2008, 2009) rather than NBD1 stability (Farinha et al., 2013; Okiyoneda et al., 2013). HTSs by Vertex Pharmaceuticals introduced new correctors (Van Goor et al., 2006), including VRT-325 (quinazolinone, C3), which stabilizes both the ER- and PM-localized F508del-CFTR by improving domain assembly (Loo et al., 2008, 2009). Both Corr-4a and VRT-325 may not function through direct binding to CFTR because they are not specific to CFTR (Van Goor et al., 2006, 2011). Although Corr-4a and VRT-325 present a correction activity *in vitro*, they did not find a pharmacological use because of the high toxic effects and low *in vivo* efficacy.

Other small-scale screenings have provided further correctors, such as the drug glafenine (Robert et al., 2010), phenylhydrazone RDR1 (Sampson et al., 2011), and candidate molecules from computational screening (Kalid et al., 2010). These compounds are relatively less efficacious and have not been tested in clinical studies. Finally, a structure-based virtual screening by Odolczyk et al. (2013) developed other small compounds that rescue F508del-CFTR functional cell surface expression by inhibiting the interaction of F508del-CFTR with keratin 8 (Colas et al., 2012).

Following HTS by Vertex Pharmaceuticals and chemical optimization, the most promising compound VX-809 (lumacaftor, VRT-826809) became the first corrector approved for clinical use as a combined oral treatment with VX-770 (trade name of combination Orkambi®). VX-809 has a higher potency and efficacy than VRT-325 and Corr-4a (Van Goor et al., 2010, 2011; Farinha et al., 2015). In F508del-homozygous HBE cells, VX-809 rescued total Cl^- secretion up to ∼14% of WT-HBE cells (Van Goor et al., 2011) and even up to ∼25%, as evaluated in HBE/HNE cells (Pranke et al., 2017). The VX-809 correction effect on F508del-CFTR is additive to VRT-325 and Corr-4a, suggesting a different mode of action (Van Goor et al., 2011). Interestingly, the correction effect of VX-809 can also be unmasked in HAE cells carrying only one copy of the F508del

mutation: F508del/D1152H, F508del/394delTT, F508del/1717-1G > A (Pranke et al., 2017), F508del/G542X (Awatade et al., 2015; Pranke et al., 2017), F508del/Y1092X (Awatade et al., 2015), F508del/R117H, F508del/W1282X, or F508del/E60X (Gentzsch et al., 2016). Conversely, N1303K-CFTR, another Class II mutant, was not corrected by VX-809 in HBE cells, but R117H- (Gentzsch et al., 2016) and A561E-CFTR (Awatade et al., 2015) efficiently improved PM localization and function. The mechanism of action is not yet fully known; however, it has been shown that VX-809 stabilizes TMD1 (Loo et al., 2013; Okiyoneda et al., 2013), improves TMD1 folding (Ren et al., 2013), and stabilizes interdomain interactions between TMDs and NBDs (He et al., 2012; Loo and Clarke, 2017). Studies on *in vitro* liposomes and C18 analogs of VX-809 (Eckford et al., 2014) applying the alkyne-containing VX-809 derivatives (Sinha et al., 2015) have shown that VX-809 may bind to CFTR directly; however, the exact binding site has not been found. Evidence that VX-809 binds directly to CFTR is based on the precipitation of the VX-809-bound CFTR and on the visualization of VX-809-CFTR association in cells, thanks to the fact that the alkyne group of VX-809 derivatives can be conjugated to biotin-azide molecules through Cu'-catalyzed cycloaddition and by applying the conjugated biotin moiety.

As a whole, F508del-CFTR correctors in a single compound-treatment present modest effectiveness in CFTR rescue. It has been proposed that this limited efficacy is caused by the necessity to rescue multiple defects in F508del-CFTR at the same time (folding, activity, and stability defects) (Sloane and Rowe, 2010). This was illustrated by a clinical trial testing efficiency of VX-809 alone in F508del homozygote patients, which failed to demonstrate any improvement (Clancy et al., 2012).

Considering the unsatisfying results of the VX-809 corrector, Vertex Pharmaceuticals proposed to combine the potentiator (VX-770) and corrector (VX-809) because the *in vitro* analysis demonstrated that ivacaftor increased the open probability of F508del-CFTR by fivefold. Acute administration of VX-770 to VX-809-corrected primary HAE cells increased the F508del-CFTR function (Van Goor et al., 2011). However, later analysis of HBE cells showed a reduction in the correction efficacy of VX-809, as well as VX-661 when VX-770 was applied chronically (Cholon et al., 2014; Veit et al., 2014). Chronic co-treatment with VX-809 and VX-770 affected the folding efficiency of F508del-CFTR at the ER and its metabolic stability in Golgi apparatus and PM, reducing the F508del-CFTR density at the apical PM and function (Veit et al., 2014).

Initially, two Phase III clinical studies (TRAFFIC and TRANSPORT) were designed to assess the efficacy and safety of two different doses of VX-809 in combination with VX-770 in F508del-homozygous patients (Wainwright et al., 2015). Phase III clinical trials were shown to provide a benefit for patients. Patients over 12 years of age treated with the VX-809/VX-770 combination therapy for 24 weeks showed a mean absolute improvement in FEV_1 between active treatment and placebo ranging from 2.6 to 4.0 percentage points ($P < 0.001$), a statistically significant weight gain, reduction in pulmonary exacerbations, and fewer hospitalizations. There were no major safety concerns. However, some increases in blood pressure and chest tightness/bronchospasm were reported (Wainwright et al., 2015). In 6–11-year-old patients, VX-770/VX-809 combination therapy demonstrated a statistically significant improvement in the lung clearance index (LCI) (−1.09 Unit; $p < 0.0001$), FEV_1 (+2.4%; $p = 0.02$), sweat chloride (−21 mm/l; $p < 0.0001$) and body mass index *Z*-scores (+0.15, $p < 0.0001$) (Ratjen et al., 2017). Importantly, long-term follow up of patients on VX-770/VX-809 now show a slower decline in lung function over the study period compared with the rate of decline anticipated from registry data of patients not on VX-770/VX-809 (Konstan et al., 2017). Although the improvements seen with VX-770/VX-809 in F508del homozygotes were lower than those seen in VX-770 responsive mutations, the FDA and EMA approved this combination therapy (trade name Orkambi®) in 2015 for patients ages 12 and older who have two copies of the F508del mutation. In 2016, the FDA extended the license to patients aged 6–11 years and in August 2018 patients 2 years of age and older. However, it must be pointed out that clinical studies with Orkambi® showed a variable clinical responsiveness among patients. Less than 50% of patients had a FEV_1 improvement by more than 5%, and only 25% of patients improved by more than 10% (Wainwright et al., 2015). This issue underlies the importance of the preclinical evaluation of CFTR modulators for each patient with the use of patient-specific biomarkers predictive of clinical efficacy.

A subsequent corrector developed by Vertex Pharmaceuticals—VX-661 (Tezacaftor)—has a structure similar to VX-809 but optimized pharmacokinetic properties. As reported by Van Goor et al., VX-661 increases Cl^- transport in F508del-homozygous HBE cells (from 2.5 to 8.1% of normal levels in WT-HBE) and in other additional CFTR mutant HBE cells (including mutations associated with gating defects and residual CFTR function) (Fidler et al., 2017). The combination of VX-661 and VX-770 increases Cl^- transport to 15.7% of normal Cl^- transport and improves ciliary beat frequency and fluid transport. Pranke et al. (2017) confirmed these results in HBE/HNE cells from F508del-homozygous patients and showed that Cl^- secretion increased up to 27.4% of normal WT cells. An increase of Cl^- transport was also measured in HBE cell cultures from heterozygous patients with genotypes F508del/394delTT and F508del/1717-1G > A (Pranke et al., 2017). After successful preclinical tests of VX-661, Vertex Pharmaceuticals next proposed a combination therapy of VX-661 and VX-770 originally for patients with two copies of the F508del mutation. Two separate multicenter clinical studies assessed the efficacy and safety of VX-661/VX-770 in patients 12 years of age and older: the EVOLVE study (Phase 3, randomized, double-blind, placebo-controlled, parallel group study) for patients homozygous for the F508del mutation; and the EXPAND study (Phase 3, randomized, double-blind, placebo-controlled, crossover study) for patients with one mutation that results in residual CFTR function in *trans* with the F508del mutation. The mean absolute improvement in FEV_1 in the EVOLVE study was 4% from baseline for those treated with active compounds compared with the placebo. The rate of pulmonary exacerbation was 35% lower in the VX-661/VX-770 group than in the placebo group (Taylor-Cousar

et al., 2017). In the EXPAND study, the combination treatment demonstrated a mean absolute improvement of 6.8% compared with the placebo. VX-770 alone improved FEV_1 only by 4.7% compared with the placebo (Rowe et al., 2017). Additionally, VX-661/VX-770 did not induce the chest tightness or drug–drug interactions observed with Orkambi®. At the beginning of 2018, the FDA approved combination VX-661/VX-770 (trade name Symdeko®) to treat CF in people ages 12 and older who have two copies of the F508del mutation and those who have at least one residual function mutation from the following: A455E, E56K, R74W, A1067T, E193K, R117C, D110E, F1052V, R347H, D110H, F1074L, R352Q, D579G, K1060T, R1070W, D1152H, L206W, S945L, D1270N, P67L, or S977F; or one following splice mutations: 711+3A→G, 3272-26A→G, E831X, 2789+5G→A, or 3849+10kbC→T. A Phase III trial is ongoing to evaluate the safety and efficacy of Symdeko®in patients ages 6–11.

A study presented by Zawistoski et al. (2016) introduced a novel F508del-CFTR corrector—FDL169—whose potency and efficacy is comparable to VX-809. FDL169's mechanism of action could possibly be similar to that of VX-809 because combining FDL169 and VX-809 does not further increase F508del-CFTR activity. Interestingly, the inhibitory effect of VX-770 on FDL169 activity is weaker than on VX-809. This new corrector could be an alternative for VX-809.

The Galapagos Company has developed novel correctors through HTS: GLPG2222 and GLPG2851 (C1). The chemical structure of GLPG2222 (Wang et al., 2018) is similar to the structures of VX-809 and VX-661; however, it was reported to be more potent. *In vitro* characterization demonstrated that GLPG2222 is highly functional in primary patient cells carrying two copies of the F508del mutation. The first one—GLPG2222—is currently being evaluated in a Phase II clinical study, whereas the second—GLPG2851—is currently in a Phase I study.

The modest efficacy of Orkambi®and Symdeko®triggered the development of next-generation drugs. Vertex Pharmaceuticals performed HTS on a cell model treated with VX-809 or VX-661 to search for more potent correctors, which yielded compounds that have additive rescue activity. VX-440, VX-152, VX-659, and VX-445 have been tested in separate Phase II clinical trials as a triple-combination therapy, together with VX-661 and VX-770, in adult CF patients carrying two copies of the F508del mutation or one copy of F508del and one copy of minimal CFTR function mutation. The initial results of the Phase II trials presented a significant increase in FEV_1 for all groups of patients treated with the triple-combination therapy when compared with the placebo (up to 13.3% for VX-659 and 13.8% for VX-445), and a significant reduction in Cl^- levels in the sweat test. The triple-combinations of VX-661/VX-770 with VX-445 or VX-659 have been tested in Phase III trials (Davies et al., 2018; Keating et al., 2018). *In vitro* functional tests in F508del-homozygous HBE cell cultures demonstrated that the triple combination treatment of VX-661/VX-770/VX-152 improved CFTR activity up to ∼75% and VX-661/VX-770//VX-440 up to ∼67% of normal HBE cells. Subsequently, HBE cells with one copy of the F508del mutation were corrected up to ∼47% with the triple combination containing VX-152 and up to ∼43% with the triple combination including VX-440. Interestingly, an important correction over 50% of normal CFTR function was also observed in cells with genotypes "F508del/minimal function" on triple-combination regiments VX-661/VX-770VX-152, -VX-440, or -VX-659, with the highest improvement measured for VX-659.

Other next-generation correctors were introduced by Galapagos and Proteostasis Therapeutics. The initial results demonstrated that adding GLPG2737 to VX-809/VX-770 enhances the effectiveness of the treatment. The GLPG3221 (Galapagos) compound is under Phase I evaluation in healthy volunteers. Galapagos reported also that combinations of their first-generation correctors (GLPG2222 and GLPG2851) with their next-generation correctors (GLPG2737 and GLPG3221) and a potentiator GLPG1837 significantly increases Cl^- transport *in vitro* compared with the effect of Orkambi®. GLPG2222 passed early phase clinical trials and displayed improvement in potency and drug–drug interaction compared with VX-809 and VX-661 (Radar, 2016). GLPG2222 and GLPG2737 correctors together with the GLPG2451 potentiator are currently being tested in Phase II clinical trials as a triple-combination therapy (FALCON).

The PTI-801 (Proteostasis Therapeutics), a third-generation corrector, showed superior *in vitro* efficacy over known correctors and synergy. PTI-801 in triple combination together with Orkambi®and the PTI-428 amplifier is currently in a Phase I clinical evaluation of safety and tolerability. Initial positive results have been announced by Proteostasis company (Proteostasis Announces Positive Data from Ongoing Phase 1 Study of PTI-801 in Cystic Fibrosis Patients on Background Orkambi®Therapy, 2018). Proteostasis Company reported that potentiator PTI-808 enhanced the function of mutated F508del-CFTR *in vitro* and restored it to almost normal levels when combined with PTI-428 and PTI-801. This second triple therapy obtained fast-track status from the FDA.

Recently, Veit et al. (2018) identified new compounds—4172, 6258, 3151—stabilizing specific folding defects of F508del-CFTR. These rationally designed compounds lead to ∼50–100% of wild-type-level correction in immortalized and primary human airway epithelia and in mouse nasal epithelia. The strategy to use compounds that synergistically aim at distinct structural defects proved to be efficient to rescue mutant expression and function at the PM. Therefore, the combination of correctors could translate into improved clinical benefit in patients with CF.

Amplifiers

Therapies to treat CF induced by mutations leading to decreased CFTR synthesis (Class V) requires agents that stimulate protein expression. The compounds enhancing the expression of CFTR protein, with a following increase of its quantity in the ER and the PM, are called amplifiers. Amplifiers could also be used as a therapy for other CFTR mutants when in combination with correctors and potentiators.

The PTI-428 compound from Proteostasis Therapeutics is a first-in-class CFTR amplifier that showed an *in vitro* increase in CFTR protein levels across genotypes. The amplifier could potentially improve mRNA stability and/or assist the processes surrounding CFTR transcription or translation. As

reported by Molinski et al. (2017), PTI amplifier enhanced correction achieved with VX-809 and VX-770 treatment in CF cells and tissues from patients with rare CFTR mutations (ΔI1234_R1239-CFTR).

Stabilizers

Class VI CFTR mutants and corrected Class II mutants, including F508del-CFTR localized to the PM present a reduced half-life because of increased endocytosis and decreased recycling. The instability of CFTR in the PM requires compounds that anchor mutant proteins in the membrane. Stabilizers are molecules that rectify the intrinsic protein instability and increase the CFTR residence time at the PM/decrease protein degradation rate from the PM.

It has been shown (Moniz et al., 2013) that hepatocytes growth factor (HGF) stimulated Rac1 signaling and contributed to F508del-CFTR anchoring at the cell surface through interactions with NHERF-1. Co-treatment of cells with HGF and lumacaftor improved the rescue of F508del-CFTR and stimulated CFTR stabilization at the apical membrane compared with lumacaftor treatment alone (Loureiro et al., 2015). An increase in CFTR interaction with NHERF-1 and subsequent stabilization of the CFTR mutant at the PM was also observed in airway cells treated with vasoactive intestinal peptide (VIP) (Rafferty et al., 2009).

Other strategies to stabilize CFTR at the PM by decreasing its endocytosis rate include cAMP signaling through EPAC1 (a guanine nucleotide exchange factor exchange protein directly activated by cAMP) (Lobo et al., 2016) and an inhibition of *S*-nitrosoglutathione reductase with *S*-nitrosylating agents, such as the endogenous *S*-nitrosoglutathione (GSNO). This latter strategy prevents CFTR interactions with Hsp70/Hsp90 chaperones (Marozkina et al., 2010; Zaman et al., 2016).

Cavosonstat (N91115, Nivalis)—an inhibitor of *S*-nitrosoglutathione reductase (GSNOR) through inhibiting GSNOR—increases *S*-nitrosoglutathione levels and leads to CFTR maturation and PM stability (Donaldson et al., 2017). A phase II clinical study is now being conducted to test Cavosonstat for patients with two copies of the F508del mutation in combination therapy with VX-809/VX-770 or for patients with gating mutants and receiving VX-770.

CONCLUSION

New light has been shed on the molecular targets and pathways for therapeutic strategy thanks to the increasing comprehension of the cellular consequences of CFTR mutations.

The astonishing results of clinical trials with protein therapy demonstrate the clinical efficacy of mutation-personalized therapy. Nowadays, scientists are convinced that an improvement of CFTR function at the molecular level can translate into an improvement in lung function and significantly improve the daily life of the patients and, most likely, their survival. It is expected that the near future will herald an era when therapeutic options will be motivated by personalized information.

Future perspectives are to develop mutation-specific and mutation-independent therapies that achieve near wild-type processing and function, as in case of the triple-combination therapy for the F508del-CFTR mutant. Further studies, however, will be needed to assess long-term efficacy and tolerance. Importantly, DNA or mRNA editing in preclinical development may allow for correct non-rescuable mutations and ultra-rare genotypes that are not targeted by current protein therapies. The next challenge is to implement those therapies in newborns with the aim of targeting the basic defect that prevents organ injury in this population.

AUTHOR CONTRIBUTIONS

IP, AG, and IS-G wrote the manuscript. AH and AE reviewed the manuscript.

FUNDING

The publication of this review has been funded by ABCF Mucoviscidose Foundation.

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Conflict of Interest Statement: IS-G is principal investigator for studies funded by Vertex Pharmaceuticals, has received research fundings from Vertex Pharmaceuticals, and is part of the scientific Advisory board of Proteostasis Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 01 February 2019
doi: 10.3389/fphar.2019.00020

Adapting Proteostasis and Autophagy for Controlling the Pathogenesis of Cystic Fibrosis Lung Disease

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Cystic fibrosis (CF), a fatal genetic disorder predominant in the Caucasian population, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*Cftr*) gene. The most common mutation is the deletion of phenylalanine from the position-508 (F508del-CFTR), resulting in a misfolded-CFTR protein, which is unable to fold, traffic and retain its plasma membrane (PM) localization. The resulting CFTR dysfunction, dysregulates variety of key cellular mechanisms such as chloride ion transport, airway surface liquid (ASL) homeostasis, mucociliary-clearance, inflammatory-oxidative signaling, and proteostasis that includes ubiquitin-proteasome system (UPS) and autophagy. A collective dysregulation of these key homoeostatic mechanisms contributes to the development of chronic obstructive cystic fibrosis lung disease, instead of the classical belief focused exclusively on ion-transport defect. Hence, therapeutic intervention(s) aimed at rescuing chronic CF lung disease needs to correct underlying defect that mediates homeostatic dysfunctions and not just chloride ion transport. Since targeting all the myriad defects individually could be quite challenging, it will be prudent to identify a process which controls almost all disease-promoting processes in the CF airways including underlying CFTR dysfunction. There is emerging experimental and clinical evidence that supports the notion that impaired cellular proteostasis and autophagy plays a central role in regulating pathogenesis of chronic CF lung disease. Thus, correcting the underlying proteostasis and autophagy defect in controlling CF pulmonary disease, primarily *via* correcting the protein processing defect of F508del-CFTR protein has emerged as a novel intervention strategy. Hence, we discuss here both the rationale and significant therapeutic utility of emerging proteostasis and autophagy modulating drugs/compounds in controlling chronic CF lung disease, where targeted delivery is a critical factor-influencing efficacy.

Keywords: proteostasis, autophagy, cystic fibrosis, CFTR, ROS, protein-misfolding

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *27 September 2018*
Accepted: *09 January 2019*
Published: *01 February 2019*

Citation:
Bodas M and Vij N (2019) Adapting Proteostasis and Autophagy for Controlling the Pathogenesis of Cystic Fibrosis Lung Disease. Front. Pharmacol. 10:20. doi: 10.3389/fphar.2019.00020

INTRODUCTION

Cystic fibrosis (CF) is one of the most common fatal autosomal recessive disorders (Zhang et al., 2018), with emerging treatment options that have prolonged survival but limited success in diminishing overall mortality. Specially, subjects with homozygous F508-del (phenylalanine-508) mutation that suffer from the most serious form of ailment, lack effective treatment options

that can warrant cure or normal survival. Briefly, chronic airway disease is a major contributor of the morbidity and mortality in CF subjects (Cantin et al., 2015; Mall, 2016), and accessibility of several novel and more potent therapeutic options has allowed substantially improved survival. Majority of these therapeutic strategies are aimed at controlling symptomatic CF-lung disease, while some of the newer strategies are designed to target the primary or "root" cause of the disease, which is the mutation(s) in the cystic fibrosis transmembrane conductance regulator (*Cftr*) gene (Esposito et al., 2016; Hudock and Clancy, 2017; Maiuri et al., 2017; Zhang et al., 2018). There are about 1700 known mutations affecting the generation (Class I), structure (Class II) or channel function (Class III–V) of the CFTR protein, and about 88% of these comprise of mutations that cause the protein misfolding defects (Class II) (C.F. Foundation, 2018). People with these mutations on both the copies of their *Cftr* gene demonstrate the classical manifestations of CF lung disease, such as a thick and sticky mucus, mucin hypersecretion, elevated inflammatory-oxidative stress and/or persistent bacterial infections, which collectively result in severe airway obstruction (Bodas and Vij, 2010; Bodas et al., 2018a). Evidence from newer animal models of CF and some clinical data indicate that symptoms of lung disease are present at very early age, or even at birth, thus proposing the concept of congenital origin of CF lung disease (Stoltz et al., 2015). With progressing age, persistent exacerbations primarily caused by *Pseudomonas aeruginosa* (*Pa*) and the ensuing IL-8 mediated persistent neutrophilic inflammation are the hallmark of clinical CF lung disease and a major contributor to irreversible lung damage as well as CF-related fatalities (Bodas and Vij, 2010; Ferrari et al., 2017; Bodas et al., 2018a).

In general, all the cellular processes that work to maintain a robust protein repertoire in the cell are collectively called the proteostasis network (PN) (Klaips et al., 2018). This complex molecular system tightly regulates the fate of a protein inside the cell, starting from its synthesis, folding, and maintenance of the folded functional state, to transport (trafficking), and eventual degradation. A vast amount of cellular energy is utilized to maintain the protein degradation machinery to get rid of misfolded, damaged, non-functional or even functional proteins that are no longer required by the cells. The main protein degradation or cellular clearance mechanisms include the ubiquitin-proteasome system (UPS) and the "autophagy-lysosomal pathway" (Korovila et al., 2017). The primary difference between proteasome and autophagy mechanisms is the type of cargo they can process as their starting material. The proteasome can only process proteins, while large protein aggregates, lipids and even damaged organelles can be processed and degraded by the autophagy pathway. Recent evidence suggests a strong inter-relationship between the proteasome and autophagy pathways (Bustamante et al., 2018) and thus it is not surprising that any disturbance in any of these mechanisms can form the basis of accumulation of aberrant proteins eventually leading to severe pathological conditions such as those seen in aging-related neurodegenerative diseases (Daniele et al., 2018; Klaips et al., 2018), proteinopathies (Chaudhuri and Paul, 2006; Hidvegi et al., 2015; Hartl, 2017), and genetic or environmentally induced chronic respiratory diseases such as CF (Lukacs and Verkman, 2012; Fraser-Pitt and O'Neil, 2015) and COPD (Tran et al., 2015; Bodas and Vij, 2017; Bodas et al., 2017; Vij, 2017), respectively. The deletion of phenylalanine-508 (F508-del) is the most common (about 80%) *Cftr* gene mutation associated with CF, which results in a misfolded CFTR protein that is unable to reach the plasma membrane (PM) (Lukacs et al., 1993; De Stefano et al., 2014). This results in the absence of mature CFTR ion-channel on the PM, leading to CFTR dysfunction, classically described as a chloride ion transport defect (Welsh et al., 1993). In addition, there is substantial evidence supporting the critical role of membrane-resident CFTR in regulating innate and adaptive immune responses in CF (Teichgraber et al., 2008; Vij et al., 2009; Bodas and Vij, 2010; Grassme et al., 2017; Svedin et al., 2017). Furthermore, a burgeoning number of studies now ascertain the crucial role of mature CFTR in regulating important cellular homeostatic processes such as proteostasis and autophagy, with a common consensus that autophagy is potentially inherently defective in CF (Gomes-Alves et al., 2010; Luciani et al., 2010, 2011; Bodas et al., 2012; Valle and Vij, 2012; Villella et al., 2013a). The genesis of defective autophagy in CF seems to be an inherent defect, as primary CF cells have diminished levels of several autophagy proteins (Abdulrahman et al., 2011, 2013), although the precise mechanism(s) are still unclear. Some interesting studies indicate the possible contribution of micro RNA's (Tazi et al., 2016) and DNA methylation (Tazi and Amer, 2015), as both could regulate the expression of autophagy proteins in CF cells. Nonetheless, it is well documented that the absence of membrane CFTR leads to ROS-mediated SUMOylation of transglutaminase 2 (TG2), which prevents its ubiquitination and subsequent degradation by the proteasome, leading to its intracellular accumulation. This results in the crosslinking of Beclin-1 (BECN1), an important protein required for autophagosome formation, leading to defective autophagy, and accumulation of SQSTM1 (p62) (Luciani et al., 2010; Bodas et al., 2017), which favors aggregation of BECN1 and other autophagy related proteins into p62+HDAC6+ aggresome bodies (**Figure 1**). The misfolded F508del-CFTR is also sequestered into aggresome bodies, as the accumulation of p62 leads to inhibition of both protein (proteasome) and aggresome clearance. This aggresome trapping of F508del-CFTR prevents its proper trafficking to the PM that contributes to the initiation and progression of inflammatory-oxidative stress responses in the CF lungs (Luciani et al., 2010).

This suggests that future strategies for managing and treating CF will need to be focused on correcting the underlying proteostasis/autophagy impairment, mainly *via* rescuing F508del-CFTR to the PM, which would simultaneously control the inflammatory-oxidative stress response in the airways of CF subjects. Lately, proteostasis-modulators and autophagy-inducers have shown encouraging results in pre-clinical studies in correcting both the F508del-CFTR trafficking to the PM (CFTR-corrector) (De Stefano et al., 2014; Tosco et al., 2016; Vu et al., 2017; Hutt et al., 2018; Stincardini et al., 2018; Zhang et al., 2018), as well as dampening the inflammatory-oxidative stress responses (anti-oxidant/anti-inflammatory) (Romani et al., 2017; Stincardini et al., 2018), although these strategies were not very efficient in restoring other rare class II CFTR mutations

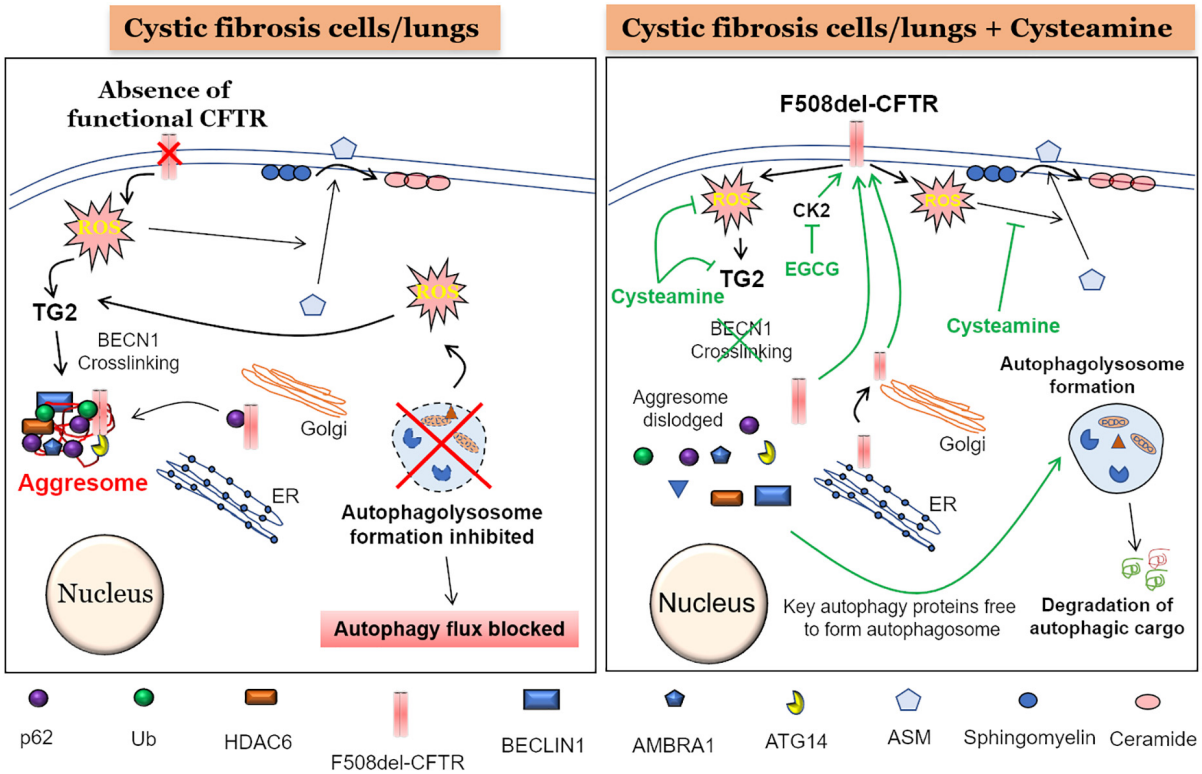

FIGURE 1 | Mechanism of cysteamine mediated autophagy induction and F508del-CFTR rescue. (Left panel) The absence of a functional CFTR at the plasma membrane (PM) leads to elevated reactive oxygen species (ROS) levels which cause activation of transglutaminase-2 (TG2). An active TG2 mediates cross-linking of crucial autophagy proteins such as Beclin1 (BECN1), ATG14, and AMBRA1 into Ub+/p62+/HDAC6+ aggresome bodies, render BECN1 and other autophagy proteins unavailable for the formation of autophagosome and thus blocking the subsequent autophagy flux process. Moreover, an accumulation of p62 could lead to aggresome sequestration of newly synthesized F508-delCFTR, thereby preventing its PM translocation. Additionally, accumulation of damaged mitochondria leads to more ROS production, further promoting TG2-mediated BECN1 crosslinking and autophagy inhibition. Further, the ROS mediated translocation of acid sphingomyelinase (ASM) from cytoplasm to PM, leads to increased conversion of sphingomyelin to ceramide, which is a deleterious sphingolipid implicated in CF pathogenesis. (Right Panel) The treatment of CF cells or mice with the autophagy inducing antioxidant drug, cysteamine, which is also an inhibitor of TG2, leads to prevention of BECN1 crosslinking. This results in dislodging of aggresome components resulting in availability of BECN1 and other key autophagy proteins to form the autophagosome, thus allowing the autophagy process to function and leading to the clearance of autophagic cargo. Moreover, decreased p62 levels due to a functional autophagy flux will possibly allow the newly synthesized F508-delCFTR to reach the PM and restore partial CFTR function, even though some of previously aggresome sequestered F508-delCFTR may be degraded by the active autophagy process. In addition to cysteamine, treatment with epigallocatechin-gallate (EGCG) at the time of cysteamine removal, potentiates the long-term stability of the PM-rescued F508-delCFTR, due to its inhibitory effect on protein kinase CK2, which would otherwise promote peripheral/PM degradation of F508-delCFTR. Additionally, a functional autophagy process means that the toxic aggregated proteins and other damaged organelles such as mitochondria are homeostatically degraded, thus decreasing overall ROS levels. Finally, cysteamine blocks the translocation of ASM from cytoplasm to the PM, thus reducing the conversion of sphingosine to ceramide, and preventing ceramide-mediated inflammatory-apoptotic signaling in CF cells and/or lungs.

(Awatade et al., 2018). Moreover, it is not known if rare CFTR mutations also lead to a autophagy defect, and thus many other CFTR modulators have been clinically tested to evaluate their efficacy in restoring the PM stability and function of different CFTR mutants (Lopes-Pacheco, 2016). This strategy is currently being developed as a personalized CF management plan and holds potential for CF patients with all classes of CFTR mutations (Lopes-Pacheco, 2016; Paranjape and Mogayzel, 2018). As an example, for people with the G551D mutation, the orally bioavailable potentiator drug, VX770, shows substantial promise as an inducer of mutant CFTR channel activity, and thus is FDA approved for clinical application in CF patients (Accurso et al., 2010). However, since about 80% of CF patients worldwide possesses the F508del-CFTR defect (Lopes-Pacheco, 2016), drugs that correct its PM trafficking, stability, and function have emerged as promising therapeutic pipeline for clinical validation in CF subjects. Since the single corrector drugs showed minimal clinical benefit, successful efforts have been made to develop combinatorial therapy for CF. In fact, several CFTR-corrector compounds have been clinically evaluated and few have even reached the CF patients in combination with CFTR-potentiator drugs Orkambi®, Symdeko® (Birault et al., 2013; Wainwright et al., 2015; Mayer, 2016), although their present costs are humongous (Mayer, 2016; Bulloch et al., 2017). Additionally, a recent study tested a triple combination of pharmacological chaperones (VX809+MCG1516A+RDR1) and demonstrated better CFTR functional correction than VX809 alone (Carlile et al., 2018), thus providing potentially

promising future therapeutics for CF subjects. Similarly, another triple combination therapy was tested using VX-659-tezacaftor-ivacaftor (Davies et al., 2018), and is currently in Phase III clinical trials (Sala and Jain, 2018).

Another important aspect of current CF drugs is the challenge of drug-delivery and/or *in vivo* bioavailability due to the notoriously thick and sticky mucus layer (Brockman et al., 2017; Vij, 2017). Considerable pre-clinical research is ongoing to address these crucial issues, and there has been some success in designing novel drug-delivery systems that achieve targeted drug-delivery and long-term bioavailability in the CF lungs (Vij et al., 2010; Vij, 2011, 2017; van Rijt et al., 2014). Although scientists and clinicians have come a long way on significantly improving the median survival age of CF patients to reach adulthood (∼40–50 years) (West and Flume, 2018), the disease is still incurable and numerous precious lives are lost at a very early age. Thus, continued basic and translational research is essential to develop a better armamentarium of preventive/therapeutic strategies to further improve patient survival and possibly find a cure to this life-restricting and life-taking genetic disease. The current perspective compiles some recent studies that have the potential to translate into emerging therapeutic strategies for CF subjects, with the focus on drugs/compounds that correct the underlying disease-promoting defect in proteostasis and autophagy, including the protein-processing defect in F508del-CFTR protein.

AUTOPHAGY INDUCERS AS EMERGING CF THERAPEUTICS

One of the foremost cellular proteostatic mechanism that regulates protein-processing is the catabolic autophagy process. There is a plethora of evidence in recent studies that a partial (in chronic obstructive pulmonary disease, COPD) (Cantin, 2016; Bodas et al., 2017; Vij, 2017; Shi et al., 2018) or complete loss (in CF) (Luciani et al., 2010; Cantin, 2016) of functional CFTR protein from the PM leads to reactive oxygen species (ROS)-mediated autophagy impairment. This results in the accumulation of misfolded CFTR in perinuclear aggresome bodies, which eventually promotes the development of the lung disease. In CF, the accumulation of misfolded F508del-CFTR leads to ROS mediated autophagy impairment that results in increased inflammatory-oxidative stress (Luciani et al., 2010) in the CF airways. Moreover, a non-functional CFTR also contributes to defective bacterial uptake, killing, and clearance (Brockman et al., 2017; Ferrari et al., 2017; Pehote et al., 2017; Shrestha et al., 2017), which contributes to persistent exacerbations and inflammation, eventually resulting in irreversible damage of the pulmonary architecture. Lately, some pre-clinical and clinical trials demonstrate the utility of autophagy inducing therapeutic compounds in controlling pathogenesis and progression of CF lung disease (Villella et al., 2013b; Junkins et al., 2014; Esposito et al., 2016; Romani et al., 2017; Stincardini et al., 2018), that have shown promise in Phase-I/II trials but none have hitherto reached the bedside yet (De Stefano et al., 2014; Devereux et al., 2015, 2016). As discussed before (Yang et al., 2013; Vu et al., 2017), some other autophagy inducing drugs are also tested as potential CF drug candidates but these may not reach the patients due to the high doses required for the treatment as well as an evidence of many off-target side effects (Yang et al., 2013; Vu et al., 2017). Thus, we highlight here the leading proteostasis/autophagy-modulating compounds/drugs, which can allow bedside translation as emerging CF drug candidates.

CYSTEAMINE: A MULTI-PRONGED DRUG FOR CF

Cysteamine is an FDA-approved drug for nephropathic cystinosis and has been very effectively used for over 25 years (Besouw et al., 2013; Fraser-Pitt et al., 2018). Chemically, cysteamine is an endogenously present, water soluble aminothiol, generated as a consequence of coenzyme A metabolism (Fraser-Pitt et al., 2018). It is commercially available as Cystagon® and Procysbi® and can be administered orally, although with well documented side effects (Cherqui, 2012; Veys et al., 2016). Over the years, cysteamine was introduced as a potential CF drug and henceforth several studies have been conducted to evaluate its efficacy in controlling CF lung disease (Besouw et al., 2013; Charrier et al., 2014; Devereux et al., 2015, 2016; Brockman et al., 2017; Shrestha et al., 2017). Mechanistically, cysteamine is a TG2 inhibitor, which dislodges the aggresome assembly, which is sequestering key autophagy proteins and F508del-CFTR, thereby restoring autophagy and decreasing p62 levels (**Figure 1**). This allows forward trafficking of misfolded F508del-CFTR to the Golgi and PM, thus reinstating its chloride channel function. Moreover, decreased p62 levels might also prevent sequestration of newly synthesized aggregation-prone F508del-CFTR protein (Luciani et al., 2010) into aggresome bodies, thus allowing its trafficking towards the PM. Additionally, knockdown of p62 also mimics the F508del-CFTR rescuing effect of cysteamine, thus confirming the crucial role of p62 in regulating the levels of F508del-CFTR on the PM. Recently, we demonstrated that cysteamine blocked translocation of acid sphingomyelinase (ASM) enzyme to the PM, thus blocking the conversion of sphingomyelin to ceramide, a pathogenic bioactive lipid implicated in CF lung disease (**Figure 1**; Bodas et al., 2018b). This study adds another novel mechanism of cysteamine action in controlling inflammatory-apoptotic signaling in CF lung disease, although further pre-clinical and clinical studies are warranted to verify these mechanisms. Nonetheless, it is encouraging that cysteamine is being developed as a delayed-release capsule form (Lynovex®) (Charrier et al., 2014) and has undergone preliminary clinical studies in CF subjects. A relatively recent small single arm, phase 1/2a open label study was conducted to evaluate the tolerance and pharmacokinetics of cysteamine in CF patients (Devereux et al., 2016). The results indicated that although some adverse reactions were observed in CF patients who were given oral cysteamine, these were similar to the side effects seen in cystinosis subjects (Devereux et al., 2016). Overall, cysteamine was well tolerated and entered the bronchial secretions at concentrations higher than plasma (Devereux et al., 2016). In

addition, a recent promising study in mice and human CF subjects was conducted using cysteamine and epigallocatechin-gallate (EGCG) as a combinatorial drug strategy (Tosco et al., 2016). The beneficial effects of this approach were attributed to autophagy-induction mediated restoration of F508del-CFTR to the PM by cysteamine, followed by enhanced stability of the PM-resident CFTR protein via inhibition of protein kinase CK2, by EGCG. Intriguingly, we and others recently described that cysteamine can be utilized as a multi-pronged CF-drug candidate, as its abilities are not restricted to just correcting the CFTR-dependent chloride ion transport defect. In fact, cysteamine possesses a diverse repertoire of beneficial properties such as anti-oxidant (Bodas et al., 2016, 2018b; Govindaraju et al., 2017; Vij et al., 2018), anti-inflammatory (Ferrari et al., 2017), autophagy-inducer (Esposito et al., 2016; Tosco et al., 2016; Ferrari et al., 2017), bactericidal (Charrier et al., 2014; Ferrari et al., 2017; Shrestha et al., 2017), mucolytic and anti-biofilm (Charrier et al., 2014; Brockman et al., 2017), which are all necessary to control acute or recurring exacerbations and CF pulmonary disease progression. Even though cysteamine is a strong CF-drug candidate, it's utility is possibly restricted to patients with only the F508del-CFTR mutation, as it was not very effective in other types of CFTR mutations such as R560S-CFTR (Awatade et al., 2018) that warrants further evaluation. Moreover, in spite of all the beneficial properties, the main caveats in the use of cysteamine is its poor bioavailability and the requirement of a high dose which is difficult to achieve *in vivo* (Vu et al., 2017). Thus, novel attempts have been made to improve the bioavailability of cysteamine, as well as to decrease the effective dose, such as by conjugating it with a [3-fatty acid (docosahexaenoic acid, DHA)], which also has its own autophagy inducing properties via the AMPK pathway (Vu et al., 2017). This conjugate could effectively rescue F508del-CFTR to the PM at a substantially lower concentration, thus warranting its further evaluation in a clinical setting. In another report, nine "prodrugs" of -glutamyl-cysteamine were tested in cultured kidney cells, to overcome its major disadvantages (Frost et al., 2016). These prodrugs could undertake successful delivery of cysteamine into kidney epithelial cells with improved bioavailability and low toxicity (Frost et al., 2016). This approach seems promising and needs further evaluation in pre-clinical CF models.

We have previously demonstrated the utility of nanotechnology in the development of novel drug delivery systems aimed at sustained and targeted delivery to the CF airways (Vij et al., 2010; Vij, 2011, 2017; Brockman et al., 2017). Using a similar approach, we recently proposed the application of dendrimer technology in designing a novel drug-delivery system to improve cysteamine's bioavailability and specificity. We developed a dendrimer-cysteamine conjugate formulation (PAMAM-DEN^{CYS}), and tested its ability to induce trafficking of F508del-CFTR to the PM in CF cells (Brockman et al., 2017). Although this was an pre-clinical early stage investigation, we were able to demonstrate key therapeutic signatures such as rescue of F508del-CFTR from the aggresome bodies and it's trafficking to the PM, as well as control of *Pa* infection and growth, and mucolytic potential (Brockman et al., 2017). Therefore, this novel PAMAM-DEN^{CYS} conjugate has a potential for further development as an emerging CF therapeutic strategy, as it corrects the proteostasis and autophagy impairment, which is the central disease-promoting mechanism in pathogenesis of chronic CF lung disease.

GSNO AND GSNOR INHIBITORS

Another interesting strategy to correct the proteostasis and autophagy defect in CF is through nitric oxide (NO)-augmentation, which facilitates the rescue of misfolded F508del-CFTR protein to the PM. Some previous reports propose the use of NO-donors (such as S-nitrosoglutathione, GSNO) or the inhibitors of GSNO-reductase (GSNOR), in controlling airway inflammation in experimental allergic asthma (Blonder et al., 2014) and CF models (Zaman et al., 2006, 2014, 2016; Rafeeq and Murad, 2017). In the lungs, NO and its reservoir, GSNO, play a very crucial role in the maintaining airway smooth muscle tone and controlling inflammation (Que et al., 2009; Sun et al., 2011). The levels of GSNO are tightly regulated by GSNOR, the enzyme which degrades GSNO (Sun et al., 2011). In fact, GSNO levels are diminished, with a concomitant increase in GSNOR levels,

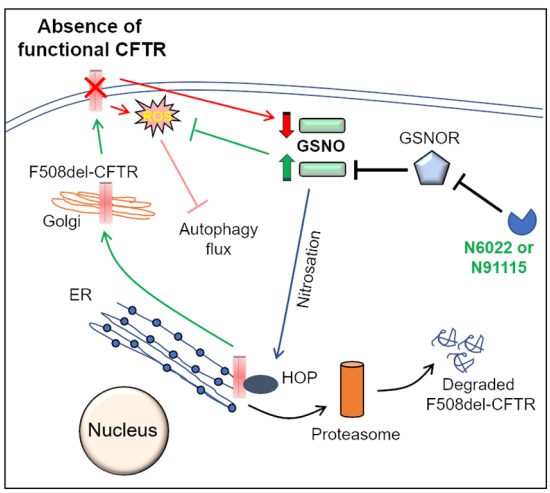

FIGURE 2 | Mechanisms of GSNOR-inhibitors mediated rescue of F508del-CFTR to the PM. Molecular chaperones such as Hsp70Hsp90 organizing protein (HOP) regulate CFTR biogenesis and proper trafficking to the PM. In the ER, association of HOP with F508del-CFTR leads to its degradation via the ER-associated proteasomal pathway. S-nitrosoglutathione (GSNO), a cellular nitric oxide (NO) donor, modulates the function of HOP by its S-nitrosation. In the absence of a functional CFTR, cellular GSNO levels are low, which results in decreased S-nitrosation of HOP, which promotes proteasome mediated degradation of F508del-CFTR. The cellular levels of GSNO are tightly regulated by the enzyme, GSNO-reductase (GSNOR), which mediates the catabolism of GSNO. Pharmacological inhibition of GSNOR using N6022 or N91115 increases GSNO levels that leads to increased S-nitrosation of HOP. It is believed that an increase in HOP S-nitrosation hampers its association with F508del-CFTR, thereby allowing the forward trafficking and maturation of F508del-CFTR. Moreover, recent studies from our group also indicate that GSNO augmentation using N6022 can control the elevated ROS levels and thus correct the ROS-mediated autophagy flux impairment in CF.

in both asthmatic and CF lungs (Que et al., 2009; Sun et al., 2011; Zaman et al., 2016), indicating that altered NO signaling contributes to asthma and CF pathogenesis. Mechanistically, the GSNO-mediated *S*-nitrosylation and subsequent degradation of Hsp70/Hsp90 organizing protein (HOP) favors the forward trafficking of CFTR to the PM (**Figure 2**; Odunuga et al., 2004; Marozkina et al., 2010). The findings that GSNO could increase the expression, maturation and function of both WT and F508del-CFTR in human bronchial epithelial cells led to clinical testing of a GSNOR-inhibitor, N91115 (Cavosonstat, Nivalis Therapeutics) (C.F. Foundation, 2015; Donaldson et al., 2017). The study reported that N91115 was well tolerated over a 28 day period, with no dose-limiting toxicities and no safety issues (Donaldson et al., 2017), albeit the study was discontinued in the Phase 2 stage as no improvement in lung function was observed in CF subjects. We recently reported that apart from its CFTR rescuing property, GSNO augmentation by using either GSNO or a GSNOR-inhibitor (N6022) effectively diminished CS-induced inflammatory-oxidative stress and also corrected the autophagy impairment (Bodas et al., 2017), thus targeting the underlying cause of CFTR dysfunction and resulting CF lung disease pathogenesis and progression. In fact, N6022 has been tested in clinical trials on CF patients with somewhat encouraging outcomes (C.F. Foundation, 2014; Rafeeq and Murad, 2017). The autophagy inducing property of GSNO or N6022 could be attributed to its rescue of CFTR to the PM (Zaman et al., 2016; Bodas et al., 2017), or other mechanisms such as its inhibitory effect on mTOR (Montagna et al., 2016), or its anti-oxidant function (Rauhala et al., 1998; Khan et al., 2011; Bodas et al., 2017). The *in vivo* application of N6022 could be restricted because of its low bioavailability, due to the presence of the highly polar imidazole group (Sun et al., 2011). Thus, GSNO-augmentation has the potential to be further tested in CF, where modifications in dosing and concurrent development of airway-delivery methodology can allow successful clinical outcomes.

POTENTIAL APPLICATION OF FISETIN AS A NUTRACEUTICAL FOR CF

A plant derived nutraceutical, Fisetin (3,3′,4′,7-tetra hydroxyflavone), demonstrates the potential to be a future CF drug candidate (Pal et al., 2016). Ongoing, experimental and clinical research is investigating the preventive and therapeutic properties of Fisetin in chronic inflammatory conditions (Pal et al., 2016), neurological diseases and various types of cancers (Pal et al., 2016). Previous studies have described Fisetin as a potent anti-oxidant (Khan et al., 2013; Pal et al., 2016; Govindaraju et al., 2017), anti-inflammatory (Khan et al., 2013; Pal et al., 2016), bactericidal (Pehote et al., 2017) and also an inhibitor of PI3K/AKT/mTOR signaling pathway (Adhami et al., 2012), which regulates key cellular processes including autophagy, and is discussed below in this article. In the context of inflammatory pulmonary diseases, Fisetin has demonstrated its therapeutic potential in murine models of allergic airway inflammation (Goh et al., 2012; Brown et al., 2016), and lipopolysaccharide (LPS) induced acute lung injury in rats, through its NFκB-targeted anti-inflammatory mechanism of action (Feng et al., 2016). Moreover, our recent report using cigarette smoke (CS)-extract and Pa model in murine macrophages, demonstrates the efficacy of Fisetin in correcting the CS-induced defect in bacterial clearance via transcription factor-EB (TFEB)-mediated autophagy-induction, and/or by restoring expression of mature (WT)-CFTR (Pehote et al., 2017). Additionally, similar to cysteamine, Fisetin also reveals direct bactericidal activity against Pa bacteria, a predominant CF-pathogen, by hitherto unknown mechanism(s) (Pehote et al., 2017). In another parallel study, using CS-exposure of retinal pigment epithelial cells (RPE) as an *in vitro* model of age-related macular degeneration (AMD), Fisetin successfully corrected the CS-induced autophagy-flux impairment and reduced the perinuclear accumulation of aggresome bodies, plausibly by controlling CS-induced ROS-activation (Govindaraju et al., 2017). Since CF lung disease is also characterized by chronic inflammatory-oxidative stress, persistent bacterial infections and autophagy impairment, proof of concept *in vitro* data warrants evaluation of the efficacy of Fisetin in pre-clinical CF-lung disease models. Although, it should be noted that similar to other promising autophagy-inducing drug candidates, the utility of Fisetin is hampered by its poor aqueous solubility (Bothiraja et al., 2014) and low oral bioavailability (Seguin et al., 2013), and thus attempts have been underway to improve its *in vivo* efficacy by the use of nanotechnology-based airway-delivery approaches (Ragelle et al., 2012; Kadari et al., 2017; Mehta et al., 2018).

A THYMIC PEPTIDE TO CORRECT THE BASIC CF-DEFECT

Recent studies have highlighted the potential of Thymosin α-1 (Tα1), a thymic peptide with broad immune-modulatory properties, in correcting the basic CF-defect, i.e., the restoration of misfolded F508del-CFTR to the PM (Romani et al., 2017; Garaci, 2018; Rubin, 2018; Stincardini et al., 2018). Mechanistically, activation of indoleamine 2, 3-dioxygenase (IDO1), and the resulting decrease in inflammation, along with autophagy-induction are proposed as the key means of Tα1-mediated F508del-CFTR rescue (Romani et al., 2017) (**Figure 3**). Tα1 was shown to rescue F508del-CFTR to the PM at a clinically achievable dose, and this was attributed to its activity as a proteostasis modulator. Tα1 act's on multiple steps of F508del-CFTR recycling such as the Rab GTPase's, the deubiquitinating enzyme USP36, and the ubiquitin-binding protein, p62, which is involved in the aggresome sequestration of F508del-CFTR (Romani et al., 2017). In Tα1 treated cells, the F508del-CFTR co-localized with Rab9, which is the marker of recycling endosome. Moreover, the co-localization of F508del-CFTR with Rab5 (early endosome marker) and Rab7 (late endosome marker) was diminished by Tα1 treatment. Thus, Tα1 reduces the endocytic recycling of F508del-CFTR into early endosomes and also prevents its transport into late endosomes and/or lysosomes, thereby promoting its forward recycling to the PM (Romani et al., 2017). Although Tα1 has a good clinical safety profile and is already available commercially as ZADAXIN® for the treatment

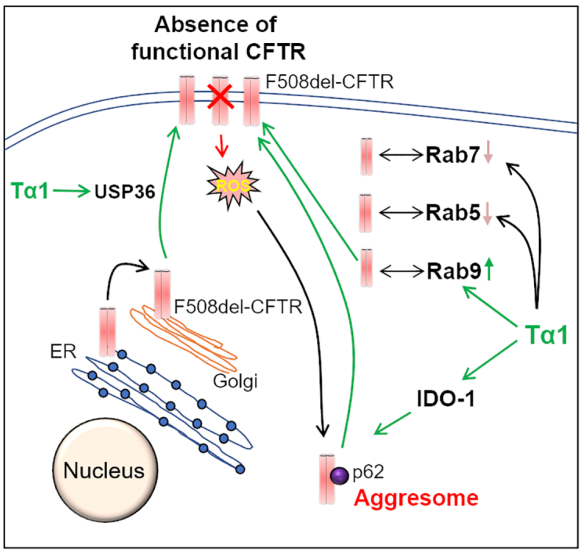

FIGURE 3 | Thymosin-α-1 acts on multiple-targets to rescue F508del-CFTR. Thymosin α-1 (Tα1) is a thymic peptide, which acts on multiple cellular pathways in the CFTR recycling and maturation process to restore the misfolded F508del-CFTR to the plasma membrane (PM). First, Tα1 can induce autophagy via activation of indoleamine 2, 3-dioxygenase (IDO-1), and reduction of p62 levels, thereby resulting in the rescue of F508del-CFTR to the PM. Additionally, Tα1 activates deubiquitinating enzyme USP36, which in turn prevents the ubiquitination and subsequent degradation of F508del-CFTR, thus improving its PM stability. Moreover, in Tα1 treated cells, F508del-CFTR is found to be associated with Rab9 GTPase (recycling endosome marker), which promotes recycling of F508del-CFTR to the PM. This happens in parallel to a decrease in co-localization of F508del-CFTR with Rab 5 (early endosome marker) and Rab7 (late endosome marker) GTPases, in Tα1 treated cells.

of several inflammatory and/or infectious diseases, such as viral infections, immunodeficiency diseases, HIV/AIDS and cancers (Romani et al., 2017), it remains to be investigated whether it possesses other important anti-CF attributes such as bactericidal and mucolytic. Moreover, some recent studies report that Tα1 failed to rescue CFTR in epithelial cells and primary bronchial epithelial cells from CF patients (Matthes et al., 2018; Tomati et al., 2018), although these effects may be due to the incorrect solvent used by these investigators (Garaci, 2018). Thus, the recent claims that Tα1 could be a potential "single-molecule" drug for preventing/treating chronic CF-lung disease seems to be a far shot that requires further in-depth studies in pre-clinical and clinical CF settings.

PI3K/AKT/mTOR INHIBITORS AS EMERGING CF DRUGS

The mammalian target of rapamycin (mTOR) is a member of the phosphatidylinositol 3-kinase (PI3K)-related kinase family of proteins that has long been implicated in regulating key cellular processes such as cell growth (Yu and Cui, 2016), survival (Yu and Cui, 2016), motility (Holroyd and Michie, 2018), metabolic pathways (Yu and Cui, 2016) and autophagy (Kim and Guan, 2015; Rabanal-Ruiz et al., 2017). The PI3K/AKT/mTOR signaling pathway is altered in several disease states such as cancer (Conciatori et al., 2018; Guri et al., 2018), immune system-related diseases (Guri et al., 2018; Jung et al., 2018), idiopathic pulmonary fibrosis (IPF) (Lawrence and Nho, 2018), COPD (Houssaini et al., 2018; Wang et al., 2018) and lymphangioleiomyomatosis (LAM) (Gao et al., 2018). Since mTOR is considered to be the master regulator of the autophagy pathway, its inhibitors have been investigated for their therapeutic potential in different types of cancers, and autophagy-induction is one of the proposed mechanisms of action (Saxton and Sabatini, 2017; Paquette et al., 2018). Moreover, in fibroblasts, higher than normal mTOR activity and the resulting autophagy-defect has been associated with pathogenesis of IPF (Lawrence and Nho, 2018), a fatal chronic restrictive lung disease. Additionally, elevated mTOR signaling mediated autophagy impairment was recently observed in lung cells and tissues isolated from COPD subjects, while the same conceptual evidence was also derived from transgenic mice with constitutive or conditional over-activation of mTOR (Houssaini et al., 2018). Interestingly, lung cell senescence and development of emphysema was found to be associated with elevated mTOR activity in these mice, as the effects could be ameliorated by rapamycin (an mTOR inhibitor) (Houssaini et al., 2018). It is also noteworthy that mTOR inhibition has been beneficial in the clearance of protein aggregates (aggresomes) in neurogenerative diseases (Heras-Sandoval et al., 2014), thus further confirming the crucial role of mTOR in regulating the autophagy process. Rapamycin mediated mTOR inhibition has been shown to reduce lung inflammatory responses in a CF mouse model (Abdulrahman et al., 2011), along with improved CFTR function (Luciani et al., 2011; Tazi and Amer, 2015). These studies confirmed that restoration of autophagy using Rapamycin, which is commercially available as Sirolimus, could benefit CF patients, although it has several limitations in clinical practice (Emoto et al., 2013; Li et al., 2014). Sirolimus has low oral bioavailability (Brasttström C. et al., 2000), poor water solubility (Kim et al., 2011), a huge pharmacokinetic variability among patients (Emoto et al., 2013), adverse side effects (Bee et al., 2018), and off-target effects (Arriola et al., 2016; Lamming, 2016; Haeri et al., 2017), which are due to its inhibition of both mTORC1 and mTORC2 (Arriola et al., 2016). The primary side effects of sirolimus include hyperglycemia, hyperlipidemia, insulin resistance and increase in new onset of type 2 diabetes (Emoto et al., 2013; Bee et al., 2018). In a national cohort study, the lung function response to rapamycin treatment and its associated side effects in women with progressive lung disease due to LAM was investigated (Bee et al., 2018). It was observed that although side effects were common, but they were manageable over several years, and improvements in lung function were evident. Overall, a low dose rapamycin was associated with fewer side effects with no difference in the beneficial effects (Bee et al., 2018), thus warranting its further clinical evaluation in CF. Moreover, several studies have been conducted to devise ways to enhance the bioavailability and improve *in vivo* delivery of sirolimus (Kim et al., 2011; Haeri et al., 2017). Considering the central role of impaired-autophagy and resulting aggresome-pathology in CF, it seems worthwhile to test the

efficacy of mTOR inhibitors in CF models. In accord with this idea, CFBE41o- cells demonstrated upregulated mTOR activity, and the resulting autophagy impairment was found to be associated with accumulation of F508del-CFTR into peri-nuclear aggresome bodies (Reilly et al., 2017). Moreover, the inhibition of PI3K/AKT/mTOR pathway by six different compounds enhanced CFTR-membrane stability and expression (Reilly et al., 2017). The study identified MK-2206 as the most potent CFTR rescuing compound, which functions through targeting Bcl-2-associated athanogene 3 (BAG3), a regulator of autophagy and aggresome clearance (Reilly et al., 2017). Thus, the efficacy of pharmacological PI3K/AKT/mTOR inhibitors warrants further evaluation as potential therapeutic candidates for chronic CF lung disease, based on their ability to rectify the disease-promoting proteostasis and autophagy defect, including the correction of underlying CFTR dysfunction.

HDAC INHIBITORS AS PROTEOSTASIS MODULATORS IN CF

Inhibition of histone deacetylases (HDAC) has been evaluated as a potential therapeutic strategy for several protein folding and other chronic inflammatory diseases such as neurodegenerative diseases (Benito et al., 2015; Rabal et al., 2016), chronic kidney disease (Liu and Zhuang, 2015), inflammatory bowel disease (Felice et al., 2015), cancer (Falkenberg and Johnstone, 2014; West and Johnstone, 2014; De Souza and Chatterji, 2015), graft-versus-host disease (Choi et al., 2014, 2017), rheumatoid arthritis (Oh et al., 2017) and CF (Hutt et al., 2010, 2011; Bodas et al., 2018a). Pharmacological studies of suberanilohydroxamic acid (SAHA, Vorinostat), a broad inhibitor of class I and II HDAC enzymes (Bubna, 2015), in different types of cancers indicate that SAHA is well tolerated and demonstrates good oral bioavailability (43%) (Kelly et al., 2005). Moreover, the major adverse effects of SAHA administration such as fatigue, diarrhea, dehydration, etc., where more prominent in the intravenous treatment route, rather than the oral treatment regime, and the more severe indications such as thrombocytopenia, were resolved upon discontinuation of treatment (O'Connor et al., 2006; Bubna, 2015). Additionally, SAHA is also an FDA approved drug for cutaneous T-cell lymphoma (Bubna, 2015). Thus, at least in patient-based studies targeting cancer, SAHA was safely administered over a prolonged period, with minimal toxicity and consistent anti-HDAC activity, thereby indicating its potential tolerance as a CF drug candidate. In CF, the pharmacological inhibition of HDACs, especially using SAHA seems encouraging as this provides twofold benefit of controlling the inflammation (Hull et al., 2016; Xu et al., 2017) and also function as a proteostasis regulator (Bouchecareilh et al., 2012; Han et al., 2015) to facilitate rescue and trafficking of F508del-CFTR to the PM (CFTR-corrector) (Bodas et al., 2018a). Indeed, we recently verified the potential utility of SAHA in rescuing the F508del-CFTR to the PM by delaying its degradation, thus confirming its potential as a CFTR-corrector (Bodas et al., 2018a). Additionally, SAHA treatment was also effective in controlling *Pa*-LPS induced inflammation and neutrophil activation in a pre-clinical CF murine model, which was possibly *via* induction of regulatory T cells (Bodas et al., 2018a). Intriguingly, this observation was CFTR-independent, as the inflammation quenching function of SAHA was evident even in C*ftr*$^{-/-}$ mice. This indicates that SAHA could provide a potential therapeutic benefit in CF irrespective of its ability to rescue mutant CFTR. An ostensibly contrasting study demonstrates the failure of SAHA to restore F508del-CFTR, albeit the cells and the dose of SAHA used in those reports are dissimilar to our studies, which possibly explains the disparity in the results (Bergougnoux et al., 2017). Moreover, in two other studies SAHA was able to increase forskolin-induced chloride secretion in cell lines expressing CFTR but failed to demonstrate the same effect in primary epithelial cells from CF patients (Sondo et al., 2011; Van Goor et al., 2011). Nonetheless, proteostasis regulators such as SAHA and specific HDAC 6/7 inhibitors, such as tubacin (Cebotaru et al., 2008) have been evaluated in rescuing misfolded F508del-CFTR from proteasomal degradation and aggresome-accumulation. Further pre-clinical studies are necessary to evaluate the therapeutic efficacy of specific HDAC inhibitors, which might be coupled with novel drug-delivery systems (Mohamed et al., 2012; Tran et al., 2014) to further enhance their *in vivo* efficacy and bioavailability in CF lungs.

Briefly, as a proof of concept in support of proposed strategy adapting proteostasis and autophagy for rescuing the CF lung disease, recent study demonstrates that VX-809 mediated CFTR rescue is proteostasis-dependent but autophagy-independent (Pesce et al., 2018), where potent autophagy augmentation will allow synergistic effects on both mutant-CFTR rescue and *other components* (Romani et al., 2017; Stincardini et al., 2018) of CF lung disease pathogenesis as discussed in detail above (De Stefano et al., 2014; Tosco et al., 2016; Vu et al., 2017; Hutt et al., 2018; Stincardini et al., 2018; Zhang et al., 2018). In this study investigators, attempted to augment autophagy using torin-1 (Pesce et al., 2018) but its effects are missed due to lack of serum-starvation and appropriate experimental conditions. Nonetheless, extensive body of experimental evidence from our group and other's suggest that adapting proteostasis and autophagy has significant potential in correcting the underlying causes of CF lung disease pathogenesis and will allow development of next generation of potent novel therapeutics as summarized below.

PERSPECTIVE

The absence of a functional membrane CFTR is the primary etiology of chronic lung disease development in CF patients, which progresses due to numerous pathological complications such as mucus-overproduction, elevated oxidative stress, chronic infections and sustained NFκB-mediated inflammation, eventually leading to early-life fatality, if left untreated. Although huge strides have been made in the development of novel "breakthrough" drug combinations such as Orkambi®, Symdeko® etc., to rectify the core CF-defect, their widespread

therapeutic advantage has been restricted due to somewhat low efficacy in maintaining sustained CFTR-activation as well as controlling other components of CF lung disease such as chronic inflammatory-oxidative stress responses and exacerbations. Therefore, alternative therapeutic methodologies using novel drugs and/or drug-delivery systems need to be concurrently developed, which can fill the gap of an affordable yet potent and effective CF treatment strategy capable of rescuing overall CF lung disease. Since, significant experimental and pre-clinical evidence suggests the key central role of proteostasis and autophagy processes in regulating most of the disease-causing pathogenic features in the CF airways, this warrants further clinical evaluation and development of proteostasis and autophagy modulating drugs, as an emerging therapeutic approach for CF lung disease. Finally, since CF subjects possesses numerous types of CFTR mutations, genotyping of the patient before deciding on the proteostasis and autophagy modulating drug(s), will allow evaluating the therapeutic advantage for the patient as a part of emerging Precision Medicine Initiative.

AUTHOR CONTRIBUTIONS

Both authors contributed to the concept, framework and writing of the manuscript for publication.

ACKNOWLEDGMENTS

NV was supported by National Institutes of Health (NIH, R03HL09631 and CTSA UL RR025005ATIP), Flight Attendant Medical Research Institute (FAMRI, YCSA082131), and Cystic Fibrosis Foundation (CFF, R025-CR07 and VIJ07IO) grants to develop proteostasis/autophagy modulation therapeutic strategy and drug-delivery systems discussed in this publication.

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Conflict of Interest Statement: NV is the lead inventor on patent targeting proteostasis mechanisms for rescuing CFTR protein-processing defect and CF lung disease and also a founder of VIJ Biotech that focuses on bench-side translation of novel CF and COPD therapeutics.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 29 January 2019
doi: 10.3389/fphar.2019.00024

Unraveling the Function of Lemur Tyrosine Kinase 2 Network

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Lemur Tyrosine Kinase 2 (LMTK2) is a recently cloned transmembrane protein, actually a serine/threonine kinase named after the Madagascar primate lemur due to the long intracellular C-terminal tail. LMTK2 is relatively little known, compared to other kinases but its role has been increasingly recognized. Published data show that LMTK2 regulates key cellular events, including endocytic trafficking, nerve growth factor signaling, apoptosis, and Cl^- transport. Abnormalities in the expression and function of LMTK2 are associated with human disease, such as neurodegeneration, cancer and infertility. We summarized the current state of knowledge on LMTK2 structure, regulation, interactome, intracellular localization, and tissue expression and point out future research directions to better understand the role of LMTK2.

Keywords: LMTK2, PP1, kinase, phosphorylation, signaling pathway

OPEN ACCESS

Edited by:
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Telethon Kids Institute, Australia

Reviewed by:
Hiroshi Hibino,
Niigata University, Japan
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *03 November 2018*
Accepted: *10 January 2019*
Published: *29 January 2019*

Citation:
Cruz DF, Farinha CM and Swiatecka-Urban A (2019) Unraveling the Function of Lemur Tyrosine Kinase 2 Network. Front. Pharmacol. 10:24. doi: 10.3389/fphar.2019.00024

INTRODUCTION

Protein phosphorylation is an important post-translational modification regulating protein–protein interactions and signal transduction (Manning et al., 2002a; Ubersax and Ferrell, 2007; Ardito et al., 2017). Indeed, the activity of enzymes and receptors is controlled by specific kinases and phosphatases. Kinases are responsible for protein phosphorylation, a reversible process that consists of the addition of a phosphate group PO_4 from the adenosine 5′-triphosphate (ATP) or guanosine 5′-triphosphate (GTP) to a polar group of various amino acids. This addition modifies the protein from hydrophobic apolar to hydrophilic polar, allowing a conformational change during interaction with other molecules (Ubersax and Ferrell, 2007; Cheng et al., 2011; Ardito et al., 2017). Most protein phosphorylation events occur on hydroxyl groups at the side chain of serine (S), threonine (T), and tyrosine (Y) residues (Ubersax and Ferrell, 2007; Schwartz and Murray, 2011; Ardito et al., 2017). In turn, phosphatases remove the PO_4 group from phosphoproteins by hydrolyzing phosphoric acid monoesters (Barford, 1996; Zhang, 2002; Ardito et al., 2017).

The human kinome comprises 518 protein kinases and 20 lipid kinases encoded by genes that correspond to 1.7% of the human genome (Manning et al., 2002b; Heath et al., 2003; Duong-Ly and Peterson, 2013). Lemur Tyrosine Kinase 2 (LMTK2) is one of the most recently cloned serine/threonine (S/T) kinases. It is also known as cyclin-dependent kinase-5 (cdk5)/p35 regulated kinase (cprk), kinase/phosphatase/inhibitor-2 (KPI2), brain-enriched kinase (BREK), and apoptosis-associated tyrosine kinase (AATYK)-2. LMTK2 was found almost simultaneously by three different groups. In 2002, Wang and Brautigan aimed to understand the protein complex composed of Inhibitor 2 (Inh2) and Protein Phosphatase 1 (PP1), specifically if Inh2 binds to other partners besides PP1, and identified LMTK2 as one of the interacting proteins by yeast two-hybrid assay (Wang and Brautigan, 2002). In 2003, Kesavapany et al. (2003) found LMTK2 as a binding partner of the cdk5/p35 kinase. In 2004, Kawa et al. (2004) identified LMTK2 as a novel

kinase expressed in the brain, using *in silico* search, confirmed by reverse-transcription polymerase chain reaction (RT-PCR) and Northern blot analysis. Here, we present a comprehensive review of published data on LMTK2, identify knowledge gaps, and point out research directions to better understand the role of LMTK2 in physiology and human disease.

STRUCTURE, SPECIFICITY, REGULATION, AND LOCALIZATION OF LMTK2

Lemur Tyrosine Kinase 2 is composed of 1503 amino acid residues forming a short soluble N-terminal domain, followed by two hydrophobic transmembrane helices (residues 11–29 and 46–63), and a kinase domain (residues 137–407) with the ATP binding site (residues 143–168) (Wang and Brautigan, 2002; Nixon et al., 2013) (**Figure 1**). N- and C-terminal domains as well as the kinase active site are located in the cytosol (Nixon et al., 2013).

Naming of LMTK2 resulted from the sequence homology of the kinase domain with tyrosine kinases. The bioinformatics analysis revealed 60% homology between the kinase domain of LMTK2 and AATYK (Wang and Brautigan, 2002). LMTK2 also shares a putative autophosphorylation site with Src-family kinases, the Y^{295} residue, while the D^{265}LALRN motif in LMTK2 is also present in non-Src tyrosine kinases (Kawa et al., 2004). Despite the initial naming, LMTK2 was found to be a serine/threonine kinase (Wang and Brautigan, 2002, 2006). First, phospho-amino acid analysis demonstrated that LMTK2 undergoes auto-phosphorylation on serine and threonine residues, while tyrosine phosphorylation was not observed (Wang and Brautigan, 2002). Second, immunoblotting with anti-phospho-threonine and anti-phospho-serine antibodies showed reactivity with LMTK2 (Wang and Brautigan, 2002). Last, phosphorylation of myelin basic protein (MBP) by LMTK2 was mostly located at serine residues, with a trace at threonine residues; once again, no tyrosine phosphorylation was found (Wang and Brautigan, 2002). Similar results were obtained using a peptide microarray, which demonstrated that LMTK2 interacts with phosphorylated serine and threonine sites in peptides from bovine MBP (Wang and Brautigan, 2006). In fact, the peptide microarray demonstrated that LMTK2 phosphorylates serine and threonine residues preceded or followed by proline (P) residues (Wang and Brautigan, 2006), suggesting similarity with proline-directed kinases. Although these kinases, such as cyclin-dependent kinase (cdk) or glycogen synthase kinase 3 beta (GSK3-β) phosphorylate only those serine/threonine residues that are followed by proline [(S/T)-P-x] (Pelech, 1995; Wang and Brautigan, 2006). LMTK2 also differs from the proline-directed kinases because it is not exclusively specific to proline sites; actually, many of the LMTK2 reactive sites have neighboring basic residues (Wang and Brautigan, 2006).

The C-terminal domain of LMTK2 contains a V^{1355}TF motif that binds the catalytic subunit of PP1 (PP1c) necessary for inhibition of PP1 activity (Wang and Brautigan, 2002). The C-terminal domain is also rich in proline residues conforming to seven PxxP-motifs, where x is a variable amino acid (Kawa et al., 2004). The PxxP domains may be involved in regulation of LMTK2 activity, intracellular localization, or substrate recognition, through interaction with SH3 domains of LMTK2-interacting proteins; however, specific SH3 domain containing partners of LMTK2 have not been identified yet.

Tissue Expression and Subcellular Localization

According to the Human Protein Atlas (HPA) database[1], LMTK2 is ubiquitously expressed in human tissues (The Human Protein Atlas, 2018a). Northern blot analysis demonstrated high levels of the LMTK2 mRNA in human skeletal muscle while low levels were observed in the brain and pancreas (Wang and Brautigan, 2002). LMTK2 protein was also experimentally detected in human bronchial epithelial (HBE) cells (Luz et al., 2014) and prostate epithelial cells (Shah and Bradbury, 2015b). LMTK2 transcripts were detected in mice, with the most prominent signal found in telenchepalon (Kawa et al., 2004). Indeed, mouse LMTK2 mRNA levels were much higher in the brain than in the skeletal muscle, in contrast to human LMTK2 (Wang and Brautigan, 2002). LMTK2 expression in the mouse brain was detected at all developmental stages, with increased expression during the early postnatal age (weeks 0–2). Similar to human, LMTK2 was found in mouse primary prostate epithelial cells (Shah and Bradbury, 2015b).

Concerning subcellular localization, LMTK2 is enriched on intracellular membranes, especially in organelles involved in the endocytic and recycling pathways (Nixon et al., 2013). Specifically, LMTK2 was found on transferrin-, Rab5-, EEA1-, and Rab11-positive vesicles (Kawa et al., 2004; Chibalina et al., 2007). Confocal imaging and subcellular fractionation studies detected LMTK2 in nuclear and non-nuclear compartments in prostate cancer cells (Shah and Bradbury, 2015b). In differentiated and undifferentiated PC12 cells, derived from pheochromocytoma of the rat adrenal medulla, LMTK2 was enriched at juxta-membranous regions while in differentiated PC12 cells it was also enhanced at the growth cone (Kawa et al., 2004). LMTK2 can also localize at the plasma membrane. It was detected at the apical and basolateral membrane domain in primary differentiated HBE cells derived from cystic fibrosis (CF) lungs and in immortalized CFBE41o- cells, a human CF bronchial epithelial cell line derived from a CF patient homozygous for the F508del-CFTR mutation (Luz et al., 2014). In HeLa cells, LMTK2 was present at the plasma membrane as well as throughout the cytoplasm and it was enriched in cellular extensions and the perinuclear region (Chibalina et al., 2007).

Regulation of *lmtk2* Gene Expression

The *lmtk2* gene promoter regulates expression of endogenous LMTK2 (Dey and Bradbury, 2017). The sequence GGTGAGTCAGTG upstream of the transcription initiation site of the *lmtk2* gene, conforms to the phorbol 12-myristate 13-acetate (TPA) response element (TRE) (Dey and Bradbury, 2017). Exposure of HEK293, HeLa, and CFBE41o- cells to low

[1] https://www.proteinatlas.org/

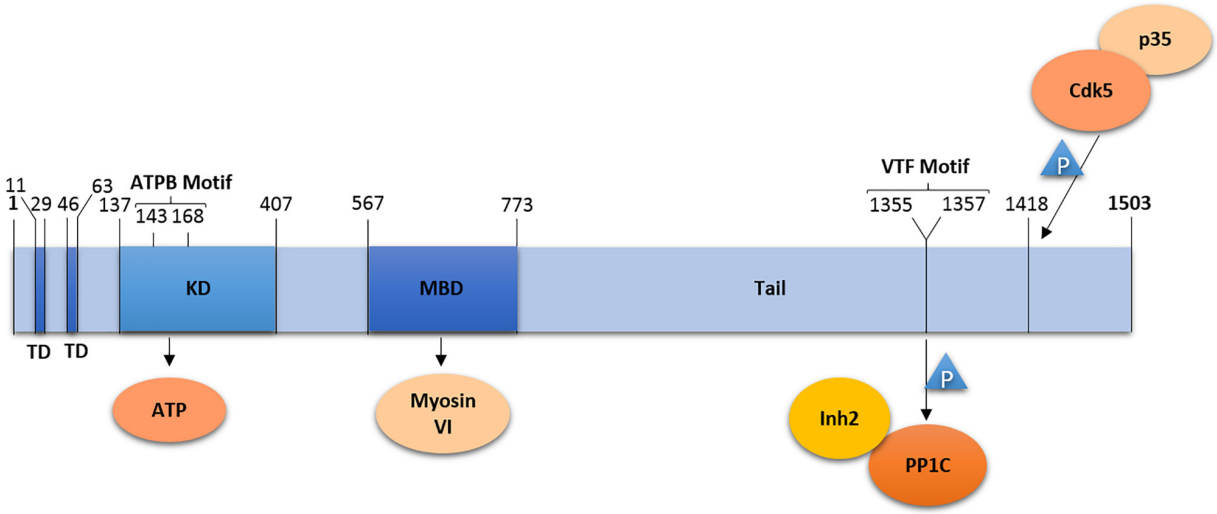

FIGURE 1 | Domain organization of LMTK2. LMTK2 contains two transmembrane domains (TD), followed by a kinase domain (KD) with an ATP binding (ATPB) motif, a Myosin VI binding domain (MBD), and a tail domain. The kinase domain residue K^{168} is critical for LMTK2 catalytic activity. LMTK2 interacts with PP1c via its VTF motif (residues 1355–1357). LMTK2 is phosphorylated in its residue S^{1418}, by the complex Cdk5/p35. Numbers indicate amino acid residues.

concentrations of TPA significantly increased LMTK2 mRNA and protein levels, that were not further increased with higher concentrations of TPA. In fact, exposure to high concentrations of TPA, in CFBE cells, decreased the LMTK2 protein expression below basal levels (Dey and Bradbury, 2017). Phorbol esters regulate gene expression by altering the protein kinase C (PKC) activity. Inhibition of PKC blunted the upregulation of LMTK2 expression by low concentrations of TPA while prolonged exposure of cells to high concentrations of phorbol esters downregulated PKC activity and inhibited LMTK2 expression (Castagna et al., 1982; Sanders and Stern, 1996; Dey and Bradbury, 2017). TPA induces binding of the transcriptional AP-1 complex c-Jun and c-Fos protein families to the TRE in *lmtk2* promoter and these proteins modulate TPA effects on LMTK2 in a PKC dependent manner (Angel and Karin, 1991; Dey and Bradbury, 2017).

Biosynthetic Processing of LMTK2

Synthesis of membrane proteins occurs on ribosomes associated with the rough ER followed by export in the coat protein complex II (COPII)-coated vesicles. To facilitate this process, LMTK2 contains three arginine residues R^8RR, located upstream of the first hydrophobic sequence, which functions as a signal peptide targeting LMTK2 to the endoplasmic reticulum (ER) and secretory pathway (Peterson et al., 2003; Kawa et al., 2004; Owji et al., 2018).

Endoplasmic reticulum export is also regulated by specific amino acid residues located in the protein cytoplasmic domain (Butler and Bradbury, 2015). One of the previously identified ER export motifs is characterized by two acidic residues separated by a variable amino acid [(D/E)×(D/E)] (Sevier et al., 2000; Butler and Bradbury, 2015). The di-acidic motif is localized within the cytosolic domain of the protein, facilitating interactions with COPII components (Butler and Bradbury, 2015).This motif, first found in the vesicular stomatitis virus glycoprotein (VSVG) (Sevier et al., 2000), was later demonstrated in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and subsequently in other mammalian proteins (Fu and Sztul, 2003; Wang et al., 2004). Sequence alignment of various mammalian orthologs of LMTK2 revealed presence of a canonical di-acidic motif in the distal portion of the C-terminal tail, with a proximal upstream tyrosine residue, as observed in VSVG sequence in all species (Sevier et al., 2000; Butler and Bradbury, 2015).

Mutations of the di-acidic ER export motif D^{1110}-S-E, with D^{1110} and E^{1112} substituted with alanines (A^{1110}SA), caused retention of LMTK2 in the ER; however, a small amount of mutant LMTK2 was exported, suggesting presence of another ER export signal (Butler and Bradbury, 2015). The A^{1110}SA mutation increased stability of LMTK2 protein compared to the wild type (WT) kinase (half-life ∼3.5 h vs. ∼2.5 h, respectively) (Butler and Bradbury, 2015). Increased half-life of the A^{1110}SA mutant suggests that its targeting to the ER-associated protein degradation (ERAD) pathway is reduced, compared to the WT LMTK2. The ER export motif is located downstream of the catalytic and membrane spanning domains, thus proper folding of the mutant LMTK2 may prevent targeting to ERAD, leading to ER accumulation and subsequent increase of the half-life (Butler and Bradbury, 2015).

THE LMTK2 INTERACTOME

Lemur Tyrosine Kinase 2 has been described to interact with different protein partners, as summarized in **Table 1**. Validation of such protein–protein interactions with endogenously expressed proteins or *in vivo* models confirms the relevance of the LMTK2 interactome in human health and disease.

TABLE 1 | LMTK2 interactome.

Interactors	Type of interaction	Function	Cell line	Reference
PP1	Phosphorylation of PP1c-T^{320}	Inhibition of PP1c	HeLa, COS7	Manser et al., 2012a,b; Wang and Brautigan, 2002
Inh2	Direct binding (dependent of PP1c)	Regulation of PP1c	HeLa, COS7	Wang and Brautigan, 2002
Cdk5/p35	Phosphorylation of LMTK2-S^{1418}	Activation of LMTK2	HeLa, CHO, rat cortical neurons, COS	Kesavapany et al., 2003; Manser et al., 2012b
GSK3-β	Mediated by PP1c	Inhibition of GSK3-β phosphorylation, leading to its activation	HeLa	Manser et al., 2012a
KLC2	Mediated by GSK3-β	Decreases KLC2 phosphorylation and promotes binding to Smad2	HeLa	Manser et al., 2012a
Myosin VI	Direct binding (no phosphorylation)	Regulation of endocytic trafficking pathway	HeLa	Chibalina et al., 2007; Inoue et al., 2008
CFTR	Phosphorylation of CFTR-S^{737}	Regulation of CFTR endocytosis	Calu-3, CFBE41o$^-$	Luz et al., 2014; Wang and Brautigan, 2006
NGF	Phosphorylation of LMTK2	Down-regulation of LMTK2 activity	PC12	Kawa et al., 2004
BCL2/BCL-xL	Indirect interaction (cell-type dependent)	Regulation of expression of anti- and pro-apoptotic proteins	HME, MCF10A, HCT116, DLD-1	Conti et al., 2017
BIM	Indirect interaction (cell-type dependent)	Regulation of expression of anti- and pro-apoptotic proteins	HME, MCF10A, HCT116, DLD-1	Conti et al., 2017
AR	Direct binding	Inhibition of AR transcriptional activity	LNCaP, Human prostate tissue, HEK293	Shah and Bradbury, 2015b

PP1, protein phosphatase 1; Inh2, inhibitor 2; Cdk5, cyclin-dependent kinase-5; GSK3-β, glycogen synthase kinase 3 beta; KLC2, kinesin-1 light chain 2; CFTR, cystic fibrosis transmembrane conductance regulator; NGF, nerve growth factor; BCL, B-cell lymphoma; BIM, Bcl-2-interacting mediator of cell death; AR, androgen receptor.

PP1, Cdk5/p35, and Cell Signaling

Protein Phosphatase 1 is an ubiquitous serine/threonine phosphatase regulating many distinct processes, including gene transcription, cell cycle progression, protein synthesis, carbohydrate metabolism, neuronal signaling, and muscle contraction (Kelker et al., 2009). The PP1 holoenzyme is composed of PP1c and diverse regulatory subunits that are cell type and subcellular compartment specific (Pelech, 1995). LMTK2 binds directly to PP1c via the C-terminal VTF motif, resulting in an inhibitory phosphorylation of the T^{320} residue in PP1c (Wang and Brautigan, 2002; Manser et al., 2012a,b). LMTK2 interacts with one of the regulatory subunits of PP1c, Inh2 (Wang and Brautigan, 2002; Bollen et al., 2010). The Inh2−LMTK2 interaction is dependent on PP1c, because both PP1 and Inh2 demonstrated default binding in cell extracts (Wang and Brautigan, 2002). In view of this finding, it was suggested that LMTK2 inhibits PP1c activity through phosphorylation of its residue PP1c-T^{320} or through the interaction with Inh2 (Wang and Brautigan, 2002). It was subsequently shown that the LMTK2 kinase activity is required to inactivate PP1c. Studies conducted in Chinese hamster ovary (CHO) cells, HeLa cells and in cultured rat cortical neurons demonstrated that phosphorylation of LMTK2 on the S^{1418} residue, in mouse sequence, activates the kinase (Manser et al., 2012b). In human sequence, this residue corresponds to S^{1450}, however, we will always mention as LMTK2-Ser^{1418} to remain consistent with the literature and to a better understanding. A bioinformatic analysis, using NetPhosK database, predicted that cdk5/p35 phosphorylates S^{1418} with the highest score. This prediction was corroborated with the observation that the LMTK2-S^{1418} precedes a proline and thus is phosphorylated by proline-directed kinases, such as the cdk5/p35 complex. Bioinformatics analysis also predicted that GSK3-β and p38 phosphorylate LMTK2-S^{1418}, although these predictions have not been experimentally validated (Manser et al., 2012b). *In vitro* studies confirmed that modulation of the cdk5/p35 activity alters LMTK2-S^{1418} phosphorylation. Studies in HeLa cells, where cdk5 is present in an inactive form due to absence of p35, demonstrated that transfection of p35 increased LMTK2-S^{1418} phosphorylation. However, LMTK2-S^{1418} was also phosphorylated at low levels in the absence of the cdk5/p35 complex, suggesting that other kinases may also phosphorylate LMTK2-S^{1418} in non-neuronal cells (Manser et al., 2012b). Although the LMTK2-S^{1418} is located only 91 amino acid (90 aminoacids in human sequence) residues apart from the PP1c-binding motif, mutation of this residue only affects the phosphorylation of PP1c-T^{320} and does not prevent the LMTK2−PP1c interaction (Manser et al., 2012b). Based on the data, a model has been proposed where cdk5/p35 activates LMTK2 by phosphorylating the S^{1418} residue that in turn stimulates LMTK2 binding and inhibition of PP1 by phosphorylation of the PP1c-T^{320} (Manser et al., 2012b).

Kinesin 1 and the TGF-β Signaling Pathway

Lemur Tyrosine Kinase 2 controls the kinesin-1 mediated transport along microtubule filaments, including the canonical TGF-β mediator, Smad2. Kinesin-1 is a heterotetramer composed of two motor subunits also known as kinesin-1 heavy chains (KHC) and two associated kinesin-1 light chains (KLC) (Rahman et al., 1998; DeBoer et al., 2008). GSK3-β phosphorylates one of the KLC, KLC2, leading to release of the protein cargo and inhibition of kinesin-1 mediated transport (Morfini et al., 2002).

The signaling pathway that controls GSK3-β phosphorylation of KLC2 involves cdk5/p35-mediated inhibitory phosphorylation of PP1c-T^{320}, which in turn induces inhibitory phosphorylation of GSK3-β-S^{9}. Several lines of evidence support the role of LMTK2 in this pathway. First, LMTK2 contains the PP1c-binding motif. Second, transfection of LMTK2 indirectly increased GSK3-β-S^{9} phosphorylation by mediating the inhibitory phosphorylation of PP1c-T^{320}. Third, transfection of LMTK2 decreased KLC2 phosphorylation and promoted binding of a known KLC2 cargo, Smad2, necessary for activation of the canonical TGF-β signaling pathway (Manser et al., 2012a).

Myosin VI and Endocytic Trafficking

Lemur Tyrosine Kinase 2 interacts with myosin VI, the minus-end directed actin-associated motor protein critical for trafficking in the endocytic and secretory pathway (Chibalina et al., 2007; Inoue et al., 2008). LMTK2 is the first transmembrane protein and kinase identified to directly bind to myosin VI. The myosin VI binding site in LMTK2 is located in a region downstream of the kinase domain (residues 567–773), first discovered by an yeast two-hybrid screen and later confirmed in cultured cells (Chibalina et al., 2007; Inoue et al., 2008). The LMTK2 role in endocytic trafficking does not involve phosphorylation of myosin VI (Tumbarello et al., 2013).

Lemur Tyrosine Kinase 2 binds to the WWY motif in the C-terminal tail of myosin VI and shares the binding site with the multifunctional adaptor protein Disabled-2 (Dab2) (Chibalina et al., 2007; Inoue et al., 2008). Myosin VI recruits Dab2 and LMTK2 to different vesicular compartments. Recruitment of myosin VI to the clathrin-coated structures requires Dab2 while LMTK2 recruitment is necessary for transport from early endosomes to the recycling compartment (Morris et al., 2002; Chibalina et al., 2007; Spudich et al., 2007). LMTK2 is present on endocytic and recycling vesicles, namely a subset of early endosomes, where it colocalizes with Rab5, early endosomal antigen 1 (EEA1) and Rab11, while myosin VI is restricted to Rab5-positive early endosomes (Hasson, 2003; Chibalina et al., 2007). Knockdown of LMTK2 resulted in enlargement, swelling, ring-like appearance, and altered function of vesicles involved in the endocytic trafficking pathways in HeLa cells (Chibalina et al., 2007). Moreover, LMTK2 binding to myosin VI was required for endocytic trafficking of the transferrin receptor (Inoue et al., 2008). Loss of LMTK2 in the appropriate vesicular regions, induced by the ER export motif mutation A^{1110}SA, affected membrane trafficking, characterized by reduced recycling of transferrin from the cells without affecting its endocytosis (Butler and Bradbury, 2015). The impact of A^{1110}SA on transferrin trafficking was similar to the results obtained after LMTK2 knockdown (Chibalina et al., 2007).

CFTR and Cl^- Transport

Lemur Tyrosine Kinase 2 interacts with CFTR and mediates its inhibitory phosphorylation and endocytosis, and inhibits the CFTR-mediated Cl^- transport. CFTR is a member of the ATP binding cassette (ABC) transporter family that functions as a cAMP-activated Cl^- ion channel (Riordan et al., 1989; Gregory et al., 1990). CFTR was identified as an interacting partner of LMTK2 through a microarray where the peptide containing the S^{737} residue, corresponding to a sequence in the regulatory domain of CFTR, showed the strongest reactivity with LMTK2 (Wang and Brautigan, 2006).

The interaction between CFTR and LMTK2 was predicted to occur at the plasma membrane (Wang and Brautigan, 2006). The prediction was later experimentally validated in human airway epithelial Calu-3 cells where the endogenous LMTK2 co-immunoprecipitated with endogenous CFTR specifically at the apical plasma membrane (Luz et al., 2014). Additionally, depletion of LMTK2 increased the steady-state plasma membrane abundance and activity of CFTR in the primary HBE cells. Increased CFTR expression at the plasma membrane, after LMTK2 knockdown resulted from decreased CFTR endocytosis, suggesting that endogenous LMTK2 decreases the CFTR-mediated Cl^- secretion, at least in part by decreasing density of the CFTR Cl^- channels at the cell surface. This is the first evidence that CFTR endocytosis can be regulated, explaining the mechanism of phospho-dependent inhibitory effect of CFTR-S^{737} on the CFTR mediated Cl^- secretion (Wilkinson et al., 1997). LMTK2 depletion increased the efficacy of the CFTR corrector VX-809 (Lumafactor), currently a component of Orkambi, a FDA approved drug for CF patients homozygous for the most common disease-causing mutation F508del (Luz et al., 2014). These data suggest that interfering with the LMTK2 mediated inhibitory phosphorylation of CFTR may increase efficacy of CF-directed therapy.

The Nerve Growth Factor (NGF) and Neuronal Differentiation

Lemur Tyrosine Kinase 2 interaction with NGF inhibits neuronal differentiation. The specific distribution and phosphorylation of LMTK2 in the brain tissue first suggested that it may regulate early postnatal brain function (Kawa et al., 2004). Endogenous LMTK2 is phosphorylated upon stimulation with NGF in PC12 cells, possibly by a PKC-dependent mechanism, although a direct interaction between LMTK2 and PKC has not been demonstrated (Kawa et al., 2004). The NGF-stimulated phosphorylation down-regulates LMTK2 activity, resulting in activation of extracellular-signal-regulated kinases (ERK), leading to neurite outgrowth (Kawa et al., 2004).

The B-Cell Lymphoma (BCL) Proteins and Apoptosis

It has been suggested that LMTK2 inhibits cytotoxicity by interacting with the apoptotic and survival pathways. A high-throughput siRNA-based screen recently identified LMTK2 as a novel anti-apoptotic sensor (Conti et al., 2017). LMTK2 knockdown reduced the expression of anti-apoptotic B-cell lymphoma-2 (BCL2) and B-cell lymphoma-extra-large (BCL-xL) proteins and increased the expression of pro-apoptotic BCL2-interacting mediator of cell death (BIM) protein. While LMTK2-dependent regulation of BIM was more evident in non-cancer cell lines, other members of the BCL2 family, such as BCL2 and BCL-xL, were regulated by LMTK2 mainly in fully transformed cancer cells. Furthermore, LMTK2 knockdown enhanced the cytotoxic effect of apoptosis inducing ligands, such as the tumor necrosis

factor-related apoptosis-inducing ligand (TRAIL) and etoposide, among others (Conti et al., 2017).

The Androgen Receptor (AR) and Prostate Development

Lemur Tyrosine Kinase 2 interacts with AR and inhibits its transcriptional activity (Shah and Bradbury, 2015a,b). AR promotes cell growth and plays an important role in prostate development (Chang et al., 1988; Mangelsdorf et al., 1995). The LMTK2−AR interaction was demonstrated by co-immunoprecipitation and Proximity Ligation Assay (PLA) in LNCaP cells, an androgen-sensitive human prostate adenocarcinoma cell line. Localization of the complex was dependent on the androgen ligands: under androgen deprivation, it was predominantly extra-nuclear and in presence of androgens the complex translocated to the nucleus. The LMTK2−AR complex was also detected in human prostate tissue (Shah and Bradbury, 2015b). Inhibition of the AR transcriptional activity by LMTK2 was demonstrated by luciferase reporter assay in HEK293 cells (Shah and Bradbury, 2015b).

ROLE OF LMTK2 IN HUMAN DISEASE: POTENTIAL BIOMARKER AND THERAPEUTIC TARGET

The role of LMTK2 in cell signaling, endocytic trafficking, apoptosis, and Cl^- transport explains why its dysregulation is associated with neurodegeneration, cancer, and infertility. Below, we discuss the role of LMTK2 in human disease and its potential to become a biomarker and a therapeutic target.

Prostate Cancer

The role of LMTK2 in prostate cancer is well established (Eeles et al., 2008; Guy et al., 2009; Waters et al., 2009; Harries et al., 2010; Wang et al., 2013; Shui et al., 2014; Shah and Bradbury, 2015a,b; Hao et al., 2016; Jiang et al., 2016). The *lmtk2* gene was identified as one of seven *loci* associated with prostate cancer in a genome-wide association study (GWAS) using blood DNA from 1854 individuals with prostate cancer detected before 60 years of age or with a family history of the disease (Eeles et al., 2008; Guy et al., 2009). The LMTK2 expression had no significant ethnic heterogeneity, as demonstrated in multi-ethnic prostate cancer populations from California with African and Latino background, or from Hawaii from native, Japanese or European descent (Waters et al., 2009). Furthermore, the LMTK2 single nucleotide polymorphism (SNP), rs6465657 was inversely associated with the risk of non-fatal prostate cancer and the cancer-specific mortality (Shui et al., 2014).

The expression of *lmtk2* gene was studied in benign prostate hyperplasia (BPH) and prostate cancer cells by RT-PCR. The *lmtk2* gene expression was decreased by 68% in tissue with prostate adenocarcinoma, when compared to BPH (Harries et al., 2010). In addition, LMTK2 protein abundance was undetectable or low in prostate cancer tissues, compared to very high expression in non-malignant tissue (Shah and Bradbury, 2015b). Additionally, a direct association between LMTK2 and tumor forming capacity was assessed in LNCaP cells. LMTK2 knockdown demonstrated significantly higher colony-forming capacity and increased cell viability, suggesting that LMTK2 plays a role in the regulation of cell proliferation (Shah and Bradbury, 2015b). This idea is corroborated by the fact that LMTK2 regulates the expression of several proteins involved in apoptosis, as described above.

In view of the above data, LMTK2 has a potential to serve as a novel biomarker in prostate cancer that, in contrast to the prostate specific antigen (PSA), can distinguish between BPH and prostate cancer. Furthermore, targeted activation of LMTK2 in the prostate tissues by small molecules could decrease the AR-proliferative activity, preventing the prostate cancer cell growth.

Other Cancers

The role of LMTK2 in apoptosis suggests that its dysregulation may be involved in other forms of cancer as well. In fact, LMTK2 mRNA is expressed in all cancer tissues according to The Cancer Genome Atlas (TCGA) (The Human Protein Atlas, 2018b). The LMTK2 protein levels differ in a variety of cancer cells. Levels are high in colorectal and ovarian cancer while they are low in lymphoma, lung, testis and renal cancer (The Human Protein Atlas, 2018b). Thus, LMTK2 has a potential to serve as a prognostic biomarker (The Human Protein Atlas, 2018b). For example, in renal cancer, LMTK2 indicates favorable prognosis because higher expression correlated with higher survival probability. By contrast, higher LMTK2 levels were associated with reduced survival in ovarian cancer (The Human Protein Atlas, 2018b).

Whole-exome sequencing (WES) followed by the Protein Variation Effect Analyzer (PROVEAN) modeling in pulmonary sarcomatoid carcinoma (PSC) cells identified deleterious effects of mutations in the *lmtk2* gene in 2 of 10 patients (Liu et al., 2016). PSC is an aggressive and poorly differentiated, non-small-cell lung carcinoma responsible for 0.1–0.4% of all lung cancer cases (Brambilla et al., 2001; Liu et al., 2016; Karim et al., 2018). A mutation in the *lmtk2* gene was also found in patients with lung adenocarcinoma (Seo et al., 2012). Future studies are needed to experimentally validate the diagnostic and prognostic potential of LMTK2 in different forms of cancer.

Male Infertility and Contraception

Animal studies suggest a strong association between LMTK2 and male fertility (Kawa et al., 2006; Sakugawa et al., 2009). Studies conducted with LMTK2 knockout (KO) mice demonstrated that LMTK2 plays an essential role in spermatogenesis. Although the KO animals had normal phenotype at birth and experienced normal growth, they had azoospermia (Kawa et al., 2006). Normal number and morphology of testicular somatic cells and intact hormonal levels in the LMTK2 KO mice suggest that impaired spermatogenesis was directly caused by a defect in germ cells. Indeed, it was observed that the first step of spermatogenesis – the differentiation of spermatogonia to round spermatids – occurred normally in these animals; however, further differentiation of spermatids was largely suppressed (Kawa et al., 2006). Thus, it has been hypothesized that LMTK2 is necessary for the morphological progression of postmeiotic

germ cells, a process that includes mitochondrial compaction and expulsion of the cytoplasm. This hypothesis was experimentally validated by examination of the wild-type mice, in which LMTK2 expression was higher between 2 and 3 weeks after birth, the time of the late phase spermatogenesis, possibly after the generation of round spermatids (Kawa et al., 2006). Additionally, the *lmtk2* gene was predicted as a target for heat-sensitive micro (mi)RNAs in pachytene spermatocytes (Yadav et al., 2018). However, a study that examined the association of LMTK2 and infertility in humans demonstrated that the nine SNPs in exon 11 of the *lmtk2* gene identified in 18 Japanese men with azoospermia were not causative (Sakugawa et al., 2009).

Lemur Tyrosine Kinase 2 is a known inhibitor of PP1 that regulates sperm motility (Chakrabarti et al., 2007; Fardilha et al., 2011). Hence, LMTK2 emerges as a potential target for both reproductive contraceptives, such as an inhibitor of sperm motility, and infertility treatment in men, by regulating the activity of PP1. However, more studies are needed to better understand how LMTK2 regulates spermatogenesis.

Cystic Fibrosis

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in Caucasians (Grody and Desnick, 2001). The most common disease-associated mutation in the *cftr* gene F508del, is caused by the in-frame deletion of three nucleotides encoding phenylalanine at position 508. This mutation, present in 90% of CF patients in at least one allele, leads to an intracellular processing defect and retention of CFTR in the ER. The corrector VX-809 (part of the FDA-approved drug Orkambi) partially rescues the processing defect of F508del-CFTR and allows maturation while passing through the Golgi complex and trafficking to the cell membrane in cultured cells (Van Goor et al., 2011, 2014; Clancy et al., 2012; Ren et al., 2013; Wainwright et al., 2015). F508del-CFTR forms a Cl^- channel regulated by cAMP but has dramatically different gating properties compared to the wild-type CFTR (Dalemans et al., 1991; Swiatecka-Urban et al., 2005). The FDA-approved drug Kalydeco^{TM} (Ivacaftor; VX-770) potentiated membrane-resident F508del-CFTR channel "open" probability (Accurso et al., 2014). Clinical trials show that the combined use of corrector VX-809 and potentiator VX-770 (Orkambi) improved the percent of predicted forced expiratory volume in 1 second (FEV1), decreased the rate of pulmonary exacerbations, and reduced the rate of events leading to hospitalization or use of intravenous antibiotics in patients with two F508del copies (Wainwright et al., 2015). Clinical trial evaluating a different corrector VX-661 (Tezacaftor) with VX-770 in patients with two F508del copies showed results similar to Orkambi [Donaldson et al., 2013; Two Phase 3 Studies of the Tezacaftor/Ivacaftor Combination Treatment Met Primary Endpoints with Statistically Significant Improvements in Lung Function (FEV1) in People with Cystic Fibrosis, 2017] – with this combination also securing FDA approval under the brand name Symdeko. Although this is seen as a very important step forward, improved treatment efficacy is still needed for these patients.

While at the cell membrane, the corrector-rescued F508del-CFTR has reduced membrane residence and current therapies do not improve this defect (Swiatecka-Urban et al., 2005). LMTK2 depletion facilitates the VX-809 mediated trafficking of F508del-CFTR to the apical plasma membrane and rescue of the CFTR-mediated Cl^- secretion across human bronchial epithelial cells (Luz et al., 2014). Since LMTK2 facilitates CFTR endocytosis, these effects could result from decreased CFTR endocytosis after LMTK2 depletion. Thus, targeting the LMTK2-mediated inhibitory phosphorylation of CFTR-S^{737} could serve as a novel approach to study the plasma membrane stability defect of F508del-CFTR. It could also help to design new pharmacological approaches to stabilize rescued F508del-CFTR and improve the efficacy of the corrector/potentiator strategy (Luz et al., 2014).

Neurodegeneration

Neurodegeneration represents an irreversible structural and functional damage of neurons that can lead to cell death. It is the hallmark of several central nervous system disorders, including Alzheimer's and Parkinson's diseases (Przedborski et al., 2003). Bencze et al. (2018) recently reviewed the role of LMTK2 in neurodegeneration. The authors proposed three LMTK2-dependent mechanisms of neurodegeneration: (i) disruption of axonal transport, mediated by aberrant LMTK2—kinesin-1 interaction; (ii) hyperphosphorylation of Tau protein by LMTK2, mediated by cdk5 and GSK3-β; and (iii) regulation of apoptosis by LMTK2 (Bencze et al., 2018). The authors proposed that modulation of LMTK2 expression could be considered as a promising novel therapeutic target for neurodegenerative states, such as Alzheimer disease (Bencze et al., 2018).

CONCLUSION

Although LMTK2 is still incompletely characterized, its role in key biological functions has been well established by strong scientific evidence. LMTK2 works as a multifunctional adaptor, being involved on very distinct signaling pathways and cell mechanisms, such as endocytosis, apoptosis, channel trafficking and cell differentiation. Improved characterization of LMTK2 holds the potential to enhance our knowledge about the molecular basis of human disease and discover novel diagnostic and prognostic biomarkers. Targeting the LMTK2 interactome could also advance treatment for cancer, neurodegeneration, infertility, and cystic fibrosis and create novel strategies for contraception.

AUTHOR CONTRIBUTIONS

DC was involved in the planning and writing of the review manuscript. CF and AS-U were involved in the planning, writing and review process.

FUNDING

This work was supported by the Portuguese FCT fellowship grant from BioSys (PD/BD/114384/2016) to DC, Portuguese FCT grant UID/MULTI/04046/2013 to CF, the NIH grants R01DK104847 and R56HL127202, and the CFF grant SWIATE18G0 to AS-U.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 13 December 2018
doi: 10.3389/fphar.2018.01464

The Autophagy Inhibitor Spautin-1 Antagonizes Rescue of Mutant CFTR Through an Autophagy-Independent and USP13-Mediated Mechanism

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: 09 October 2018
Accepted: 29 November 2018
Published: 13 December 2018

Citation:
Pesce E, Sondo E, Ferrera L, Tomati V, Caci E, Scudieri P, Musante I, Renda M, Baatallah N, Servel N, Hinzpeter A, di Bernardo D, Pedemonte N and Galietta LJV (2018) The Autophagy Inhibitor Spautin-1 Antagonizes Rescue of Mutant CFTR Through an Autophagy-Independent and USP13-Mediated Mechanism. Front. Pharmacol. 9:1464. doi: 10.3389/fphar.2018.01464

The mutation F508del, responsible for a majority of cystic fibrosis cases, provokes the instability and misfolding of the CFTR chloride channel. Pharmacological recovery of F508del-CFTR may be obtained with small molecules called correctors. However, treatment with a single corrector *in vivo* and *in vitro* only leads to a partial rescue, a consequence of cell quality control systems that still detect F508del-CFTR as a defective protein causing its degradation. We tested the effect of spautin-1 on F508del-CFTR since it is an inhibitor of USP10 deubiquitinase and of autophagy, a target and a biological process that have been associated with cystic fibrosis and mutant CFTR. We found that short-term treatment of cells with spautin-1 downregulates the function and expression of F508del-CFTR despite the presence of corrector VX-809, a finding obtained in multiple cell models and assays. In contrast, spautin-1 was ineffective on wild type CFTR. Silencing and upregulation of USP13 (another target of spautin-1) but not of USP10, had opposite effects on F508del-CFTR expression/function. In contrast, modulation of autophagy with known activators or inhibitors did not affect F508del-CFTR. Our results identify spautin-1 as a novel chemical probe to investigate the molecular mechanisms that prevent full rescue of mutant CFTR.

Keywords: CFTR, chloride channel, cystic fibrosis, autophagy, ubiquitination, spautin-1

INTRODUCTION

CFTR, a plasma membrane chloride channel with a main role in epithelial cells, is mutated in cystic fibrosis (CF), one of the most frequent genetic diseases (Stoltz et al., 2015; Castellani and Assael, 2017). Loss of CFTR-dependent chloride transport affects multiple organs, including lungs, pancreas, liver, and sweat glands. Among the many types of mutations that affect the *CFTR* gene, the loss of phenylalanine 508 (F508del) is the most frequent. F508del impairs the folding and stability of CFTR protein (Lukacs and Verkman, 2012). Consequently, F508del-CFTR trafficking to the cell surface is severely altered. The mutant protein is retained in the endoplasmic reticulum and degraded by the ubiquitin-proteasome system (Lukacs and Verkman, 2012). A small fraction of the protein may reach the plasma membrane where, however, it is rapidly removed and eliminated by peripheral quality control mechanisms (Sharma et al., 2001; Okiyoneda et al., 2010; Fu et al., 2015).

Recently, pharmacological correctors of F508del defect have been developed (Galietta, 2013; Quon and Rowe, 2016). Such molecules favor F508del-CFTR trafficking with different mechanisms. One of the most advanced molecules is the corrector VX-809 (Van Goor et al., 2011). This small molecule partially rescues F508del-CFTR by possibly binding to the mutant protein itself (Ren et al., 2013; Hudson et al., 2017). VX-809 is presently used, in combination with the potentiator VX-770, to treat CF patients homozygous for the F508del mutation (Wainwright et al., 2015). However, the extent of clinical benefit obtained with the VX-809/VX-770 combination is relatively modest. The low efficacy of the combination is believed to be due to the partial activity of VX-809 as a corrector (Okiyoneda et al., 2013). A negative interaction between VX-809 and VX-770 can be also involved (Cholon et al., 2014; Veit et al., 2014). It has been shown that more marked levels of F508del-CFTR rescue can be obtained with combinations of correctors having complementary mechanisms of action (Farinha et al., 2013; Okiyoneda et al., 2013). Such other correctors may work by binding to a second site in the CFTR protein or by modulation of the cell machinery responsible for CFTR processing and degradation. Several proteins, including RNF5/RMA1, gp78, CHIP, CAL, Dab2, and cCBL, have been identified to affect CFTR processing but the list is probably far from being complete (Cheng et al., 2002; Younger et al., 2006; Morito et al., 2008; Okiyoneda et al., 2010; Ye et al., 2010; Fu et al., 2015; Tomati et al., 2015, 2018; Sondo et al., 2017). It has been shown that the ubiquitin specific peptidase 10 (USP10) is an important factor that controls CFTR degradation (Bomberger et al., 2009). Interestingly, a small molecule inhibitor of USP10, spautin-1, has been recently described. This compound also inhibits another ubiquitin peptidase, USP13 (Liu et al., 2011). By inhibiting USP10 and USP13, spautin-1 is also an inhibitor of autophagy, a process that has a possible important relationship with CFTR (Luciani et al., 2010). Therefore, we were interested in evaluating spautin-1 as a possible pharmacological tool to perturb CFTR processing. We found that spautin-1 antagonizes the rescue by VX-809 causing a rapid rundown of F508del-CFTR at the functional and molecular level. This effect may involve USP13 inhibition but is independent from autophagy block. USP13 appears as an important protein regulating the fate of mutant CFTR while spautin-1 may become an interesting probe for mechanistic studies and the search of new therapeutic agents.

RESULTS

CFBE41o- cells expressing F508del-CFTR and the halide-sensitive yellow fluorescent protein (HS-YFP) were treated for 24 h with 1 μM VX-809 or vehicle (DMSO) alone. F508del-CFTR function was then determined with the HS-YFP assay in microplate reader. The treatment with VX-809 caused a nearly three-fold increase in F508del-CFTR function, as indicated by the faster fluorescence quenching caused by iodide influx (**Figure 1A**). We tested spautin-1 at 10 μM, the concentration previously found to affect USP10 and USP13 activity (Liu et al., 2011). When spautin-1 was added in the last 3 h of incubation, the rescue induced by VX-809 was reduced by nearly 40% (**Figure 1A**). The decrease in CFTR function by spautin-1 was paralleled by an altered pattern of F508del-CFTR maturation as indicated by immunoblot experiments. In lysates from untreated cells, the mutant CFTR migrates as a core-glycosylated protein (band B) with an apparent size of 150 kDa (**Figure 1B** and **Supplementary Datasheet 2**). Treatment with the corrector VX-809 is known to improve F508del-CFTR maturation with appearance of a fully glycosylated version of the protein (band C) with mobility corresponding to 170 kDa. VX-809 also increases band B abundance. Importantly, treatment with spautin-1 for 3 h antagonized the rescue by VX-809 as evident from the downregulation of band C and of total CFTR (band C plus band B; **Figure 1B**). Interestingly, the ratio band C/band B was not altered since both bands were downregulated by spautin-1 to a similar extent.

Using the HS-YFP functional assay, we determined the time-dependence of spautin-1 effect. Treatments with spautin-1 (10 μM) as short as 5–15 min were enough to cause a significant inhibition (20–30%) of F508del-CFTR function (**Figure 1C**). Larger effects were observed with longer incubations.

To estimate spautin-1 potency, we tested multiple concentrations in the range 0.08–20 μM. We found that micromolar concentrations were needed to inhibit F508del-CFTR function, with 20 μM causing a nearly total reversal of VX-809 rescue (**Figure 2A**). A similar dose-dependence was found by examining F508del-CFTR protein maturation (**Figure 2B**). Importantly, at the same concentrations, spautin-1 caused no inhibition of wild type CFTR activity, irrespective of presence or absence of VX-809 (**Supplementary Figure 1A**). This result was consistent with unaltered pattern of CFTR protein maturation even with 20 μM of spautin-1 (**Supplementary Figure 1B**).

F508del-CFTR can be also rescued by cell incubation at low temperature. Cells were incubated for 21 h at 32°C, a condition that increases F508del-CFTR function as a result of enhanced expression on cell surface (Sondo et al., 2011). Then, cells were kept for three additional hours at 32°C or at 37°C, with and without spautin-1 (20 μM). As shown in **Figure 2C**, spautin-1 inhibited F508del-CFTR function also in cells rescued by hypothermia.

We also investigated F508del-CFTR protein expression by cell surface biotinylation. Intact cells were labeled with biotin and then lysed. Biotinylated proteins were isolated by pull-down and then revealed by immunoblot. Spautin-1 decreased F508del-CFTR but not Na^+/K^+-ATPase expression on cell surface (**Supplementary Figure 2**). It may be noted that both bands C and B are revealed by this method. The presence of core-glycosylated F508del-CFTR on cell surface, due to an unconventional route of trafficking, has already been shown in CFBE41o- cells (Gee et al., 2011; Tomati et al., 2015). In this respect, absence of calnexin and 14-3-3 proteins in the biotinylated fraction indicates that intracellular proteins were not labeled by our procedure.

We asked whether spautin-1 effects could be due to a block in protein synthesis. CFBE41o- cells were treated with VX-809 for 24 h and, in the last 3 or 6 h, with cycloheximide (CHX)

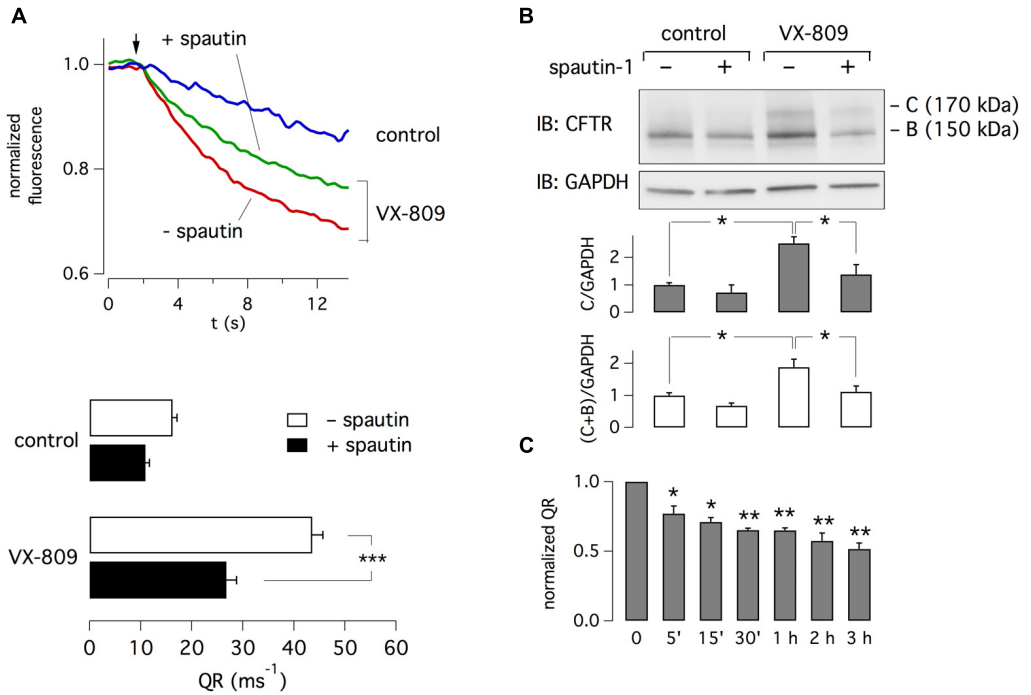

FIGURE 1 | Inhibition of F508del-CFTR function and expression by spautin-1. **(A)** Representative traces and bar graphs showing F508del-CFTR activity measured with the HS-YFP microplate reader assay. The arrow indicates the time of addition of extracellular I^-. CFBE41o- cells were treated for 24 h with vehicle (DMSO) or VX-809 (1 μM). In the last 3 h, cells also received spautin-1 (10 μM) or vehicle. Data in the graph are reported as quenching rate (QR) of HS-YFP fluorescence (n = 14–23 independent experiments; ***$p < 0.001$). **(B)** Immunoblot analysis of F508del-CFTR expression. The image shows that both band C and band B, particularly in VX-809 treated cells, are decreased by spautin-1 (10 μM, 3 h). The bar graphs show the densitometric analysis of band C or band C plus band B, normalized for GAPDH (n = 3 independent experiments; *$p < 0.05$). **(C)** Time-course of spautin-1 effect. Bars report the normalized QR of HS-YFP fluorescence in cells treated with VX-809 for 24 h and then with spautin-1 (10 μM) for the indicated time (n = 3 independent experiments; *$p < 0.05$; **$p < 0.01$).

plus/minus spautin-1. In the presence of CHX and VX-809, we noted a strong reduction in both band C and band B levels (**Figure 3A**). However, the immature form was more markedly affected. It is interesting to note that the smear of F508del-CFTR signal, evident in cells treated with VX-809 alone, disappeared in CHX-treated cells. We interpret such results as an effect of rapid F508del-CFTR degradation (despite the presence of the corrector) that is not compensated by novel protein synthesis. Importantly, in the presence of CHX, spautin-1 was able to further decrease F508del-CFTR expression (**Figure 3A**).

We also investigated the effect of spautin-1 on F508del-CFTR ubiquitination (**Figure 3B**). After immunoprecipitation of F508del-CFTR, ubiquitin levels were assessed by immunoblot. Treatment with the proteasome inhibitor MG-132 increased the levels of ubiquitinated F508del-CFTR, also in cells corrected with VX-809. The lysosome inhibitor bafilomycin A1 was instead ineffective. Surprisingly, spautin-1 decreased the ubiquitin signal despite the presence of MG-132 or both inhibitors together (**Figure 3B**). This finding suggests that the F508del-CFTR disappearance elicited by spautin-1 may still occur independently of proteasome and lysosome.

The effect of spautin-1 was also checked on other cell types and with other assays. We studied FRT cells with expression of F508del-CFTR (**Figure 4A**). Such cells form tight epithelia when seeded on porous membranes (Snapwell supports). Therefore, the function of F508del-CFTR in the plasma membrane can be simply evaluated by measuring transepithelial electrical resistance (TEER), which is then converted, for convenience, to transepithelial electrical conductance, TEEC (Xue et al., 2014). Indeed, a rescue of F508del-CFTR results in higher number of chloride-conducting channels and therefore increased TEEC. It should be noted that this method measures both membranes, apical and basolateral, as two electrical resistances in series. Therefore, the detection of a drop or increase in resistance, reflecting activation or inhibition of CFTR respectively, does not distinguish whether this occurs on one or the other membrane. In this respect, it is known that CFTR traffics to both membranes in FRT cell (Sheppard et al., 1994). Therefore, TEEC changes are a reasonably good indicator of CFTR function in the plasma membrane (Xue et al., 2014).

For TEEC measurements, FRT cells were incubated for 24 h with and without VX-809 to rescue F508del-CFTR. In the last 3 h, cells were treated with or without spautin-1. At the time of the experiment, TEEC was measured under resting conditions, after maximal stimulation of F508del-CFTR with forskolin (20 μM) plus genistein (50 μM), and finally after block with PPQ-102 (30 μM), a CFTR inhibitor. **Supplementary Figure 3** precisely describes the procedure. F508del-CFTR function was reported as ΔTEEC, i.e., the difference between TEEC measured with forskolin plus genistein and TEEC measured after PPQ-102

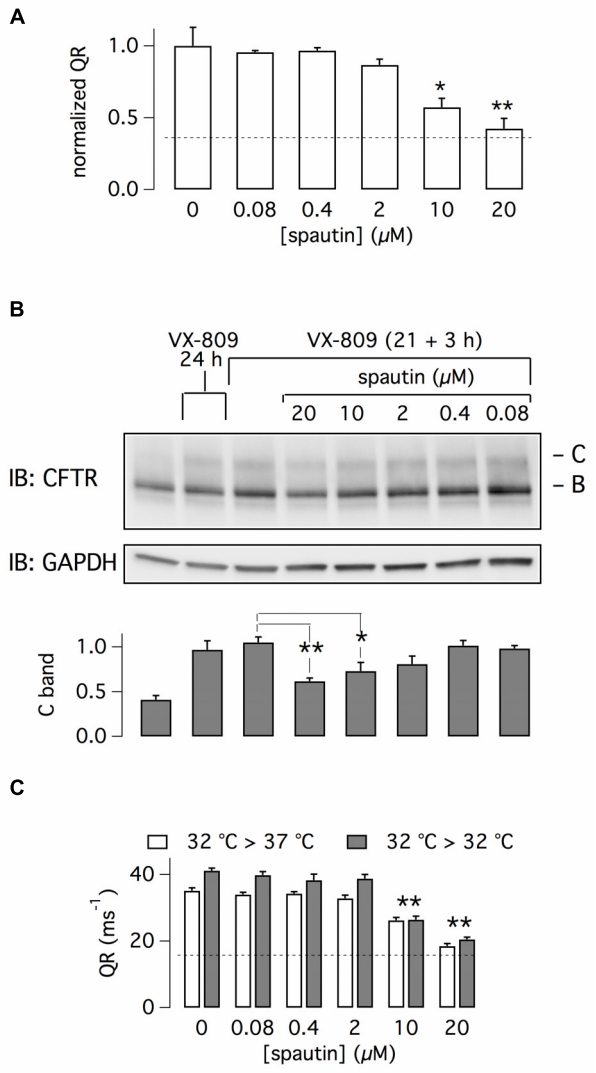

FIGURE 2 | Characterization of spautin-1 effect. **(A)** Dose-response of spautin-1. The graph reports the normalized QR of HS-YFP in CFBE41o- cells treated with VX-809 (1 μM) and then, in the last 3 h, with vehicle or spautin-1 at the indicated concentrations (n = 5 independent experiments; *$p < 0.05$; **$p < 0.01$). The dashed line indicates the level of F508del-CFTR activity in uncorrected cells (no VX-809). **(B)** Immunoblot analysis of F508del-CFTR expression (representative image and densitometric analysis of band C; n = 3 independent experiments). CFBE41o- cells were treated with the indicated conditions (*$p < 0.05$; **$p < 0.01$). **(C)** F508del-CFTR activity (QR) in CFBE41o- cells incubated at 32°C. In the last 3 h, cells were kept at 32°C or shifted to 37°C and treated with vehicle or the indicated concentrations of spautin-1 (n = 3 independent experiments; **$p < 0.01$ vs. vehicle).

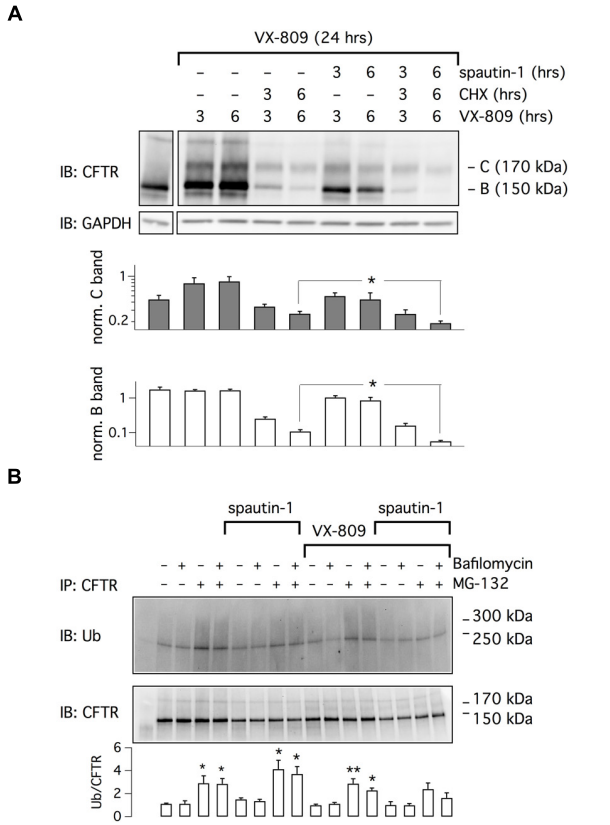

FIGURE 3 | Analysis of spautin-1 mechanism of action. **(A)** Spautin-1 in the presence of protein synthesis block. The image shows a representative western blot experiment done on lysates of CFBE41o- cells treated with VX-809 (1 μM) for 24 h and, in the last 3 or 6 h, with CHX (150 μg/ml), with spautin-1 (20 μM), or with both compounds together. To apply the different conditions, the entire medium was replaced at 3 or 6 h with fresh medium containing the required compounds, including VX-809. Therefore, the times indicate for VX-809 (3 and 6 h) simply indicate the time of medium replacement (the total time of VX-809 treatment was kept at 24 h). The pattern of lysates from cells treated with vehicle alone (no corrector) for 24 h is also shown (first separate lane on the left). Bar graphs show densitometry of bands C and B, normalized for GAPDH intensity, from three independent experiments. Spautin-1 caused a significant downregulation of F508del-CFTR signal in CHX-treated cells (*$p < 0.05$). **(B)** Evaluation of spautin-1 on ubiquitinated F508del-CFTR. The image shows immunodetection of CFTR and ubiquitin in cell lysates after immunoprecipitation using an anti-CFTR antibody. Where indicated, cells where treated with VX-809 (1 μM) for 24 h and, in the last 3 h, with MG-132 (10 μM), bafilomycin A1 (100 nM), and/or spautin-1 (20 μM). The image is representative of three similar experiments. The bar graph shows the ratio of ubiquitin to CFTR signals (n = 3 separate experiments; *$p < 0.05$; **$p < 0.01$ vs. corresponding condition without MG-132 and bafilomycin).

addition. Treatment with VX-809 increased ΔTEEC by more than three-fold, reflecting F508del-CFTR rescue (**Figure 4A**). Spautin-1 decreased the effect of VX-809 by nearly 75 and 95% at 10 and 20 μM, respectively (**Figure 4A**).

Effect on spautin-1 in FRT cells was also assessed using the patch-clamp technique in the whole-cell configuration (**Figure 4B**, top part). FRT cells expressing F508del-CFTR were treated with VX-809 (1 μM) for 24 h. During whole-cell patch-clamp recordings, F508del-CFTR was activated with forskolin (20 μM) plus genistein (50 μM). We monitored membrane currents for 15 min to check that they were stable. Then, we added spautin-1 (20 μM) by extracellular perfusion. We observed a progressive decline in F508del-CFTR function that reached a stable level (nearly 30% of original value) in 30–40 min (**Figure 4B**). This decline was not observed in experiments in which membrane currents were monitored for a comparable time

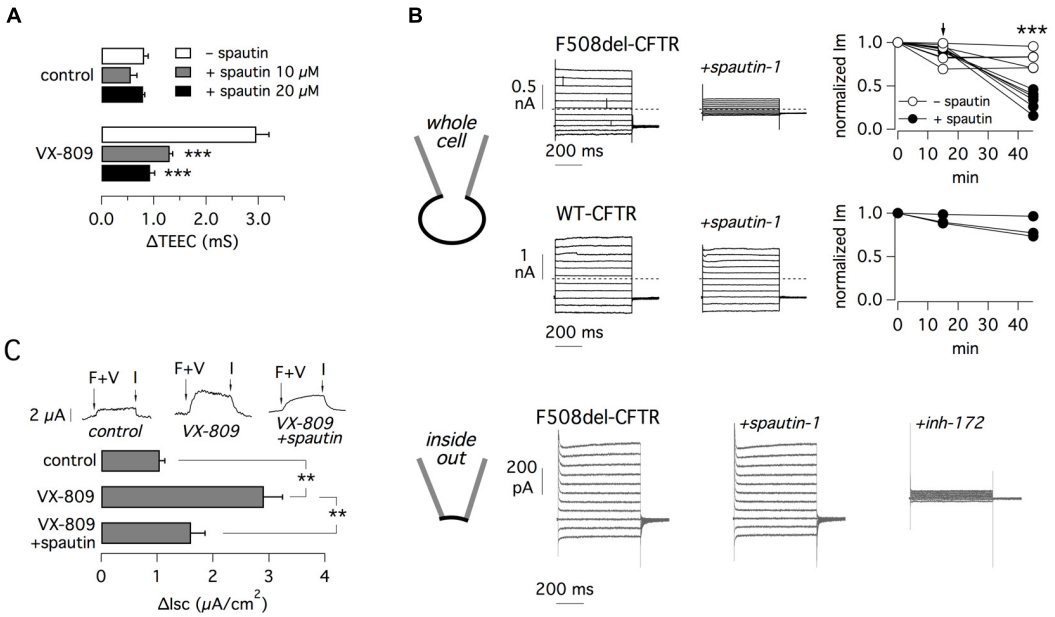

FIGURE 4 | Electrophysiological evaluation of F508del-CFTR inhibition by spautin-1. **(A)** TEEC measurement in FRT cells expressing F508del-CFTR. Cells were treated with or without VX-809 (1 μM) and, in the last 3 h, with spautin-1 (10 or 20 μM) or vehicle. The graph reports the ΔTEEC value, i.e., the difference between TEEC after maximal stimulation with forskolin plus genistein and TEEC after block with PPQ-102 (see text and **Supplementary Figure 3** for further details). Data are from four independent experiments (***$p < 0.001$). **(B)** Results from patch-clamp experiments. The top part shows data obtained in the whole-cell configuration on FRT cells expressing F508del or wild type CFTR (representative traces and graphs reporting the normalized current measured at +100 mV). Cells expressing mutant CFTR were previously treated for 24 h with VX-809 (1 μM). During experiments, cells were acutely stimulated with forskolin (20 μM) plus genistein (50 μM). After full stimulation of mutant or wild type CFTR, the currents were measured for 15 min to check that the activity was stable. Then spautin-1 (20 μM) was added to the extracellular solution and the currents were measured for further 30–40 min. Spautin-1 elicited a significant decrease in membrane currents in F508del-CFTR but not in wild type CFTR cells. The graphs show the currents measured at the three relevant time points. Arrow shows the time of spautin-1 addition. Spautin-1 caused a significant decrease in F508del-CFTR currents (***$p < 0.001$). The bottom part of panel B shows representative currents obtained in the inside-out configuration on FRT cells expressing F508del-CFTR. Currents were significantly inhibited by $CFTR_{inh}$-172 (10 μM) but not by spautin-1 (data are representative of three similar experiments). **(C)** Effect of spautin-1 (20 μM) on human bronchial epithelial cells from a F508del-CFTR patient. Representative traces show short-circuit recordings from epithelia treated with/without VX-809 (1 μM) for 24 h. Where indicated, spautin-1 was added in the last 3 h. During recordings, F508del-CFTR was activated with 20 μM forskolin plus 1 μM VX-770 (F+V) and then blocked with 10 μM $CFTR_{inh}$-172 (I). The graph reports the amplitude of the current drop elicited by the CFTR inhibitor ($n = 4$ separate experiments per condition; **$p < 0.01$).

in the absence of spautin-1 (**Figure 4B**). Addition of $CFTR_{inh}$-172 (10 μM), another specific inhibitor of CFTR channel, strongly blocked the residual current (not shown). We also tested spautin-1 on cells expressing wild type CFTR (**Figure 4B**). In agreement with HS-YFP data, spautin-1 was largely ineffective on the normal protein (**Figure 4B**).

To check the dependence of spautin-1 on an intact intracellular environment, we carried out patch-clamp experiments in the inside-out configuration (**Figure 4B**, bottom part). After excision of the membrane patch, F508del-CFTR channels were activated by phosphorylation. Subsequent addition of spautin-1 (20 μM), kept for the duration of the recording (up to 30 min) did not significantly inhibit the current. Membrane currents were 370 ± 79 and 403 ± 113 pA before and after spautin-1 ($n = 3$), respectively. After spautin-1, block by $CFTR_{inh}$-172 demonstrated that the recorded currents were in large part due to CFTR activity (**Figure 4B**, bottom).

Spautin-1 was also tested in primary bronchial epithelial cells from a F508del/F508del patient (**Figure 4C**). F508del-CFTR function was determined with short-circuit current recordings. The ENaC sodium channel was first blocked with amiloride (10 μM) and then F508del-CFTR was stimulated with CPT-cAMP (100 μM) and VX-770 (1 μM). Stimulation resulted in an increase in transepithelial current due to F508del-CFTR function which was subsequently blocked with $CFTR_{inh}$-172 (10 μM). The amplitude of the current blocked by $CFTR_{inh}$-172 indicates the amount of functional F508del-CFTR in the plasma membrane. This value increased from nearly 1 to 2.9 $\mu A/cm^2$ after treatment for 24 h with VX-809 (1 μM). Rescue by VX-809 was decreased by 70% in cells treated for 3 h with 10 μM spautin-1 (**Figure 4C**).

To elucidate the mechanism of action of spautin-1, we separately silenced USP10 and USP13, the possible targets of this small molecule. We transfected CFBE41o- cells with three different DsiRNAs against USP13 or control DsiRNA. Cells were then treated with VX-809. Two of the anti-USP13 molecules caused a significant decrease in anion transport (**Figure 5A**). In particular, USP13A DsiRNA decreased the corrector-dependent fraction of F508del-CFTR function by ∼50%. Importantly, the functional effect on mutant CFTR correlated with the extent

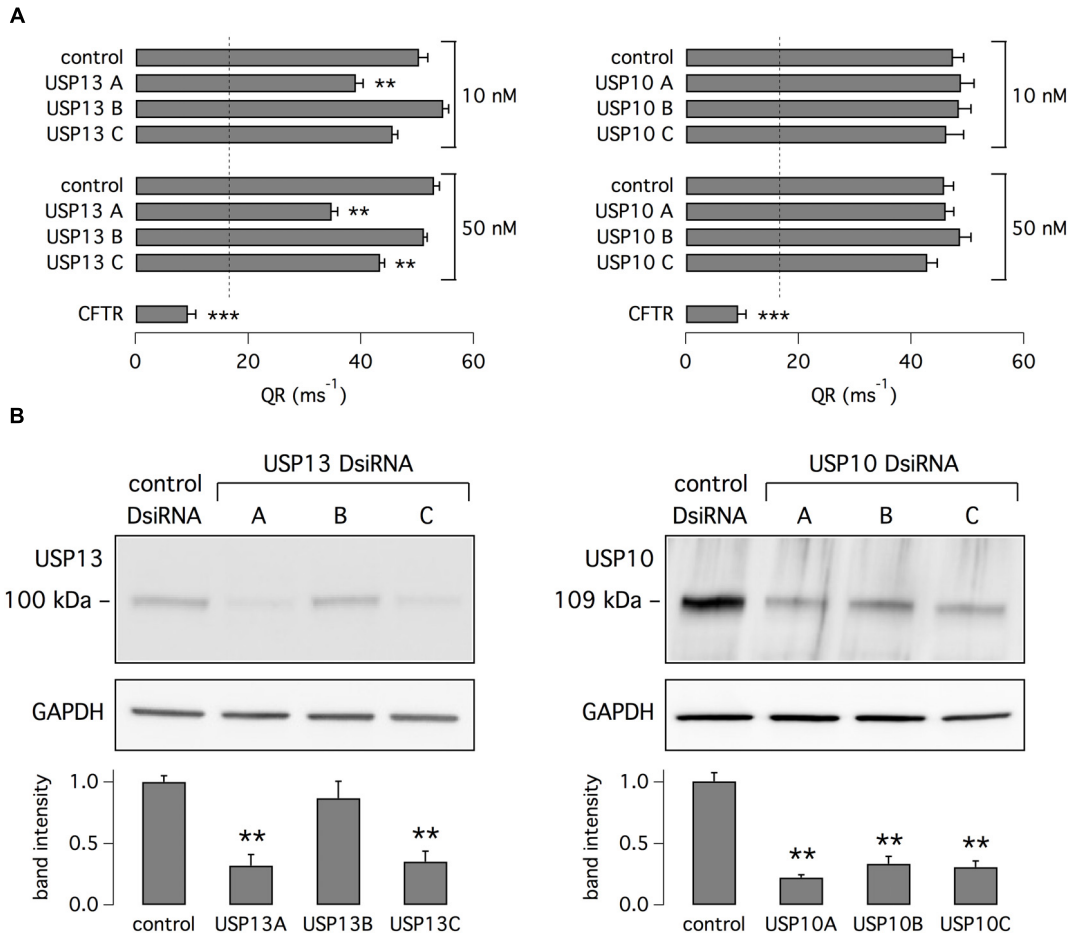

FIGURE 5 | Analysis of USP13 and USP10 relationship with F508del-CFTR expression and function. **(A)** Evaluation of F508del-CFTR activity in CFBE41o- cells treated with VX-809 (1 μM). Data were obtained with the HS-YFP assay after transfection of cells with DsiRNAs (10 or 50 nM) against USP13, USP10, or CFTR. Non-silencing DsiRNA was also used as control. Dashed line reports the value of activity in cells without VX-809 treatment. Silencing of USP13 but not of USP10 significantly decreased F508del-CFTR function. Silencing of CFTR also caused a marked decrease in activity (n = 4–7 independent experiments; $^{**}p < 0.01$; $^{***}p < 0.001$). **(B)** Immunoblot analysis of USP13 and USP10 expression in cells transfected with indicated DsiRNAs (50 nM). Bar graphs report densitometric analysis of USP13 and USP10 bands normalized for GAPDH (n = 3 separate experiments; $^{**}p < 0.01$ vs. control).

of USP13 protein knockdown as revealed by western blot experiments (**Figure 5B**). We also transfected CFBE41o- cells with DsiRNAs against USP10. Unexpectedly, given that USP10 has been shown to control CFTR processing (Bomberger et al., 2009), USP10 knockdown (confirmed by western blot) did not affect F508del-CFTR function (**Figures 5A,B**). As a control, cells were also treated with siRNAs against CFTR. Anion transport was reduced by more than 80% following CFTR knockdown (**Figure 5A**).

The possible role of USP13 and USP10 was also investigated by transfection in CFBE41o- cells. For this purpose, we used plasmids coding for USP13 and USP10 tagged with the mCherry red fluorescent protein. Therefore, the HS-YFP assay was carried out with a microscope and fluorescence quenching, reflecting F508del-CFTR activity, was analyzed in mCherry-positive cells. Cells were treated with VX-809 or vehicle alone. Importantly, overexpression of USP13 did not rescue F508del-CFTR by itself but significantly amplified the effect of VX-809 (**Figure 6A**). In contrast, overexpression of USP10 was ineffective (**Figure 6A**). Importantly, the effect of USP13 overexpression on F508del-CFTR function was nearly abolished by spautin-1 (**Figure 6B**).

The relationship between F508del-CFTR and USP13 was further investigated by analyzing their subcellular localization with immunofluorescence. Endogenous USP13 appeared to be expressed in nucleus and cytosol (**Figure 7A**), in agreement with previous studies (Zhang et al., 2013; Li et al., 2017). Interestingly, cytosolic USP13 showed significant co-localization with F508del-CFTR. In particular, in cells treated with VX-809, regions of the cell periphery showing F508del-CFTR expression also showed staining for USP13 (**Figure 7A**, arrows). We also noticed peripheral regions with USP13 and no F508del-CFTR (**Figure 7A**, arrowheads) but not the opposite situation, thus suggesting that mutant CFTR at the plasma membrane or close compartments is always accompanied by USP13. The dot plots in **Figure 7B** show that peripheral colocalization of USP13

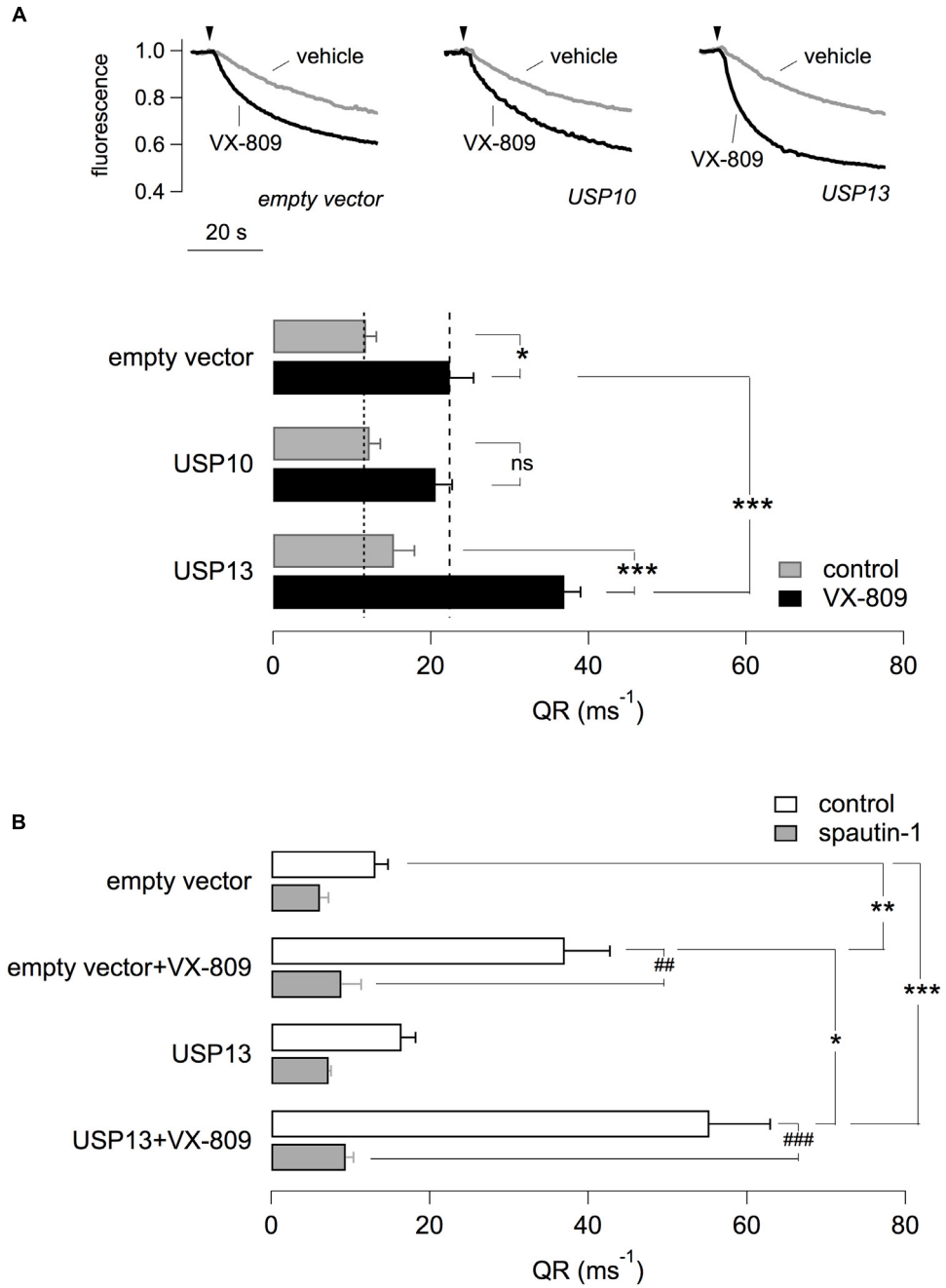

FIGURE 6 | Effect of USP13 or USP10 overexpression. **(A)** Functional analysis in CFBE41o- transiently transfected with plasmids expressing USP13/USP10 or with a control plasmid. Cells also stably expressed F508del-CFTR and the HS-YFP. After transfection, cells were treated with VX-809 (1 μM) or with vehicle. The figure shows representative traces (top) and summary of QR for the different conditions (n = 4 independent experiments; *$p < 0.05$; ***$p < 0.001$). **(B)** Effect of spautin-1 on cells transfected with USP13 and treated with/without VX-809. Data obtained with the HS-YFP assay. Spautin-1 markedly abolishes F508del-CFTR function in USP13-transfected cells (n = 4–7 independent experiments; *$p < 0.05$; ##/**$p < 0.01$; ###/***$p < 0.001$).

and F508del-CFTR is significantly increased following VX-809 treatment (**Figure 7B**).

It has been reported that spautin-1 is an autophagy inhibitor through the ubiquitin-dependent degradation of beclin-1, a subunit of the Vps34 PI3K complex (Liu et al., 2011). On the other hand, F508del-CFTR trafficking was connected to autophagy (Luciani et al., 2010). Therefore, to further investigate the mechanism of action of spautin-1 on F508del-CFTR, we also used SAR-405, another recently identified autophagy inhibitor (Ronan et al., 2014). While spautin-1 causes degradation of Vps34 complex, SAR-405 directly inhibits the catalytic activity of Vps34. Importantly, SAR-405, tested at concentrations between 10 nM

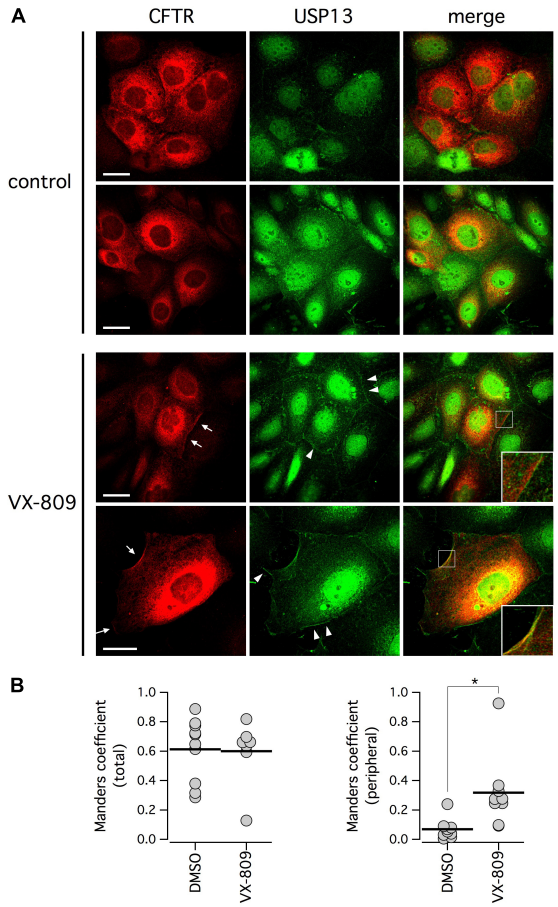

FIGURE 7 | Subcellular localization of F508del-CFTR and endogenous USP13. **(A)** Confocal microscope images taken from CFBE41o- cells stained with antibodies against CFTR and USP13. Cells were treated with VX-809 (1 μM) or vehicle. Arrows indicate examples of cells with peripheral co-localization of F508del-CFTR and USP13. Arrowheads show peripheral regions with USP13 alone. Insets depict magnified cell areas of cell periphery with colocalization of USP13 and F508del-CFTR. **(B)** Colocalization analysis of F508del-CFTR and USP13 in cells treated with vehicle alone (DMSO) or with VX-809. Analysis was done on total cell area (left) or on cell periphery (right; $*p < 0.05$).

and 10 μM did not affect the function of F508del-CFTR rescued by VX-809 (**Figure 8A**). In agreement with functional data, the expression of F508del-CFTR protein was also not affected by SAR-405 in contrast to spautin-1 (**Figure 8B**). To further explore the link between autophagy and F508del-CFTR, we used torin-1 as an autophagy inducer (Thoreen et al., 2009). Torin-1, tested at multiple concentrations, did not alter F508del-CFTR rescue (**Figure 9A**) despite being effective in increasing the expression of LC3-II, a marker of autophagy (**Figure 9B**). In separate experiments, we confirmed that spautin-1 and SAR-405, as autophagy inhibitors, antagonize the effect of torin-1 on LC3-II (**Figure 9C**).

To check the effect of autophagy modulators, we also used an assay based on monodansylcadaverine (MDC). This fluorescent probe is a specific marker for autolysosomes (Biederbick et al., 1995). Indeed, MDC is accumulated inside autophagosomes. After autophagosomes fusion with lysosomes, MDC fluorescence increases due to the acidic environment (Biederbick et al., 1995; Munafó and Colombo, 2001). As shown by representative images (**Figure 10A**) and bar graphs (**Figure 10B**), torin-1 markedly increases the signal of MDC, in particular the total number and brightness of spots. This effect is significantly inhibited by spautin-1, SAR405, and wortmannin, another autophagy inhibitor acting on the Vps34 complex. Similarly to SAR405, and in contrast to spautin-1, wortmannin did not affect F508del-CFTR function (**Supplementary Figure 4A**). Also, the silencing of beclin 1, confirmed at the protein level, did not reduce F508del-CFTR function and expression in VX-809 treated cells (**Supplementary Figures 4B–D**).

In a further attempt to elucidate spautin-1 mechanism of action, we investigated the effect of dynasore, an endocytosis inhibitor. Treatment with this compound did not block the effect of spautin-1 (**Supplementary Figure 5**).

DISCUSSION

Our study reveals novel mechanisms that affect the therapeutic correction of mutant CFTR. There is a strong interest in the development of novel strategies to maximize the rescue of CFTR with the F508del mutation. This is particularly important for CF patients with a single copy of the mutation. Indeed, the drug available for clinical use, VX-809, is only modestly effective on F508del homozygotes (in combination with the potentiator VX-770) and ineffective on individuals with a single F508del allele (Rowe et al., 2017). As shown previously, F508del-CFTR, even in the presence of VX-809 or other correctors, is still intrinsically unstable and therefore recognized by quality control mechanisms and eliminated (He et al., 2013). Enhanced rescue could be obtained by combining VX-809 (or another similarly active compound) with another small molecule acting on a second site in the F508del-CFTR protein (Farinha et al., 2013; Okiyoneda et al., 2013). Alternatively, pharmacological modulation of another protein that is responsible for mutant CFTR elimination could improve the efficacy of correctors.

In the present study, we have investigated spautin-1 as a possible probe to perturb F508del-CFTR rescue. There were two reasons for testing this small molecule. First, spautin-1 is an inhibitor of USP10 (Liu et al., 2011), a deubiquitinating enzyme that is important for CFTR homeostasis (Bomberger et al., 2009). Second, spautin-1, by acting on USP10 and USP13, is also an inhibitor of autophagy (Liu et al., 2011), a cell process that has been proposed to be crucial for F508del-CFTR fate and CF pathogenesis (Luciani et al., 2010).

Short-term pre-incubation of cells with spautin-1 significantly decreased the activity of F508del-CFTR in cells treated with VX-809 or low temperature. An acute effect of spautin-1 was also observed during electrophysiological recordings of rescued F508del-CFTR activity. Importantly, we found no inhibitory effect of spautin-1 in cells expressing wild type CFTR. This finding indicates that spautin-1 is not acting as a direct blocker of CFTR or by interfering with the process of CFTR channel

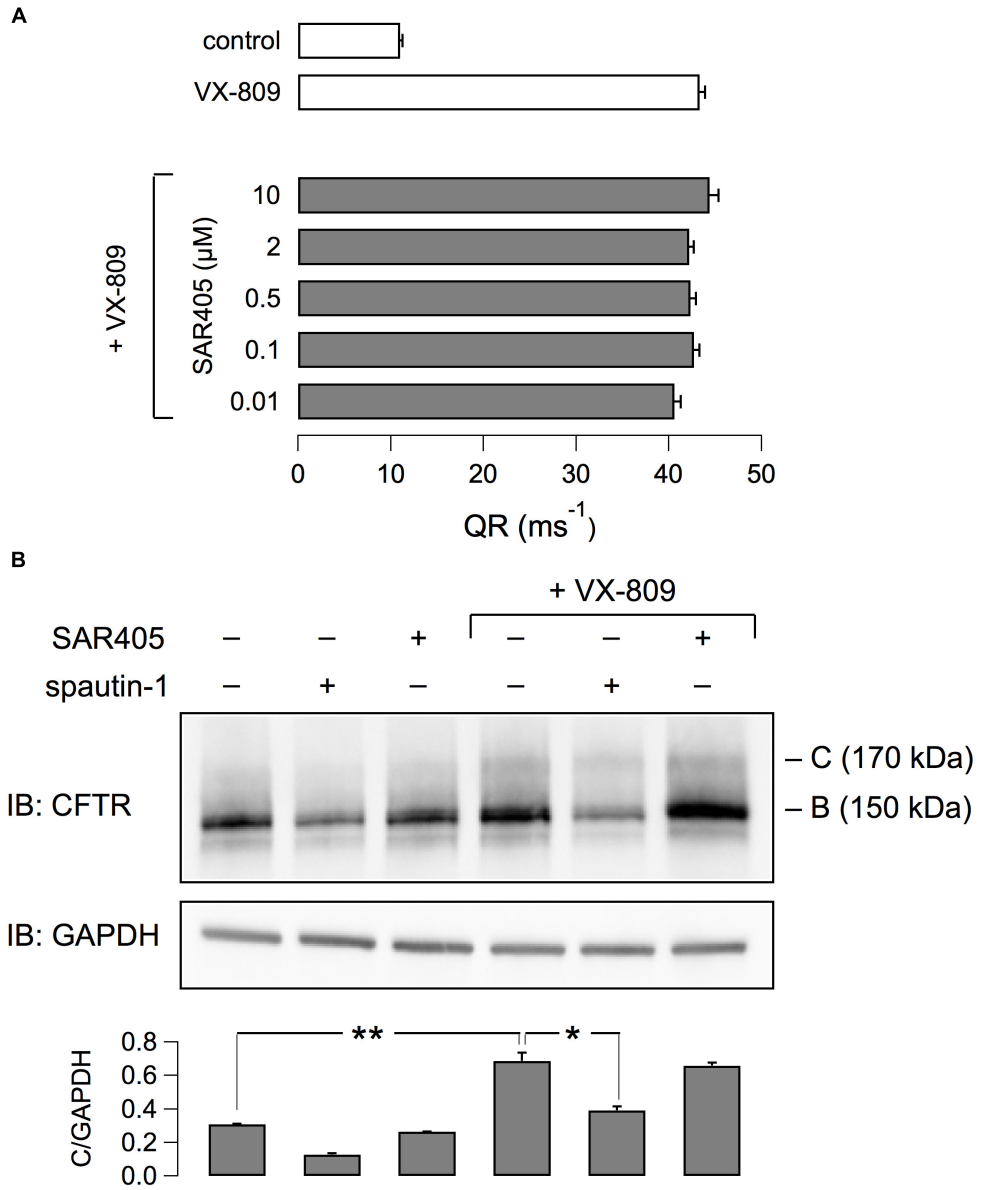

FIGURE 8 | Evaluation of SAR405 on F508del-CFTR expression and function. **(A)** F508del-CFTR activity determined as QR with the HS-YFP assay. CFBE41o- cells expressing F508del-CFTR were treated with VX-809 (1 μM) and various concentrations of SAR405 or vehicle. **(B)** Immunoblot analysis of F508del-CFTR expression in cells treated (3 h) with spautin-1 or SAR405 in the presence or absence of VX-809 (1 μM, 24 h). Bar graph shows normalized densitometric analysis of band C for the different conditions ($n = 3$ independent experiments; $^{*}p < 0.05$; $^{**}p < 0.01$).

opening but with a mechanism that is linked with the intrinsic instability of F508del-CFTR. We also found that spautin-1 is effective in different cell types, including primary CF bronchial epithelial cells, and using different assays.

We investigated the effect of spautin-1 at the protein level. In agreement with functional data, spautin-1 altered the expression of F508del-CFTR but not of wild type CFTR. In particular, spautin-1 decreased the expression of both mature and immature forms of the mutant protein. This type of effect was also observed when the protein in the plasma membrane was detected by cell surface biotinylation. The presence of partially glycosylated F508del-CFTR (i.e., band B) in the plasma membrane is not surprising since it has been attributed to an unconventional route of protein trafficking that bypasses the Golgi (Gee et al., 2011; Tomati et al., 2015). Therefore, the decrease in F508del-CFTR function caused by spautin-1 could be explained with a decreased expression of both forms of F508del-CFTR in the plasma membrane, either by reduced trafficking to the cell surface and/or accelerated internalization. In this respect, it is particularly interesting to note the results from patch-clamp experiments. In the whole-cell configuration, spautin-1 caused a significant decrease in the activity of F508del-CFTR. This

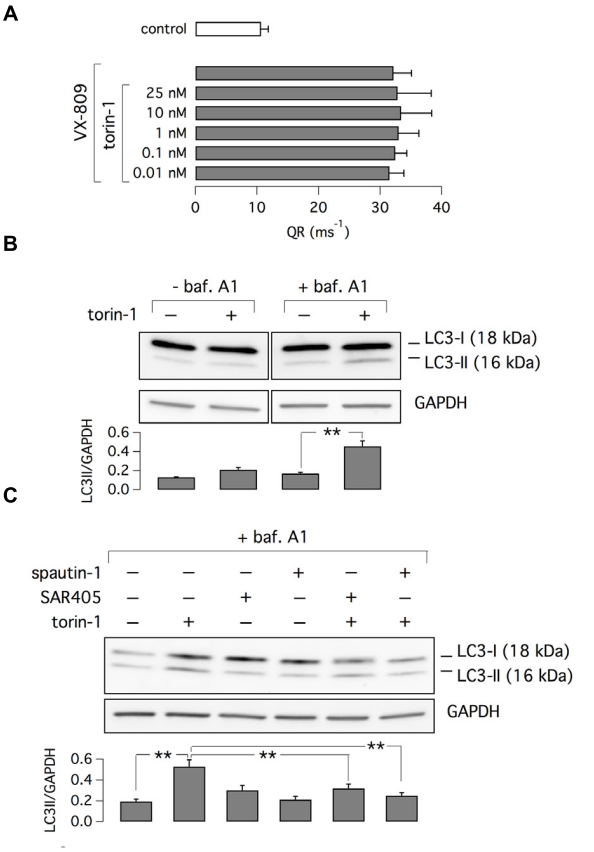

FIGURE 9 | Evaluation of torin-1 as an autophagy inducer. **(A)** Test of different torin-1 concentrations on F508del-CFTR function. Cells were treated with VX-809 (1 μM) or vehicle (n = 3 separate experiments). **(B)** Analysis of LC3-I and LC3-II expression in cells treated with/without torin-1, to induce autophagy, and bafilomycin A1, to block lysosome-dependent degradation. **(C)** Analysis of LC3-I and LC3-II expression in cells treated with torin-1 (or vehicle) in presence/absence of SAR405 or spautin-1. Bar graphs in **(B,C)** show normalized densitometry analysis of LC3-II (n = 3 separate experiments; $^{**}p < 0.01$).

effect was not detected in inside-out patch recordings from the same cell type. Such results indicate that inhibition by spautin-1 requires an intracellular machinery that is lost upon membrane patch excision. The fast effect induced by spautin-1, with a decrease in function starting a few minutes from compound addition, is intriguing. It is possible that this initial effect is due to rapid internalization of F508del-CFTR protein. However, we could not block the effect of spautin-1 with dynasore. Therefore, if internalization is involved, it should occur through a mechanism insensitive to dynasore. Another possibility is that the initial decrease in function is caused by F508del-CFTR channel inactivation by ubiquitination at the plasma membrane.

We investigated the involvement of USP10 and USP13 in the effect of spautin-1 on mutant CFTR. We silenced USP10 and USP13 expression by transfection with specific DsiRNAs of cells that were also treated with VX-809. Surprisingly, USP10 knockdown did not result in alteration of F508del-CFTR activity. This seems to be in contrast with previous studies in which function of USP10 was found to be important in CFTR trafficking at the cell periphery (Bomberger et al., 2009). However, those studies were done on cells expressing wild type CFTR. Therefore, it is possible that USP10 is much less involved in mutant CFTR processing. Instead, we found that silencing of USP13 inhibited F508del-CFTR function. In agreement with RNAi transfection experiments, overexpression of USP13, but not of USP10, increased F508del-CFTR function above the level achieved with VX-809 incubation and this effect was blocked by spautin-1. These results indicate that USP13 is an important deubiquitinase that controls the extent of F508del-CFTR expression in the plasma membrane. Consequently, inhibition of endogenous USP13 could be the mechanism through which spautin-1 affects F508del-CFTR function and processing. It should be emphasized, however, that most small molecules are rarely absolutely specific for a single target. Therefore, we cannot exclude that spautin-1 also acts on another deubiquitinase. This issue will require further studies.

By inhibiting a deubiquitinase, spautin-1 treatment should result in increased F508del-CFTR ubiquitination, an effect that should lead to enhanced degradation. However, combined inhibition with MG-132 and bafilomycin did not prevent spautin-1 effect. In particular, MG-132 alone was sufficient to markedly enhance ubiquitination of F508del-CFTR. However, addition of spautin-1 did not further enhance ubiquitination but actually had the opposite effect. This unexpected finding could suggest that F508del-CFTR, similarly to other proteins, is also eliminated through a proteasome- and lysosome-independent pathway (Donoso et al., 2005; Mbonye et al., 2008; Lee et al., 2014). Alternatively, it should be noted that pro-collagen was found to be degraded by the lysosome with a non-conventional mechanism insensitive to bafilomycin (Omari et al., 2018). This issue needs further investigation to clarify the molecular mechanisms involved in this type of protein degradation.

We also asked whether spautin-1 affects F508del-CFTR by inhibiting autophagy. In a recently published paper, which investigated the molecular mechanisms of unconventional CFTR trafficking, spautin-1 was found to decrease F508del-CFTR in the plasma membrane (Noh et al., 2018). This effect was interpreted as an evidence of the involvement of autophagy machinery in CFTR trafficking. According to this model, autophagosomes could be used, under particular conditions, to transport immature CFTR to the plasma membrane (Noh et al., 2018). In our study, we tested two other small molecules, torin-1 (Thoreen et al., 2009) and SAR405 (Ronan et al., 2014), which act respectively as an activator and an inhibitor of autophagy. Importantly, neither SAR405 and wortmannin, as autophagy inhibitors, nor torin-1, as autophagy activator, affected F508del-CFTR in a positive or negative way. In particular, SAR405 and wortmannin act at the same early stage of autophagic process as spautin-1 (Vps34 complex). Therefore, we conclude that the effect of spautin-1 on F508del-CFTR is independent of autophagic machinery. In general, our results also indicate that pharmacological modulation of autophagy does not affect *per se* the function/trafficking of F508del-CFTR nor its rescue by VX-809.

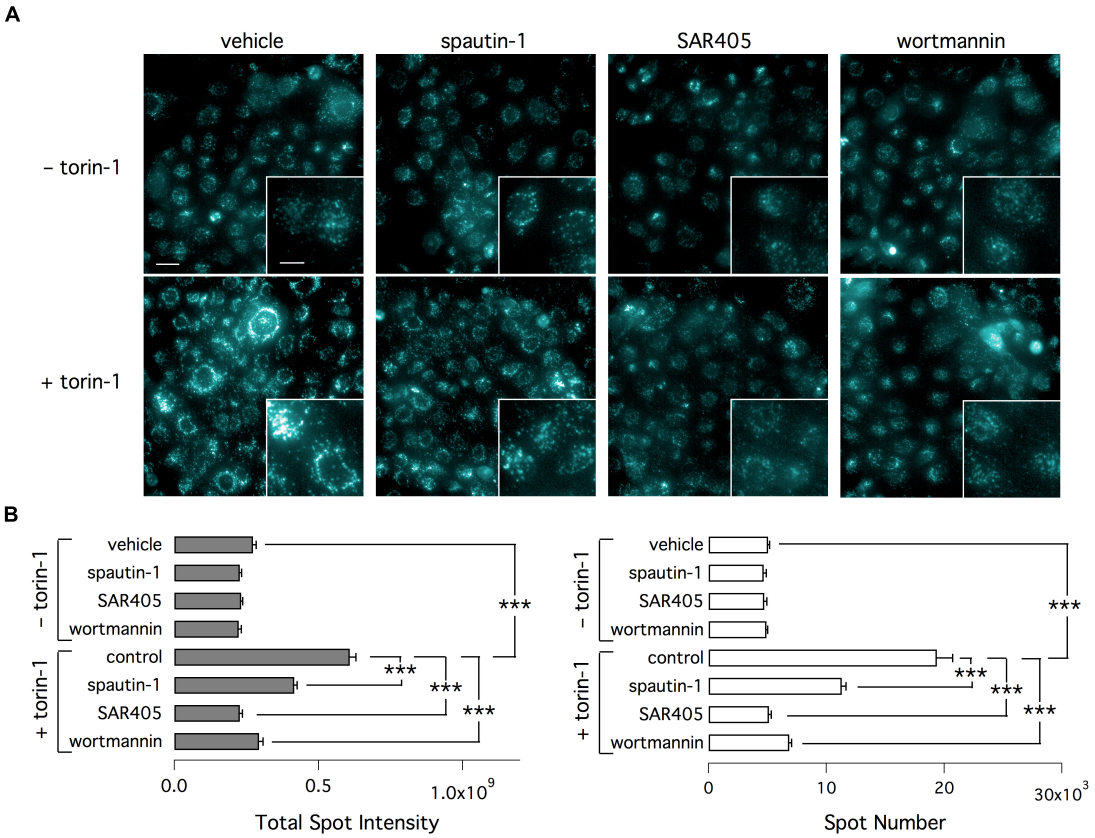

FIGURE 10 | Determination of autophagy with MDC. **(A)** Representative microscopy images of CFBE41o- cells exposed to indicated treatments (torin-1, spautin-1, SAR405). Cells were stained with MDC. **(B)** Data (total spot intensity, left, and spot numer, right) obtained from MDC-stained cells (n = 3 separate experiments; $^{***}p < 0.001$).

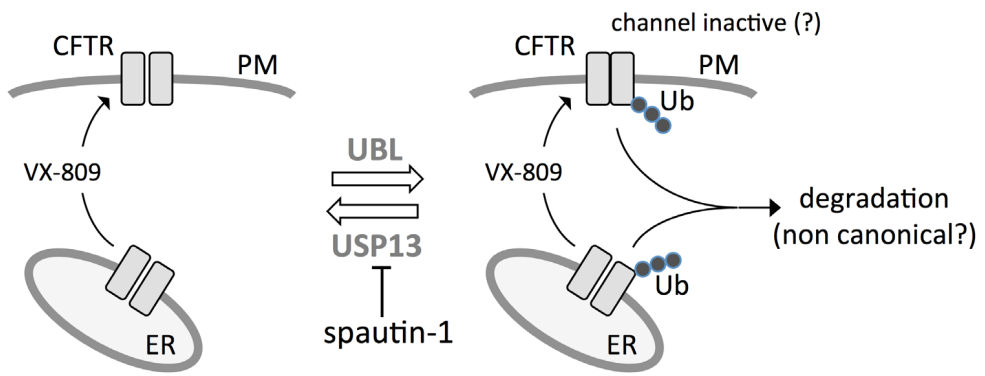

FIGURE 11 | Postulated model for spautin-1 mechanism of action. F508del-CFTR is ubiquitinated at the ER and plasma membrane by ubiquitin ligases. At the plasma membrane, ubiquitination may cause internalization and, possibly, as an initial effect, also channel inactivation. Mutant CFTR is partially protected from degradation by the activity of USP13. Inhibition of USP13 with spautin-1 blocks deubiquitination thus increasing the fraction of F508del-CFTR undergoing elimination.

Summarizing, our results reveal spautin-1 as an interesting probe that works as an "anti-corrector." In the presence of this compound, the effect of VX-809 and of another rescue maneuver, low temperature incubation, is significantly diminished. A possible explanation is that spautin-1 blocks a protective mechanism, possibly deubiquitination by USP13, which shields F508del-CFTR from degradation through a still unknown process (**Figure 11**). Therefore, spautin-1 can be used to reveal the underlying degradation mechanisms that limit F508del-CFTR rescue. In future studies, elucidation of spautin-1 mechanism of action may lead to the identification of novel therapeutic targets to improve rescue of F508del-CFTR and to

the discovery of novel biological pathways involved in protein degradation.

MATERIALS AND METHODS

Cell Culture Conditions

The bronchial epithelial cell line CFBE41o- with and without stable co-expression of F508del-CFTR or wild type CFTR was cultured with MEM medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The generation of CFBE41o- cells co-expressing F508del-CFTR and the HS-YFP was previously described (Sondo et al., 2011). The FRT cells expressing F508del-CFTR and HS-YFP (Pedemonte et al., 2010) were cultured in Coon's modified Ham's F-12 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Fluorescence Assay for CFTR Activity (Microplate Reader Version)

CFBE41o- cells with co-expression of F508del-CFTR or wt-CFTR and the HS-YFP were treated for 24 h with DMSO or VX-809 1 μM. In some experiments, rescue of F508del-CFTR was obtained by incubation at low temperature for 24 h. Where needed, spautin-1 was added at different concentrations in the last 3 h of incubation. Shorter times of incubation with spautin-1 were also tested. After treatments, cells were washed with PBS (containing: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) and then stimulated for 30 min with forskolin (20 μM) and genistein (50 μM). Cells were then transferred to a microplate reader (FLUOstar Galaxy; BMG Labtech) for CFTR activity determination. The plate reader was equipped with high-quality excitation (HQ500/20X: 500 ± 10nm) and emission (HQ535/30M: 535 ± 15 nm) filters for YFP (Chroma Technology). Each assay consisted of a continuous 14 s fluorescence reading including 2 s before and 12 s after injection of an iodide-containing solution (PBS with Cl^- replaced by I^-; final I^- concentration 100 mM). Data were normalized to the initial background-subtracted fluorescence. To determine I^- influx rate, the last 11 s of data for each well were fitted with an exponential function to extrapolate initial slope (dF/dt).

Fluorescence Assay for CFTR Activity (Microscope Version)

CFBE41o- cells with stable co-expression of F508del-CFTR and HS-YFP, were transfected 6 h after plating with plasmids coding for USP13 or USP10 tagged with the mCherry red fluorescence protein (Origene). The day after, the cells were treated with DMSO or VX-809 (1 μM) for 24 h and, where needed, spautin-1 (20 μM) was added in the last 3 h of incubation. At the time of the assay, cells were stimulated with forskolin (20 μM) and genistein (50 μM) for 30 min and transferred to a fluorescence microscope (IX50 Olympus) equipped with a 20× objective and excitation/emission optical filters for YFP and mCherry. Images of HS-YFP fluorescence were taken with a digital camera (CoolSNAP; Photometrics) at four frames per second for a total of 40 s. At 5 s from beginning of acquisition, the high I^- solution was added in the well (same volumes and concentrations as indicated for the plate reader assay). A single image of mCherry-expressing cells was taken at the end of assay. For analysis of results, the MetaMorph software was used to determine the time-course of HS-YFP fluorescence quenching in mCherry-positive cells. For each well, 10 cells were considered for analysis.

Gene Silencing With DsiRNAs

CFBE41o- cells were reverse transfected with DsiRNAs (Origene) at the time of plating. For this purpose, complexes were formed by combining DsiRNAs at the desired concentration with 0.25 μl Lipofectamine 2000 in 50 μl of Optimem synthetic medium for each well. The DsiRNA-lipofection agent complexes were pipetted in each well of a 96-well microplate together with 100 μl of MEM without antibiotics containing 50,000 cells. After 24 h of cell incubation at 37°C, the medium was removed and replaced with fresh MEM plus serum and antibiotics. After additional 24 h, the functional YFP-based assay was performed to determine the extent of F508del-CFTR activity in the plasma membrane.

Transepithelial Electrical Conductance (TEEC)

FRT cells expressing F508del-CFTR were plated on HTS Transwell-24 well permeable supports (Code 3379, Corning Life Sciences) at a density of 200,000 cells/well. After 6 days, cells were incubated for 24 h with vehicle (DMSO) or VX-809 (1 μM) in both basolateral (800 μl) and apical (300 μl) culture medium. Where needed, spautin-1 was included in the last 3 h of treatment. After treatment, the culture medium was removed and replaced on both sides with a saline solution (100 μl apical, 800 μl basolateral) containing (in mM): 130 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, 10 glucose, 10 Na-Hepes (pH 7.4). The 24-well tray with cells was placed on a block heater (SBH 130D, Stuart) to keep the temperature at 37°C during the entire experiment. After 10 min, the basal TEER across each layer of FRT cells was measured with a STX100C electrode pair connected to an EVOM2 voltohmeter (World Precision Instruments). To stimulate F508del-CFTR, each well received (apical side) 50 μl of saline solution containing 60 μM forskolin and 150 μM genistein (final concentrations: 20 μM forskolin, 50 μM genistein). Forskolin was added to the basolateral medium to obtain the 20 μM concentration. After 10 min, TEER was measured again in each well. To block F508del-CFTR function, the inhibitor PPQ-102 was used at the final concentration of 30 μM. To achieve the desired concentration, 75 μl of the apical solution in each well was replaced with an equal volume of saline solution containing 20 μM forskolin, 50 μM genistein, and 60 μM PPQ-102. After further 10 min, the TEER was measured. All values of TEER were converted to TEEC using the formula TEEC = 1/TEER. The parameter to indicate activity of F508del-CFTR in each well, ΔTEEC was calculated from the difference in TEEC measured after maximal stimulation of F508del-CFTR with forskolin plus genistein and after block with PPQ-102 (see

Supplementary Figure 3). Two particular conditions used in these experiments need to be discussed in detail. These conditions were defined during the setting of this technique. First, we used genistein instead of VX-770 as a potentiator. Indeed, the very high potency and "stickiness" of VX-770 caused problems of contamination and carry over due to adhesion to electrodes. Second, we used PPQ-102, instead of $CFTR_{inh}$-172, to block CFTR, because the former compound is more water soluble. Therefore, it is more adequate for a procedure in which the CFTR inhibitor has to be prepared in a saline solution at three times the final concentration. For $CFTR_{inh}$-172, this would mean 30 μM, which appeared to cause problems of solubility.

Whole-Cell and Inside-Out Patch-Clamp Recordings

Experiments were done on FRT cells stably expressing F508del-CFTR or wild type CFTR. For whole-cell experiments, the extracellular (bath) solution had the following composition: 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM mannitol, and 10 mM Na-Hepes (pH 7.4). The pipette (intracellular) solution instead contained 120 mM CsCl, 10 mM TEA-Cl, 0.5 mM EGTA, 1 mM $MgCl_2$, 40 mM mannitol, 1 mM ATP, and 10 mM Cs-Hepes (pH 7.2). Acute stimulation was done by perfusion with forskolin (20 μM) plus genistein (50 μM).

For inside-out experiments, the bath solution contained: 150 mM NMDG-Cl, 2 mM $MgCl_2$, 10 mM EGTA, 1 mM MgATP, 10 mM HEPES (pH 7.35). The bath solution also contained 5 μg/ml (125 nM) of protein kinase A catalytic subunit of (PKA, Promega). The pipette was instead filled with: 150 mM NMDG-Cl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES (pH 7.35). After excision of inside-out membrane patches, CFTR Cl^- channels were activated by the activity of the kinase.

During experiments, the membrane capacitance and series resistance were analogically compensated using the circuitry provided by the EPC7 patch-clamp amplifier. The usual protocol for stimulation consisted in 600 ms-long voltage steps from −100 to +100 mV in 20 mV increments starting from a holding potential of −60 mV. The waiting time between steps was 4 s. Membrane currents were filtered at 1 kHz and digitized at 5 kHz with an ITC-16 (InstruTech) AD/DA converter. Data were analyzed using the Igor software (Wavemetrics) supplemented by custom software kindly provided by Dr. Oscar Moran.

Short-Circuit Current Recordings on Human Bronchial Epithelial Cells

Human bronchial epithelial cells obtained from a CF patient (F508del/F508del genotype) were plated on Snapwell inserts (Code 3603, Corning Life Sciences) at a density of 500,000 cells per insert. Cells were cultured for 2 weeks in a differentiating medium whose compositions has been previously described (Scudieri et al., 2012). For the first week, the medium was kept on both apical and basolateral sides of inserts (liquid–liquid condition). For the second week, the apical medium was removed (air-liquid condition, ALC). At the end of second week, cells were treated for 24 h in the basolateral medium with vehicle (DMSO) or VX-809 (1 μM). Where needed, spautin-1 was added in the medium for the last 3 h. After treatment, Snapwell inserts carrying differentiated bronchial epithelia were mounted in a vertical chamber resembling an Ussing system with internal fluid circulation. Both apical and basolateral hemichambers were filled with 5 ml of a Krebs bicarbonate solution containing (in mM): 126 NaCl, 0.38 KH_2PO_4, 2.13 K_2HPO_4, 1 $MgSO_4$, 1 $CaCl_2$, 24 $NaHCO_3$, and 10 glucose. Both sides were continuously bubbled with a gas mixture containing 5% CO_2 – 95% air and the temperature of the solution was kept at 37°C. The transepithelial voltage was short-circuited with a voltage-clamp (DVC-1000, World Precision Instruments) connected to the apical and basolateral chambers via Ag/AgCl electrodes and agar bridges (1 M KCl in 1% agar). The offset between voltage electrodes and the fluid resistance were canceled before experiments. The short-circuit current was recorded with a PowerLab 4/25 (ADInstruments) analogical to digital converter connected to a Macintosh computer. During recordings, cells were sequentially treated with: amiloride (10 μM, apical side) to block Na^+ absorption through ENaC channel; CPT-cAMP (100 μM, apical and basolateral side) plus VX-770 (1 μM) to stimulate F508del-CFTR activity; $CFTR_{inh}$-172 (10 μM, apical side) to inhibit F508del-CFTR. The difference between the current measured with CPT-cAMP plus potentiator and the current remaining after $CFTR_{inh}$-172 activity was taken as the parameter reflecting F508del-CFTR expression in the apical membrane.

Labeling of Autophagic Vacuoles With Monodansylcadaverine (MDC)

CFBE41o- cells stably expressing F508del-CFTR and the HS-YFP were plated (50,000 cells/well) on high quality clear-bottom 96-well black microplates suitable for high-content imaging. After 24 h, cells were treated with test compounds or DMSO (as negative control). After 24 h, cells were washed and incubated with 50 μM MDC (Sigma-Aldrich) in PBS at 37°C for 10 min (Biederbick et al., 1995). After incubation, cells were washed three times with PBS and immediately analyzed. High-content imaging and data analysis were performed using an Opera Phenix (PerkinElmer) high-content screening system. Wells were imaged in confocal mode, using a 40X water-immersion objective with high numerical aperture. MDC signal was laser excited at 405 nm and the emission was collected between 435 and 550 nm.

Data analysis of MDC signal spots was performed using the Harmony software (version 4.5) of the Opera Phenix high-content system. Briefly, for each field of view, the analysis algorithm detected the signal spots, and quantified the total number of spots, single spot intensity, and total spot intensity.

Western Blot

Cells were grown to confluence on 60-mm diameter dishes and lysed in RIPA buffer containing a complete protease inhibitor (Roche). Cell lysates were centrifuged at 12000 rpm at 4°C for 10 min. Supernatant protein concentration was calculated using the BCA assay (Euroclone) following the manufacturer's instructions. Equal amounts of protein (30 μg) were separated onto gradient (4–15% or 4–20% depending on target protein molecular weight) Criterion TGX Precast

gels (Bio-Rad Laboratories Inc.), transferred to nitrocellulose membrane with *Trans*-Blot Turbo system (Bio-Rad Laboratories Inc.) and analyzed by Western blotting. Primary antibodies and dilutions were: mouse monoclonal anti-CFTR (596, Cystic Fibrosis Foundation Therapeutics, University of North Carolina, Chapel Hill) 1:5000; mouse monoclonal anti Na^+/K^+-ATPase α1 (cl. C464.6; Millipore) 1:6000; mouse monoclonal anti-GAPDH (cl.6C5; Santa Cruz Biotechnology, Inc.) 1:10000; rabbit polyclonal anti-USP13 (Abcam) 1:1000; rabbit polyclonal anti-USP10 (Abcam) 1:1000; rabbit monoclonal anti-calnexin (Abcam) 1:5000; rabbit polyclonal anti-14-3-3 epsilon (Abcam) 1:1000; rabbit polyclonal anti-LC3B (Sigma, L7543) 1:1000; rabbit polyclonal anti-Beclin-1 (Cell Signaling Technology, 3738) 1:1000. As secondary antibodies, we used anti-rabbit HRP-conjugated antibody (Abcam) diluted 1:5000 and anti-mouse HRP-conjugated secondary antibody (Ab 97023, Abcam) diluted 1:10000. Results were subsequently visualized by chemiluminescence using the SuperSignal West Femto Substrate (Thermo Scientific) and the Molecular Imager ChemiDoc XRS System. Images were analyzed with ImageJ software (National Institutes of Health). Intensity of bands was analyzed as Region-Of-Interest (ROI). The background was subtracted sand intensity was normalized against the GAPDH loading control. Data are presented as mean ± SEM of independent experiments.

Detection of CFTR Ubiquitination

CFBE41o- cells stably expressing F508del-CFTR were grown to confluence on 60-mm diameter dishes, treated for 24 h with VX809 (1 μM) and in the last 3 h with MG132 (10 μM), bafilomicyn A1 (100nM) and/or spautin-1 (20 μM). Then, cells were rinsed twice with ice-cold Ca^{2+}/Mg^{2+}-free PBS and then lysed with IP Lysis Buffer (#87788 Thermo Sci.) containing EDTA-free complete protease inhibitor (Roche), N-ethylmaleimide (5 mM) and MG-132 (20 mM). Nuclei were pelleted by centrifugation at 12000 rpm at 4°C for 20 min. Supernatant protein concentration was calculated using the BCA assay (Euroclone) following the manufacturer's instructions. An aliquot of supernatant corresponding to 500 μg of protein was incubated for 1 h with 2 μg/sample of mouse monoclonal anti-CFTR R24-1 antibody (R&D), rocking at room temperature. Antibody-antigen mixture was precipitated with 25 μl/sample of Pierce Protein A/G Magnetic Beads (Thermo Sci.) for 1 h rocking at RT, following supplier instructions. Immunoprecipitated proteins were eluted from the resin under reducing conditions with 100 μl Laemli Sample Buffer 1X, at RT. Equal amount of IP products (20 μl) were subjected to SDS-PAGE for immunoblotting analysis. Detection of CFTR and ubiquitin was performed using mouse monoclonal anti-CFTR (596, Cystic Fibrosis Foundation Therapeutics, University of North Carolina, Chapel Hill) 1:5000 and anti mouse monoclonal anti-ubiquitin (ub-P4D1, Santa Cruz Biotechnology) 1:1000. As secondary antibody, we used anti mouse-HRP-conjugated secondary antibody (Ab 97023, Abcam).

Cell Surface Biotinylation

CFBE41o- cells were processed as previously described (Tomati et al., 2015). Briefly, cells were seeded on 100 mm dishes and treated with 1 μM VX-809 (or vehicle) for 24 h and with 20 μM spautin-1 (or vehicle) in the last 3 h. At the end of treatments, cells were washed twice with ice-cold PBS and incubated with biotin (0.35 mg/ml in PBS) for 25 min each time on a shaker at 4°C. After three washes in PBS, biotin was quenched with two washes of 50 mM NH_4Cl in PBS (15 min each) on a shaker at 4°C. Cells were then washed three times in PBS without Ca^{2+} and Mg^{2+} and then scraped into Lysis Buffer (50 mM Hepes pH 7, 150 mM NaCl, 1% Glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 5 mM EGTA). Cell lysates were collected in an Eppendorf tube and rocked for 30 min at 4°C. Nuclei were then pelleted by centrifugation at 10,000 rpm at 4°C for 20 min. Supernatant protein concentration was calculated using the BCA assay (Euroclone) according to manufacturer's instructions. An aliquot of supernatant corresponding to 600 μg of proteins was precipitated by rotation at 4°C for 6 h using high capacity streptavidin agarose resin (Thermo Fischer Scientific Inc.), following the manufacturer's recommendation. The resin was then washed with the following solutions: once with Lysis Buffer, twice with Buffer 1 (150 mM NaCl, 20mM Tris-HCl, pH 8, 5 mM EDTA, 1% Triton X-100, 0.2% BSA), once with Buffer 3 (150 mM NaCl, 20 mM Tris-HCl, pH 8, 5 mM EDTA, 0.5% Triton X-100), and once with Buffer 4 (50 mM Tris–HCl, pH 8). Biotinylated proteins were eluted from the resin with reducing Sample Buffer 4X and 30 μl of each sample were separated on a 4–15% or 4–20% gradient Criterion TGX gel (Biorad) and analyzed by Western blotting.

Immunofluorescence Detection of CFTR and USP13

CFBE41o- cells seeded in a 12-well μ-chamber (81201, Ibidi) at a density of 25.000 cells per well and treated with VX-809 and spautin-1 as for functional assays, were rinsed with PBS and fixed by adding 100 μl per well of 10% neutral buffered formalin for 5 min at room temperature. After three washes with 300 μl of PBS, cells were permeabilized with PBS-Triton X-100 0.3% for 5 min, blocked with PBS-BSA 1% for 2 h, and then incubated overnight at 4°C with 100 μl of primary antibodies diluted in blocking solution. The following antibodies and dilutions were used: ab570 mouse IgG1 anti-CFTR (J. R. Riordan, University of North Carolina at Chapel Hill, and Cystic Fibrosis Foundation Therapeutics) at 1:250 and ab99421 rabbit anti-USP13 (Abcam) at 1:200. Following incubation with primary antibodies, cells were rinsed three times with PBS and incubated with 100 μl of a solution of secondary goat anti-rabbit Alexa Fluor-488 and goat anti-mouse Alexa Fluor-546 antibodies (Invitrogen) diluted in PBS-BSA 1% for 1 h in the dark. After three further washes in PBS, cells were covered with mounting medium and coverslip, and then analyzed using a laser-scanning confocal microscope SPE (Leica Microsystems). Image analysis was performed using Leica and ImageJ software. Fractional overlap between CFTR and USP13 was quantified via Manders' colocalization coefficient in total cell ROIs or in peripheral ROIs by using Coloc2 plugin.

Statistics

Data are shown as representative traces/images or as a mean values and SEM of independent experiments. Significance of differences was established with ANOVA (with Tukey or Dunnett *post hoc* tests, as appropriate) except for experiments shown in **Figures 4B**, **7B** in which Student's *t*-test was used. Analysis was done with InStat (GraphPad) software.

ETHICS STATEMENT

The protocols to isolate, culture, store, and study bronchial epithelial cells from patients undergoing lung transplant (described in Scudieri et al., 2012) were approved by the Regional Ethical Committee (Comitato Etico Regionale) under the supervision of the Italian Ministry of Health (registration number: ANTECER, 042-09/07/2018). Informed and written informed consent was obtained from all patients using a form that was also approved by the same Ethical Committee.

AUTHOR CONTRIBUTIONS

EP, ES, NP, AH, and LG conceived the study. EP, ES, LF, VT, EC, PS, IM, MR, NB, NS, DdB, and NP investigated and validated the data. EP, ES, NP, AH, and LG wrote the manuscript. NP and LG acquired the funding. NP, DB, AH, and LG provided the resources.

FUNDING

This study was supported by grants from Telethon Foundation (TMLGCBX16TT), from Fondazione Italiana per la Ricerca sulla Fibrosi Cistica (FFC#2/2017, grant adopted by FFC delegations from Bologna and Ferrara), from ERA-Net for Research Programmes on Rare Diseases (01GM1601), and from "Ricerca Corrente" from the Italian Ministry of Health (Linea 1).

ACKNOWLEDGMENTS

We thank Dr. John Riordan (University of North Carolina – Chapel Hill), and Cystic Fibrosis Foundation Therapeutics for kindly providing us with anti-CFTR antibodies.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01464/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

MINI REVIEW
published: 27 September 2018
doi: 10.3389/fphar.2018.01100

Peripheral Protein Quality Control as a Novel Drug Target for CFTR Stabilizer

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Conformationally defective cystic fibrosis transmembrane conductance regulator (CFTR) including rescued ΔF508-CFTR is rapidly eliminated from the plasma membrane (PM) even in the presence of a CFTR corrector and potentiator, limiting the therapeutic effort of the combination therapy. CFTR elimination from the PM is determined by the conformation-dependent ubiquitination as a part of the peripheral quality control (PQC) mechanism. Recently, the molecular machineries responsible for the CFTR PQC mechanism which includes molecular chaperones and ubiquitination enzymes have been revealed. This review summarizes the molecular mechanism of the CFTR PQC and discusses the possibility that the peripheral ubiquitination mechanism becomes a novel drug target to develop the CFTR stabilizer as a novel class of CFTR modulator.

Keywords: peripheral QC, CFTR, stabilizer, ubiquitination, RFFL

OPEN ACCESS

Edited by:
Nicoletta Pedemonte,
Istituto Giannina Gaslini (IRCCS), Italy

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *19 July 2018*
Accepted: *10 September 2018*
Published: *27 September 2018*

Citation:
Fukuda R and Okiyoneda T (2018) Peripheral Protein Quality Control as a Novel Drug Target for CFTR Stabilizer. Front. Pharmacol. 9:1100. doi: 10.3389/fphar.2018.01100

INTRODUCTION

Cystic fibrosis (CF) is one of the most lethal autosomal-recessive diseases caused by mutation in CFTR (Lopes-Pacheco, 2016). CFTR mutations are classified as I–VII according to their properties (I–protein synthesis defect, II-maturation defect, III-gating defect, IV-conductance defect, V-reduced quantity, VI-reduced PM stability, VII-no mRNA transcription). The most prevalent CF causing mutation, ΔF508, was classically categorized as class II mutation. However, rescued ΔF508 (rΔF508)-CFTR by corrector (e.g., VX-809/lumacaftor) or low temperature culture shows class III and VI phenotypes (Dalemans et al., 1991; Veit et al., 2016a). Although drug targets of the class II or III mutations are well studied, that of the class VI mutation are not because the mechanism of CFTR PM stability regulation are still veiled by numerous undefined molecules involved in CFTR PQC system. In this review, we summarize accumulated findings regarding the CFTR PQC from the molecular and environmental aspects and also discuss the potential of recently identified PQC machineries including endocytic adaptors and ubiquitination enzymes as targets for CFTR stabilizer which anchors the functional channel at the PM and reduces the degradation (**Figure 1**).

CFTR INSTABILITY AT THE PM

Nascent wild-type (WT) CFTR is *N*-glycosylated at the endoplasmic reticulum (ER) during translation and folded by the aid of chaperones such as calnexin (CNX), HSP70 and HSP90 (Amaral, 2004; Kleizen et al., 2005; Okiyoneda et al., 2008; Rosser et al., 2008; Glozman et al., 2009; Kim and Skach, 2012). Properly folded CFTR is then sorted to the Golgi apparatus and

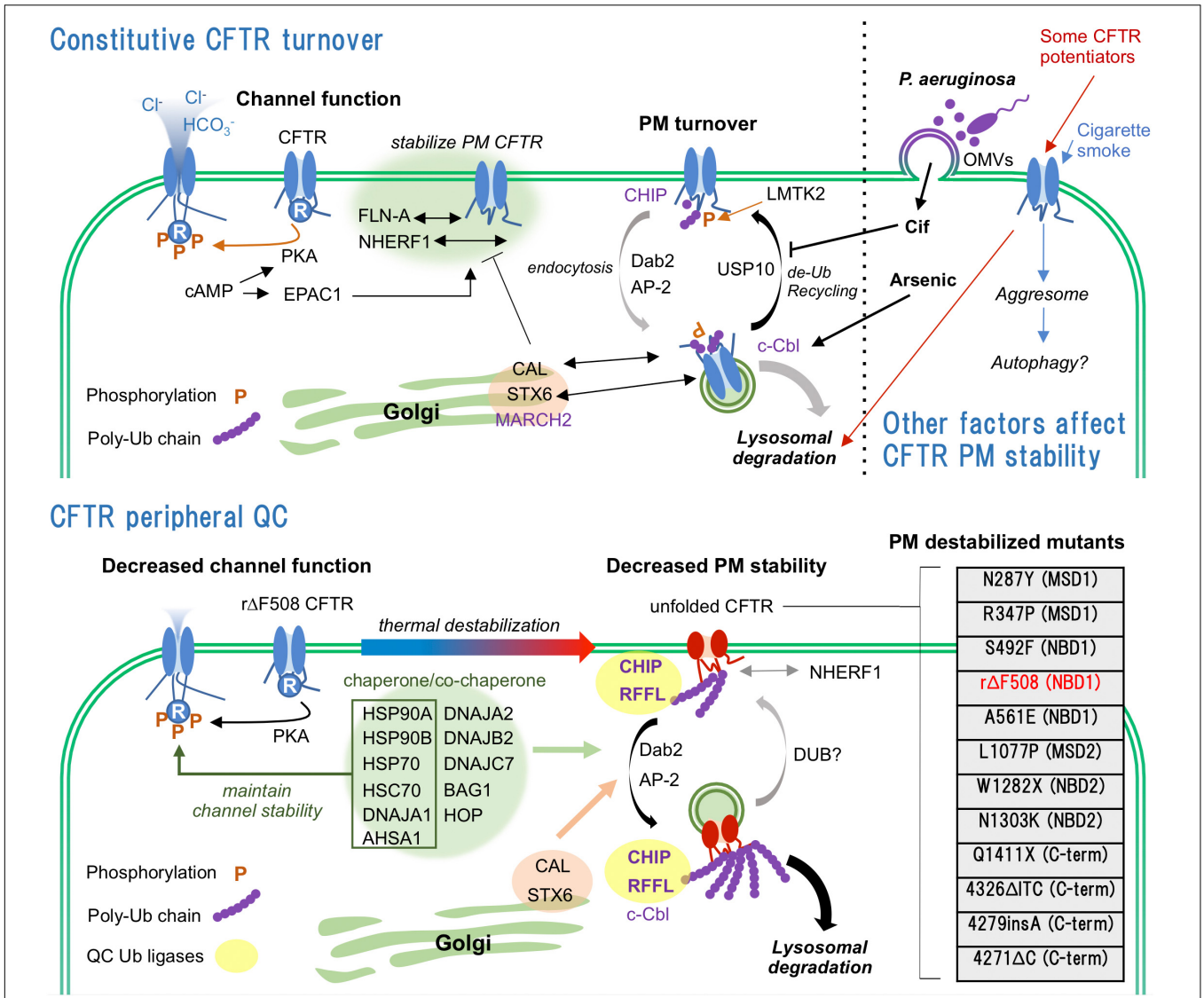

FIGURE 1 | Constitutive turnover and PQC of CFTR. **(Upper)** WT-CFTR is stabilized at the PM by interaction with FLN-A and NHERF1. Internalization of WT-CFTR is regulated by LMTK2-mediated phosphorylation and CHIP-mediated ubiquitination. Predominant endocytosed WT-CFTR is recycled back to the PM by USP10 mediated de-ubiquitination. Golgi localized CAL complex promotes lysosomal sorting of CFTR. CFTR PM stability is deteriorated by infection, CS and CFTR potentiators. **(Lower)** Unfolded rΔF508-CFTR at the PM is ubiquitinated by PQC Ub ligases CHIP and RFFL, and rapidly internalized probably by CME mediated by DAB2 and AP-2. Chaperone/co-chaperone complex also facilitates the unfolded CFTR internalization while it helps the channel functionality at the PM. Internalized unfolded CFTR could be ubiquitinated at endosomes and targeted to lysosomal degradation. CAL also facilitates the lysosomal degradation of internalized CFTR. PM destabilizing CFTR mutations and their locations are listed (Veit et al., 2016a, Cystic Fibrosis Genetic Consortium Database). N287Y mutation increases CFTR endocytosis without affecting maturation (Silvis et al., 2003). R347P, S492F, rΔF508, A561E, L1077P, and N1303K mutations induce severe maturation defect and PM instability (Van Goor et al., 2014). N287Y and L1077P are localized at intracellular loop 2 (ICL2) of MSD1 and ICL4 of MSD2, respectively. Both mutations are predicted to destabilize the MSD1-NBD2 and MSD2-NBD1 interactions that define CFTR conformational stability.

processed to complex glycosylation while misfolded CFTR is retained in the ER and consequently degraded by ER-associated degradation (ERAD). The CFTR ERAD is associated with several ER QC processes such as chaperones binding, ubiquitination and retro-translocation from the ER to cytosol (Younger et al., 2006; Turnbull et al., 2007; Morito et al., 2008; Grove et al., 2011; Nery et al., 2011; El Khouri et al., 2013; Matsumura et al., 2013; Ernst et al., 2016; Gong et al., 2016; McClure et al., 2016; Ramachandran et al., 2016; Hou et al., 2018). Properly folded and matured CFTR is trafficked to the PM to function as an ATP-regulated ion-channel (Chen et al., 2000).

The CFTR is internalized by clathrin-mediated endocytosis (CME) and recycled back to the PM. Conformationally defective CFTR produced by genetic mutations (e.g., ΔF508, T70) and/or environmental stresses (e.g., heat) selectively undergoes ubiquitination at the PM by PQC machineries. The ubiquitinated CFTR is rapidly internalized and delivered to lysosome for degradation (Sharma et al., 2004; Okiyoneda et al., 2010).

Internalized CFTR could be de-ubiquitinated at endosomes by deubiquitinase (DUB) and recycled back to the PM depending on the conformational states. The class VI mutations render the CFTR unstable at the PM. Additionally, the class I, II and some class III CFTR mutants also show PM instability (Lukacs et al., 1993; Haardt et al., 1999; Silvis et al., 2003; Wang et al., 2014; Veit et al., 2016a). *N*-glycosylation, especially the core-glycosylation, determines the CFTR PM stability likely by affecting the CFTR conformational stability (Glozman et al., 2009; Cholon et al., 2010). Protein translation kinetics is also a significant factor that modulates proper co-translational folding. Knock down (KD) of ribosomal protein L12 (RPL12) increases ΔF508-CFTR PM expression and stability (Veit et al., 2016b). RPL12 KD might affect protein translation kinetics associated with co-translational protein folding efficiency (Buhr et al., 2016) and thereby improve CFTR thermodynamic stability which also determines the CFTR PM stability (Okiyoneda et al., 2010; Rabeh et al., 2012). Thus, correcting the CFTR structural defects at the ER could improve the PM stability.

ENVIRONMENTAL STRESSES AFFECTING THE CFTR PM STABILITY

Infection and Inflammation

The CFTR loss of function induces airway surface liquid (ASL) dysregulation which impairs clearance of infected bacteria and/or fungi, and increases the concentration of other soluble signal mediators such as cytokines, chemokines and growth factors. *Pseudomonas aeruginosa* (PA) is one of the most common bacteria found in CF respiratory tissue and responsible for lung injury in CF (Koch, 2002; Bhagirath et al., 2016). PA destabilizes PM CFTR by inhibiting endocytic recycling (Swiatecka-Urban et al., 2006). PA secretes CFTR inhibitory factor (Cif) that stabilizes complex formation of ubiquitin (Ub) specific peptidase 10 (USP10) and GTPase activating protein (SH3 domain) binding protein 1 (G3BP1) and inhibits CFTR-USP10 interaction. Cif inhibits internalized CFTR sorting to recycling pathway by suppressing USP10 dependent CFTR de-ubiquitination at endosome, resulting in the lysosomal degradation of WT-CFTR (Bomberger et al., 2011). PA also activates transforming growth factor β1 (TGF-β1) signaling that is an important modifier of lung disease severity in CF (Harris et al., 2011). TGF-β1 inhibits functional PM expression of WT-CFTR and ΔF508-CFTR by reducing mRNA level (Snodgrass et al., 2013; Sun et al., 2014) although its role in the PQC remains unknown.

Heavy Metals

More than 10 ppb of arsenic induces the WT-CFTR ubiquitination and lysosomal degradation via c-Cbl in CF bronchial epithelial (CFBE) cells (Bomberger et al., 2012). Importantly, the phenotype of arsenic toxicity overlaps with CF patient (Bomberger et al., 2012; Mazumdar et al., 2015). Cadmium (Cd) is a major component of cigarette smoke (CS), and its inhalation is associated with decreased pulmonary function and chronic obstructive pulmonary disease. Cd reduces CFTR PM level, but it remains unknown if it reduces the PM stability (Rennolds et al., 2010).

Cigarette Smoke

Cigarette smoke is a major risk factor of chronic obstructive pulmonary disease and interferes with CFTR functionality. Ten minutes of CS exposure transiently suppresses CFTR function, induces internalization and decreases ASL height in human bronchial epithelial (HBE) cells (Clunes et al., 2012). CS promotes CFTR internalization in BHK cells and results in increased insolubility of CFTR and colocalization with vimentin, a filament protein associated with aggresome Ca^{2+} dependently. This observation suggesting that CS induces PM CFTR destabilization by stimulating internalization and aggregation in addition to suppressing CFTR functionality (Clunes et al., 2012; Rasmussen et al., 2014).

MOLECULAR MACHINERIES DETERMINING THE CFTR PM STABILITY

Endocytosis Adaptors and Tethering Factors

Endocytosis is the critical step of elimination of PM CFTR as a part of PQC and is regulated by several molecules. WT-CFTR is internalized slowly by CME while misfolded rΔF508-CFTR endocytosis is accelerated (Sharma et al., 2004; Swiatecka-Urban et al., 2005; Varga et al., 2008; Okiyoneda et al., 2010). KD of CME adaptor AP-2 μ2 subunit or disabled 2 (DAB2) stabilizes rΔF508-CFTR at the PM by inhibiting endocytosis (Fu et al., 2012, 2015).

CFTR has a postsynaptic density 95, disks large, zonula occludens-1 (PDZ) binding motif at C-terminus and binds with Na^+/H^+ exchanger regulatory factor (NHERF1) PDZ domain. NHERF1 tethers CFTR with Ezrin and works as a scaffold protein that supports CFTR efficient channel activation and apical PM localization (Favia et al., 2010; Arora et al., 2014; Loureiro et al., 2015). NHERF1 also binds to misfolded ΔF508-CFTR and increases the PM stability by inhibiting carboxy terminus of HSP70-interacting protein (CHIP) Ub ligase interaction (Loureiro et al., 2015). An exchange protein directly activated by cAMP1 (EPAC1) selective activating cAMP analog 007-AM promotes WT-CFTR and NHERF1 interaction and increases CFTR PM stability in CFBE cells by suppressing endocytosis (Lobo et al., 2016). EPAC1 activation can rescue ΔF508-CFTR PM expression, and its effect is further improved with VX-809 combination (Lobo et al., 2016).

The CFTR-associated ligand (CAL) negatively regulates ΔF508-CFTR PM abundance through its PDZ domain (Wolde et al., 2007). CAL inhibition enhances the functional stability of ΔF508-CFTR at the apical PM, implying an attractive therapeutic target for CFTR PM stabilizer (Cushing et al., 2010). However, CAL also interacts with syntaxin 6 (STX6) and Golgi-localized E3-ligase membrane associated RING-CH type finger

2 (MARCH2) and regulates WT-CFTR PM expression (Wolde et al., 2007; Cheng and Guggino, 2013).

Filamin-A (FLN-A) is a membrane tethered actin adaptor protein and interacts with CFTR N-terminus region. S13F mutation of CFTR compromises FLN-A binding and consequently destabilizes the PM CFTR (Thelin et al., 2007). FLN-A binds with both WT and rΔF508-CFTR at similar level, however, its contribution to the CFTR PQC remains unclear.

Protein Kinases

The CFTR PM stability is regulated by phosphorylation. CFTR is predominantly phosphorylated at the R domain and also at nucleotide binding domain 1 (NBD1) and C-terminus residues by protein kinase A (PKA), protein kinase C (PKC), casein kinase II (CK2) and AMP-activated protein kinase (AMPK) for the channel function (Chappe et al., 2003; Kongsuphol et al., 2009; Luz et al., 2011). CK2 is predicted to regulate CFTR PM stability by phosphorylation at Thr-1471 where NHERF1 could interact (Venerando et al., 2013). Lemur tyrosine kinase 2 (LMTK2) phosphorylates CFTR at Ser-737 (Wang and Brautigan, 2006) and its KD or mutation at CFTR Ser-737 suppresses the endocytosis and increases CFTR PM density and stability (Luz et al., 2014). However, LMKT2 KD only modestly improves the PM function of rΔF508-CFTR (Luz et al., 2014). Spleen tyrosine kinase (SYK) phosphorylates CFTR at Tyr-512 and decreases CFTR PM levels possibly by triggering endocytosis (Luz et al., 2011; Mendes et al., 2011). Mixed-lineage kinase 3 (MLK3) pathway regulates not only ΔF508-CFTR ERQC, but also the PQC by regulating the CFTR proteostasis (Hegde et al., 2015). Inhibition of MLK3 pathway could regulate ΔF508-CFTR folding/degradation switch by impairing interaction with PQC machinery such as HSP70/HSP90 Organizing Protein (HOP) (Hegde et al., 2015).

Chaperones

Molecular chaperones selectively interact with and stabilize unfolded or partially folded protein to acquire a functionally active conformation. Nascent CFTR interacts with a panel of chaperones and co-chaperones including HSC70, HSP70, HSP90, and CNX at the ER (Yang et al., 1993; Pind et al., 1994; Loo et al., 1998; Meacham et al., 1999; Okiyoneda et al., 2004). Even at the post-ER compartments, conformationally defective CFTR such as unfolded rΔF508-CFTR is recognized by chaperone/co-chaperone complex (Okiyoneda et al., 2010). HSC70/HSP90 complex selectively interacts with unfolded rΔF508-CFTR at the post-Golgi and this interaction is crucial for the unfolding dependent ubiquitination (Okiyoneda et al., 2010). KD of HSC70/HSP90 complex (HSP90, HSC70, HOP, AHA1, DNAJB2, DNAJA1, BAG1) increases the rΔF508-CFTR PM stability in HeLa cells (Okiyoneda et al., 2010). The HSC70/HSP90 complex is also essential for maintaining kinetic and thermodynamic stability of rΔF508-CFTR at the PM by reshaping the CFTR conformation during energetic destabilization (Bagdany et al., 2017). This chaperone activity also maintains rΔF508-CFTR channel function at the PM (Bagdany et al., 2017). Thus, modulating the chaperone activity would be a viable target for attenuating the ubiquitination and for stabilizing the CFTR function at the PM.

Ubiquitination Enzymes

Ubiquitination determines CFTR elimination not only at the ER, but also from the PM. Ubiquitination is mediated by a sequential action of E1, E2, and E3 enzymes and this modification could be removed by DUB. Specifically, E3 Ub ligase has been proposed to determine the substrate specificity. CHIP is the first identified E3 ligase responsible for the CFTR PQC (Okiyoneda et al., 2010). Consistent with the action at the ER (Meacham et al., 2001), CHIP selectively interacts with unfolded ΔF508-CFTR at the post-Golgi through the HSC70/HSP90 chaperones. CHIP KD reduces the ubiquitination of unfolded ΔF508-CFTR, resulting in the decelerated endocytosis and lysosomal delivery in HeLa cells (Okiyoneda et al., 2010). CHIP KD also stabilizes rΔF508-CFTR at the PM of polarized CFBE cells (Fu et al., 2015).

E3 ligase c-Cbl may play a role in the CFTR peripheral QC, but its contribution could be modest since its KD slightly increases rΔF508-CFTR PM stability in CFBE cells (Cihil et al., 2013; Fu et al., 2015). c-Cbl also binds with WT-CFTR and decreases the PM stability without affecting the ubiquitination, suggesting that c-Cbl could regulate constitutive PM turnover of folded CFTR by inducing endocytosis through its C-terminus adaptor function (Ye et al., 2010).

Nedd4-2 is a member of homologous to the E6-AP carboxyl terminus (HECT) E3 which may regulate the CFTR PM expression. Nedd4-2 KD reduces ΔF508-CFTR ubiquitination at the ER, and increases the PM expression and function in CF pancreatic adenocarcinoma cell 1 (CFPAC1) and IB3-1 cells (Caohuy et al., 2009). Nedd4-2 binds both WT- and ΔF508-CFTR while its role in the WT-CFTR ubiquitination remains controversial (Koeppen et al., 2012). However, Nedd4-2 KD does not stabilize the PM rΔF508-CFTR in CFBE cells, implying its marginal contribution to the CFTR PQC (Koeppen et al., 2012; Fu et al., 2015). Nedd4-2 is unlikely a viable CF drug target because its knock out (KO) induces CF-like lung phenotype by excessive function of epithelial Na^+ Channel (ENaC) (Kimura et al., 2011; Rotin and Staub, 2012).

A number of DUBs regulate the CFTR turnover. USP10, a DUB localized at early endosomes, interacts with WT-CFTR and reduces the CFTR poly-ubiquination in CFBE cells. The USP10-mediated deubiquitination enhances the endocytic recycling of WT-CFTR (Bomberger et al., 2009). The role of USP10 in the PM stability of conformationally defective CFTR such as rΔF508-CFTR remains unclear.

Recently, we have discovered RING finger and FYVE like domain containing E3 Ub protein ligase (RFFL) as a novel component of the CFTR PQC machineries by a comprehensive siRNA screen in CFBE cells (Okiyoneda et al., 2018). RFFL selectively recognizes unfolded rΔF508-CFTR through the disordered regions. RFFL promotes K63-linked poly-ubiquitination of the unfolded CFTR in post-Golgi, resulting in accelerated endocytosis and lysosomal degradation. Importantly, RFFL directly interacts with conformationally defective CFTR such as rΔF508-CFTR, but not with folded WT-CFTR at the PM and endosomes. Moreover, the RFFL-mediated ubiquitination is

conformation dependent as it selectively ubiquitinates thermally unfolded NBD1. RFFL KD enhances the functional PM expression of rΔF508-CFTR in the presence of VX-809, and this effect is further improved by inhibiting the HSC70-dependent ubiquitination machinery. Thus, RFFL plays an important role in the chaperone-independent CFTR PQC mechanism in HBE cell models.

CFTR MODULATORS AFFECTING THE CFTR PM STABILITY

Pharmacological Chaperones and Chemical Chaperones

Pharmacological chaperones affect the CFTR PM stability by direct stabilization. CFTR corrector VX-809 is the first food and drug administration (FDA) approved CFTR corrector in combination with VX-770/ivacaftor (known as Orkambi). VX-809 selectively improves the processing of misfolded CFTR by stabilizing NBD1-membrane spanning domain (MSD) interface but not other misfolded proteins such as human ether-à-go-go-related gene (hERG) mutants (Van Goor et al., 2011; Okiyoneda et al., 2013; Ren et al., 2013; Farinha et al., 2015). VX-809 repairs not only the CFTR folding defect at the ER but also the CFTR PM instability. VX-809 washout prolongs ΔF508-CFTR functional sustainability (Van Goor et al., 2011), suggesting that improvement of the CFTR folding at the ER could increase the thermal stability and proper co- and/or post-translational modifications that renders CFTR more energetic robust conformations even at the PM. VX-809 also promotes ΔF508-CFTR and NHERF1 interaction, that may increase the PM stability (Arora et al., 2014). C3 (CFcor-325/VRT-325) and C4 (Corr-4a) also extend rΔF508-CFTR PM stability in CFBE cells probably by directing binding (Wang et al., 2007; Varga et al., 2008) although their effect could be not specific to the conformationally defective CFTR (Van Goor et al., 2011). Chemical chaperones such as glycerol also increases the rΔF508-CFTR PM stability probably by non-specifically improving the conformational stability (Okiyoneda et al., 2013).

CFTR Potentiators

The first FDA approved CFTR potentiator VX-770 improves the gating defect of some CFTR mutants. However, chronic VX-770 treatment destabilizes the PM rΔF508-CFTR in CFBE and ΔF508 homozygous CF patient HBE (CF-HBE) cells (Cholon et al., 2014; Veit et al., 2014). Importantly, chronic VX-770 treatment diminishes the VX-809 therapeutic efficacy by stimulating the elimination of PM rΔF508-CFTR (Cholon et al., 2014; Veit et al., 2014). In addition to VX-770, several CFTR potentiators including P1 (VRT-532) and P2 (PG-01) also decrease the rΔF508-CFTR PM stability (Veit et al., 2014). VX-770 and other potentiators could destabilize a variety of CFTR rare mutants referred to as CFTR2 mutants including E92K and L1077P at the PM (Avramescu et al., 2017). Thus, several CFTR potentiators may decrease the thermal stability of metastable mutant CFTR at the PM by inducing conformational change that positively affects for channel gating but negatively affects stability. High-throughput screening has identified several novel CFTR potentiators such as class A analog 4 (A04) and class P analog 12 (P12) that could not destabilize the PM rΔF508-CFTR (Phuan et al., 2015).

Proteostasis Regulating Drugs

Proteostasis regulating drugs that affect array of proteins regulating CFTR folding and QC also affect the CFTR PM stability. Histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) alters expression of a subset of CF-interacting gene products (e.g., chaperones and DAB2) and sustains PM expression of ΔF508-CFTR in CFBE cells (Hutt et al., 2010). Tissue transglutaminase (TGM2) inhibitor cystamine also stabilizes ΔF508-CFTR at the PM of airway epithelial cells by restoring BECN1 interactome which is sequestrated by CFTR dysfunction (Luciani et al., 2012; Villella et al., 2013). MLK3 pathway inhibitor oxozeaenol has been reported to be effective in correcting the ΔF508-CFTR proteostasis defect in the primary HBE cells (Trzcinska-Daneluti et al., 2012). Oxozeaenol could stabilize ΔF508-CFTR at the PM as MLK3 KD reduces mature ΔF508-CFTR elimination by PQC (Hegde et al., 2015).

Cavosonstat and CAL Inhibitor

HSP70/HSP90 Organizing Protein is adaptor protein which coordinates HSP70 and HSP90 function in protein folding and regulates CFTR maturation and PM stability (Odunuga et al., 2004; Okiyoneda et al., 2010). HOP *S*-nitrosylation by *S*-nitrosoglutathione (GSNO) induces HOP degradation and increases ΔF508-CFTR PM expression (Marozkina et al., 2010). Levels of *S*-nitrosothiols such as GSNO are low in CF airway (Grasemann et al., 1999) and *S*-nitrosothiol decreases the internalization rate of rΔF508-CFTR in HBE cells (Grasemann et al., 1999, 2000; Zaman et al., 2014). Cavosonstat (N91115) is an orally bioavailable inhibitor of GSNO reductase and restores GSNO levels (Donaldson et al., 2017). Cavosonstat is the first CFTR stabilizer in phase II trials, but it was not beneficial for improvement of lung function in combination with ivacaftor.

CFTR-associated ligand binds CFTR via a PDZ interaction domain and targets CFTR for lysosomal degradation (Cheng et al., 2004). CAL inhibition increases the PM stability of ΔF508-CFTR (Cushing et al., 2010) and cell penetrating CAL inhibiting peptide is established (Qian et al., 2015). CAL inhibitor has been developed as a cell surface CFTR stabilizer in pre-clinical level while its therapeutic efficacy and conformational selectivity remain unclear.

Ub Ligase Inhibitors

RING finger protein 5 (RNF5/RMA1) is an ER associated E3 Ub ligase that regulates early stage CFTR proteostasis at the ER (Younger et al., 2006). A RNF5 inhibitor Inh-2 identified by homology modeling and virtual ligand screening causes

significant rescue of ΔF508-CFTR in immortalized and primary HBE cells from CF patients (Sondo et al., 2018). Intriguingly, Inh-2 modestly increases mature ΔF508-CFTR half-life and this stabilization effect is further improved by VX-809. While the contribution of RNF5 in the CFTR peripheral QC remains unclear, RNF5 inhibitor may be useful to overcome the CFTR instability.

Currently, CHIP and RFFL are the only Ub ligases responsible for the CFTR peripheral QC (Okiyoneda et al., 2010, 2018). Thus, inhibiting their activity could selectively reduce the ubiquitination and elimination of unfolded CFTR from the PM, improving the limited efficacy of CF combination therapy. CHIP binds and regulates a number of substrates via chaperones (Connell et al., 2001). Moreover, inhibiting the CHIP activity induces deleterious effect as the CHIP KO mice result in the abnormal phenotypes including ataxia and pre-mature death[1]. In contrast, RFFL could bind and regulate a limited number of substrates because of its nature of direct binding to the CFTR through the disordered regions (Okiyoneda et al., 2018). More importantly, inhibiting the RFFL activity seems to have no venomousness since the RFFL KO mice exhibit no abnormal phenotype (Ahmed et al., 2009). Therefore, counteracting RFFL activity may provide a preferable therapeutic approach as a CFTR stabilizer that is a class of drugs that extends the PM resident time of CFTR class VI mutants. Although future studies are needed to validate the impact on ΔF508-CFTR in CF-HBE cells, developing agents selectively inhibiting RFFL-mediated CFTR ubiquitination may help improve the efficacy of CF pharmacological therapy.

[1] http://www.informatics.jax.org/marker/phenotypes/MGI:1891731

CONCLUSION AND PERSPECTIVE

Beside the progresses of CF pharmacological therapy, stabilizing the cell surface CFTR remains challenging and is necessary to improve the limited therapeutic efficacy. Recent studies have revealed some of the CFTR PQC mechanism eliminating the functional but conformationally defective CFTR from the PM. Understanding the CFTR PQC mechanism help the development of the CFTR stabilizer, a novel class of CFTR modulator necessary to establish the robust CF pharmacological therapy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by JSPS KAKENHI grant numbers JP25893275, 15H05643, and 15H01192.

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Conflict of Interest Statement: TO has a patent pending in Japan for methodology to identify inhibitors of RFFL-mediated CFTR ubiquitination (2017-047626).

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 04 June 2019
doi: 10.3389/fphar.2019.00619

Network Biology Identifies Novel Regulators of CFTR Trafficking and Membrane Stability

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In cystic fibrosis, the most common disease-causing mutation is F508del, which causes not only intracellular retention and degradation of CFTR, but also defective channel gating and decreased membrane stability of the small amount that reaches the plasma membrane (PM). Thus, pharmacological correction of mutant CFTR requires targeting of multiple cellular defects in order to achieve clinical benefit. Although small-molecule compounds have been identified and commercialized that can correct its folding or gating, an efficient retention of F508del CFTR at the PM has not yet been explored pharmacologically despite being recognized as a crucial factor for improving functional rescue of chloride transport. In ongoing efforts to determine the CFTR interactome at the PM, we used three complementary approaches: targeting proteins binding to tyrosine-phosphorylated CFTR, protein complexes involved in cAMP-mediated CFTR stabilization at the PM, and proteins selectively interacting at the PM with rescued F508del-CFTR but not wt-CFTR. Using co-immunoprecipitation or peptide–pull down strategies, we identified around 400 candidate proteins through sequencing of complex protein mixtures using the nano-LC Triple TOF MS technique. Key candidate proteins were validated for their robust interaction with CFTR-containing protein complexes and for their ability to modulate the amount of CFTR expressed at the cell surface of bronchial epithelial cells. Here, we describe how we explored the abovementioned experimental datasets to build a protein interaction network with the aim of identifying novel pharmacological targets to rescue CFTR function in cystic fibrosis (CF) patients. We identified and validated novel candidate proteins that were essential components of the network but not detected in previous proteomic analyses.

Keywords: CFTR, interactome, membrane traffic, network biology, plasma membrane

OPEN ACCESS

Edited by:
Anthony Kicic,
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *11 March 2019*
Accepted: *15 May 2019*
Published: *04 June 2019*

Citation:
Loureiro CA, Santos JD, Matos AM, Jordan P, Matos P, Farinha CM and Pinto FR (2019) Network Biology Identifies Novel Regulators of CFTR Trafficking and Membrane Stability. Front. Pharmacol. 10:619. doi: 10.3389/fphar.2019.00619

INTRODUCTION

CFTR is a polytopic integral membrane protein that belongs to the ATP-binding cassette (ABC) transporter superfamily functioning as cAMP- and phosphorylation-regulated chloride and bicarbonate at the apical membrane of epithelial cells. Mutations in the *CFTR* gene cause cystic fibrosis, the most common autosomic recessive disorder among Caucasians (Riordan, 2008). The most prevalent CF-causing mutation is F508del, which is present in about 90% of CF patients and generates a mutant protein recognized by endoplasmic reticulum (ER) quality control (ERQC)

mechanisms (Farinha and Amaral, 2005; Farinha et al., 2013; Farinha and Canato, 2017) and targeted for proteasomal degradation (Jensen et al., 1995).

Although most F508del-CFTR protein is only detected as a non-native (misfolded) core-glycosylated ER-specific intermediate, a small amount reaches the plasma membrane (PM) and is partially functional as a channel (Dalemans et al., 1991; Swiatecka-Urban et al., 2005). Thus, one important focus of basic research in CF involves attempts to rescue F508del-CFTR to the PM, by identifying either the key molecular factors involved in its ER retention or small molecules that promote its folding and release from the ERQC. Recent advances in the field allowed the identification of small molecules with potential to treat the basic defect in CF—either correctors, promoting the relocation of F508del-CFTR from the ER to the PM or potentiators, promoting channel activity (for variants already located at the PM). Among these, the potentiator VX-770 alone or its combinations with the correctors VX-809 or VX-661 made their way to the clinical practice, securing approval from the EMA and the FDA. However, results of these modulators in patients bearing F508del suggest that a physiologically relevant restoration of function will require additional small molecules with distinct corrective properties (Farinha and Matos, 2016). This may be due to the fact that F508del-CFTR, despite its partial pharmacological rescue of intracellular retention and impaired channel regulation, still presents decreased stability at the PM (Sharma et al., 2004; Swiatecka-Urban et al., 2005). Whereas the retention and the gating defects have been extensively studied, and both small-molecule correctors and potentiators are available, CFTR membrane stability is a less understood aspect, albeit crucial to restore F508del-CFTR to levels that correspond to clinical benefit.

CFTR traffic and activity at the PM is the result of numerous interactions that function as molecular switches (Amaral, 2005; Farinha et al., 2013) modulating channel activity and controlling the number of channel present at the membrane, through regulation of the secretory and membrane recycling pathways. This complex network of proteins is the last step in the control of the overall biogenesis/function of the protein and includes other transporters, channels, and trafficking machinery components (Rab GTPases, SNAREs, PDZ-domain-containing proteins) as well as different types of molecular switches (such as kinases, phosphatases, and small GTPases) (Guggino and Stanton, 2006; Farinha et al., 2013; Farinha and Canato, 2017).

Several mechanisms have been described to regulate CFTR anchoring and trafficking at the PM, which include CFTR phosphorylation by different kinases, interaction with cAMP sensors, or response to small GTPase signaling.

Phosphorylation in the regulatory region of CFTR is known for long to be required for its activity and it involves protein kinases A (PKA) and C (PKC) (Schultz et al., 1999). Together with PDZ-domain containing proteins, phosphorylation is responsible for the formation of multiprotein signaling complexes that provide spatial and temporal specificity to CFTR function (Guggino and Stanton, 2006). Besides this well-documented role on function, phosphorylation by spleen tyrosine kinase (Syk) in NBD1 (Luz et al., 2011; Mendes et al., 2011) or by lemur tyrosine kinase 2 LMTK2 in the R region (Luz et al., 2014) was shown to control the levels of CFTR at the PM.

cAMP levels in the cell lead to PKA activation and hence promote CFTR function, as described above. cAMP also activates members of guanine nucleotide-exchange factors, called EPACs (exchange proteins directly activated by cAMP), whose activation does not lead to PKA activation (Bos, 2006). Activation of EPAC1, the most abundant EPAC family member expressed in the lung, promotes its interaction with CFTR, leading to its stabilization at the PM through interaction with the PDZ-containing anchor protein NHERF-1 (Lobo et al., 2016).

Overexpression of NHERF1 promotes apical expression of the F508del-CFTR mutant channel (Guerra et al., 2005). This effect relies on the activation of endogenous RAC1 signaling and may also contribute to the increase of F508del-CFTR PM levels (Moniz et al., 2013; Loureiro et al., 2015).

Here, we used network biology to identify relevant protein nodes that may connect the interactomes obtained by analyzing different mechanisms regulating CFTR stability, namely, through phosphorylation by SYK or activation of EPAC1 or RAC1. We focused the network analysis on proteins never described to interact with or regulate CFTR and show that this approach is a valid strategy for finding novel regulators of CFTR function and traffic. Network biology thus proves to be a tool capable of contributing insights into the etiology of other human diseases linked to chloride transport or membrane protein traffic.

MATERIALS AND METHODS

Protein Interaction Networks

Human physical protein–protein interaction data were extracted from HuRI (Human Reference Protein Interactome Mapping Project) (interactome.baderlab.org) (Rual et al., 2005; Venkatesan et al., 2009; Yu et al., 2011; Rolland et al., 2014; Yang et al., 2016) and APID (Agile Protein Interaction DataAnalyzer) (apid.dep.usal.es) (Alonso-Lopez et al., 2016) databases (accessed in June 2018). APID gathers literature-reported protein interactions, while HuRI results from unbiased large-scale screens for binary interactions. We constructed undirected and unweighted networks using Igraph R-package (Csardi and Nepusz, 2006). Loop and multiple edges were eliminated and only the main component of the network was selected. The network was further filtered to contain only proteins that are not exclusively located in nucleus, mitochondria, or peroxisomes. Gene ontology cellular component annotations obtained from UNIPROT were used to define protein subcellular location. This filter was necessary since the relevant interactions affecting CFTR stability at the PM should not occur within these three cellular compartments. The resulting operational network contained 17,218 proteins and 252,472 interactions.

Network Analysis

Candidate proteins were selected through a neighbor enrichment test and a bridge score. The neighbor enrichment test evaluates if the set of direct neighbors of a candidate protein is enriched in members of an input protein set using a hypergeometric test. The bridging score quantifies for every node in a network its

relevance in connecting two other nodes (provided as inputs) through short paths of length 2, 3, or 4. It counts the fraction of paths of length 2, 3, or 4 linking the two input nodes that contain a candidate node. These fractions for each length are used in a weighted sum where paths of length 2 have weight 1, paths of length 3 have weight 0.5, and paths of length 4 have weight 0.25. Both methods are implemented as R functions (*specific_neighbors* and *bridge_score*, respectively) publicly available in the NetSetStat repository (github.com/GamaPintoLab/NetSetStat). Proteins selected through both methods were analyzed for biological process enrichment using the DAVID tool (Huang Da et al., 2009a; Huang Da et al., 2009b) with the *Homo sapiens* genome as background and testing terms from the FAT subset of the Biological Process branch of Gene Ontology. Enriched GO terms were considered significant with a False Discovery Rate lower than 0.05 and were excluded if their background frequency was greater than 10% or lower than 5%.

Preparation of siRNA-Coated 96-Well Plates

Two siRNAs against each of the following targets (CUL3, GABARAP, GABARAPL1, GABARAPL2, ILK, IQGAP1, LRRK2, NF2, NOS2, SMURF1, and UBASH3B) were acquired as a cherry-pick siGenome library (Dharmacon, Cambridge, UK); 96-well plates were coated with customized siRNAs for solid-phase reverse transfection. For that, a transfection mixture of 300 µl was prepared containing 161.5 µl of the Lipofectamine 2000 (Invitrogen #11668019) and 138.5 µl of 0.4 M sucrose in OptiMEM solution. Then, 1.25 µl of siRNA 20 µM was added per well into 96-conic well plates (siRNA plate); 1.75 µl of transfection mix was added per well to siRNA plate, followed by mixing and centrifugation for 1 min at 50×*g*. After 30 min incubation at room temperature, 1.75 µl of 0.2% (w/v) gelatin solution was added to the siRNA plate and centrifuged at 50×*g*. Then, the total of 4.25 µl of the mix in siRNA plate (siRNA plus transfection mix plus gelatin) was diluted 1:50 in a 96-conic well plate using double-distilled water. Finally, 50 µl from the diluted mix was lyophilized and stored in an anhydrous atmosphere before cell seeding.

Reverse Transfection With siRNA

CFBE cells expressing an inducible doxycycline promotor for mCherry-Flag-CFTR (kind gift of Prof. Margarida Amaral, Lisbon) were grown to confluence and split. Then, after 24 h, cells were seeded in a siRNA-coated 96-well plate (100 µl of cell suspension/well, 7×10^7 cells/well) using a Multidrop™ Combi Reagent Dispenser (Thermo Scientific #5840300). After 24 h of reverse transfection, CFTR expression was induced for 24 h for wt-CFTR and 48 h for F508del-CFTR, using 1 µg/ml doxycycline (Sigma #9891) in antibiotic-free medium.

CFTR Trafficking Assay

The CFTR trafficking assay was performed as previously documented (Almaca et al., 2011; Botelho et al., 2015). This assay uses a double-tagged version of CFTR with mCherry at the N-terminus and FLAG at the fourth extracellular loop. Usage of the mCherry tag allows for quantification of the total amount of CFTR protein expressed by each individual cell. The Flag tag allows one to quantify CFTR that is exclusively localized at the cell surface by usage of an antibody applied extracellularly without cell permeabilization. Hence, 72 h after seeding and siRNA knockdown, cells were washed once with cold $PBS^{+/+}$ (1× PBS supplemented with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2) using Tecan Hydrospeed™ and immunostained for the extracellular Flag-tag (in non-permeabilized cells) for 1 h at 4°C with 1:500 anti-Flag® M2 monoclonal antibody (Sigma-Aldrich #F1804). Then, cells were washed three times in ice with cold $PBS^{+/+}$ and incubated 20 min with 3% (w/v) paraformaldehyde (PFA) at 4°C. Cells were then washed three times in ice with cold $PBS^{+/+}$ and incubated with anti-mouse Cy5 conjugated secondary antibody (Invitrogen, #A10524). Cells were washed three times in ice with cold $PBS^{+/+}$ and incubated for 1 h with 1:5000 Hoechst 33342 solution (Sigma #B2261) for nucleus staining. Finally, cells were washed in ice with cold $PBS^{+/+}$ and immersed in $PBS^{+/+}$. All solutions were prepared in $PBS^{+/+}$ and primary and secondary antibodies were diluted in $PBS^{+/+}$ supplemented with 1% (v/v) BSA.

Image Acquisition

Cell imaging was performed at room temperature with an inverted wide-field fluorescence microscope Leica DMI6000 equipped with a 12-bit 1,344 × 1,024 pixel resolution DFC360FX camera (Leica) and a 10× objective. Exposure times at maximum light brightness for Hoechst, mCherry, and Cy5 were 50, 1,300, and 8,000 ms, respectively. The Hoechst channel was used for contrast-based autofocus. The experiment was conducted in triplicate and five images per well were collected.

Image Analysis

Automatic image analysis was performed using open-source software tools [cell image analysis software CellProfiler[1] (Carpenter et al., 2006) and R[2]] and a pipeline to measure CFTR traffic efficiency developed previously (Botelho et al., 2015). Using this pipeline, the background subtraction was first applied to each image to correct the image illumination and background fluorescence. Then, a quality control was also applied for each image to exclude cells that do not significantly express CFTR, present abnormal morphology, or contain a significant number of saturated pixels. Finally, CFTR traffic was measured in each cell using the fluorescence quantification according to the following formula:

$$\text{CFTR Traffic Efficiency} = \frac{\text{PM CFTR}}{\text{Total CFTR}} = \frac{\text{Cy5 Integrated Fluorescence}}{\text{mCherry Integrated Fluorescence}} \quad \text{(Formula 1)}$$

CFTR traffic efficiency was calculated using the median CFTR traffic efficiency for all cells in the image. Using this pipeline, we

[1] CellProfiler, http://cellprofiler.org/ (last accessed October 19, 2017).
[2] R program, https://www.r-project.org/ (last accessed March 09, 2018).

also remove out-of-focus images, images with high background fluorescence, and images with a lower number of cells (less than 20 cells). Then, the average of CFTR traffic efficiency for all images in wells transfected with siRNAs, which passed the quality control (traffic efficiency test), was compared with the one measured under control conditions—siRNA EGFP (Traffic $\text{Efficiency}_{\text{control}}$) according to the following formula:

$$\text{Deviation Score} = \frac{\text{Traffic Efficiency}_{\text{test}} - \text{Traffic Efficiency}_{\text{control}}}{2 \times \text{SEM}_{\text{control}}} \text{ (Formula 2)}$$

The standard error of the mean for the Traffic Efficiency is given by the $\text{SEM}_{\text{control}}$ recorded for siRNA EGFP. Deviation scores (DSs) that are positive or negative correspond to traffic enhancers or inhibitors, respectively. The DS equation normalizes the Traffic Efficiency measures to the mean and variation of the negative controls included in the same 96-well plate. This normalization facilitates the comparison between independent replicates, as all values are relative to the mean and standard error of the mean (SEM) of the negative controls in the respective plate. A DS of 1 is the double of the negative control SEM, which means that the latter has a normalized value of 0.5.

Analysis of siRNA Knock-Down Efficiency

CFBE cells constitutively expressing F508del-CFTR were seeded in 96-well dishes and transfected with the panel of siRNAs described above (Dharmacon, Cambridge, UK), using Lipofectamine 2000 (Invitrogen). All cells were incubated for 48 h at 37°C with 5% CO_2 and then RNA was extracted with the NucleoSpin RNA kit (Macherey Nagel, Düren, Germany). For cDNA synthesis, 0.5 µg of total RNA was then reverse transcribed using random primers (Invitrogen/Life Technologies) and Ready-to-Go You-Prime beads (GE Healthcare, Buckinghamshire, UK). Real-time PCR quantification (qPCR) was performed as previously described (Matos et al., 2008). Primers used in qPCR for each of the targeted transcripts are described in **Table 1**. Each cDNA sample was diluted fivefold to guarantee accurate pipetting and 5 µl was added to each real-time reaction together with 250 nM primers and SYBR Green Master Mix (Applied Biosystems/Life Technologies). Data were analyzed with the ABI Prism 7000 SDS 1.1 RQ Software (ΔΔCT method, Applied Biosystems/Life Technologies).

CFTR Functional Assay by Halide-Sensitive YFP (HS-YFP)

CFBE cells constitutively expressing wt- or F508del-CFTR together with HS-YFP-F46L/H148Q/I152L (Galietta et al., 2001) were seeded in eight-well chamber slides. F508del-CFTR cells were seeded in duplicate slides. Cells were then transfected with either siLUC (Eurofins MWG Operon), siGABARAP_1, siSMURF1_2, or siENOS_2 (Dharmacon) using Lipofectamine 2000 (Invitrogen). All cells were incubated for 48 h and VX-809 (3 µM; Gentaur) was added to one of the F508del-CFTR cell sets. Cells were carefully washed twice with isomolar PBS (WPBS: 137 mM NaCl, 2.7 mM KCl, 0.7 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4), and wt-CFTR cells were incubated for 15 min in WPBS containing 1 µM indomethacin to reduce endogenous cAMP levels. Cells were then transferred to a Leica TCS-SPE confocal microscope for time-lapse analysis. Each well was assayed individually for iodide influx by recording fluorescence continuously (500 ms per point), first for 10 s (baseline) and then for 60 s after the rapid (≤1 s) addition of isomolar PBS, containing either 5 µM Forskolin (Fsk) (wt-CFTR cells) or 5 µM Fsk plus 20 µM Gen (F508del-CFTR cells), in which 137 mM Cl^- was replaced by I^- (IPBS; final NaI concentration of 100 mM/plate well). Cells were kept at 37°C up until being assayed at room temperature. After background subtraction, HS-YFP fluorescence recordings (F) were normalized to the initial average value measured before addition of I^- (F_0). Quantification of fluorescence decay was performed on at least 24 individual cells per well, using ImageJ (NIH) as previously described (Matos et al., 2018). The average fluorescence decay was fitted to an exponential decay function to derive the maximal slope $[d(F/F_0)/dt$, at $t = 0]$ that is proportional to the initial influx of I^- into the cells. (Loureiro et al., 2015).

Protein Thermal Destabilization Assay (Thermal Shift Assay, TS)

After Dox-induced CFTR expression (1 µg/ml; Sigma-Aldrich), mCherry-Flag-F508del-CFTR CFBE cells were transfected with either siLUC (Eurofins MWG Operon) or siGABARAP_1 (Dharmacon), using Lipofectamine 2000. Cells were then incubated for 24 h with VX-809 (3 µM; Gentaur) at 37°C and then moved for another 24 h to 30°C, to increase the thermal stability of rescued F508del-CFTR

TABLE 1 | Primers used for qPCR quantification of siRNA efficiency.

Gene name	Transcript_id	Forward primer sequence	Reverse primer sequence
CUL3	NM_003590.4	ACAGCTATGGTGATGATTAGAGAC	TACGACTTCTCCTTTCCGCT
GABARAP	NM_007278.1	GGCTCCCAAAGCTCGGATAG	GCAGCTTCACAGACCGTAGA
GABARAPL1	NM_001363598	TGCCTGATCTGGACAAGAGG	AACGCATCTAGAACAAGGGCT
GABARAPL2	NM_007285.6	TGTTCAAGGAGGACCACTCG	CCACAAACAGGAAGATCGCC
ILK	NM_004517.3	GCTTGGGGTTCATCCTCCTT	TTACATTGATCCGTGCCCCC
IQGAP1	NM_003870.3	GCACTGGCTAAGACGGAAGT	CGGATAGCACGTCTCTGCAT
LRRK2	XM_005268629.4	GGCCCTCCTCACTGAGACTA	TGCATCAGCATGGAGAGCAT
NF2	NM_181832.2	AGAGGAGCTGGTTCAGGAGA	GCCAAAAATCCCCGCTTGTG
NOS2	NM_000625.4	CCTCGCTCTGGAAAGACCAG	GGGACAGGACGTAGTTCAGC
SMURF1	NM_181349.2	CGTGGGGAAGAAGGTTTGGA	AAGCCCCCGTTGATGTAGTG
UBASH3B	NM_032873.4	TGGACGTGCTCCTCTCCAT	GGGGAAGATGTTGTGTGCCT

at the PM and allow its accumulation at the cell surface. For the thermal shift assay, cells were again transferred to 37°C for 4 h to induce F508del-CFTR thermal destabilization and PM removal, as described previously (Okiyoneda et al., 2010). Cells were then placed on ice, washed three times with ice-cold WPBS, and left 5 min in cold WPBS to fully arrest CFTR trafficking. Afterwards, they were incubated on ice with anti-Flag M2 Ab (F3165, Sigma-Aldrich) in WPBS for 1 h at 4°C, without permeabilization. Cells were washed three times with ice-cold WPBS and incubated with anti-mouse Alexa Fluor 488 secondary Ab (Thermo Fisher Scientific) for 1 h at 4°C. Cells were then fixed with 4% formaldehyde for 15 min, thoroughly washed in PBS containing DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich), mounted on microscope slides with Vectashield (Vector Laboratories), and analyzed by confocal microscopy.

Confocal Microscopy Imaging

Slides of mCherry-Flag-F508del-CFTR CFBE cells immunolabeled with anti-Flag followed by Alexa Fluor 488 were analyzed by confocal microscopy. Images were acquired on a Leica TCS-SPE confocal microscope, where mCherry fluorescence is proportional to the total amount of CFTR, Alexa Fluor 488 fluorescence is proportional to the amount of CFTR present at the cell surface, and nuclei were stained with DAPI. A 0.2-µm Z-stack of 1-Airy confocal images was acquired to select the best *XY* plane to analyze the CFTR protein at the cell surface. ImageJ software (NIH) was then used to delimitate each single cell as individual ROIs (regions of interest, to allow measurement of mCherry and Alexa488 fluorescence signal intensities). Quantification of fluorescence was performed on at least 24 individual cells per field, as previously described (Matos et al., 2018), and expressed as mean ± SEM fold variation over control cells.

Statistical Analysis

Multiple treatment data analysis—Statistical significance of the observed variations between different treatments was assessed using two-tailed Kruskal–Wallis tests, followed by paired comparisons using the Mann–Whitney *U* test, considering a 0.05 significance level for both tests. All observations were confirmed in at least three independent experiments, each with two replicates for each individual condition tested.

Statistical analysis of CFTR trafficking assay—Each siRNA was used to measure traffic efficiency of wild-type CFTR, F508del-CFTR, and F508del-CFTR rescued with VX-809. For each siRNA and CFTR type, three independent replicate DSs were obtained. The DS mean was considered a measure of the siRNA capacity to influence the traffic efficiency of a particular CFTR type ($<DS_i>$, i corresponding to CFTR type), and the sum of the three DS mean absolute values ($\Sigma|<DS_i>|$) was considered a global measure of the siRNA effect. To evaluate statistical significance of these effects, a random permutation test was performed, where the data matrix containing all the individual DS (all replicates for all CFTR types and all siRNAs, including siRNAs without knockdown effect, functioning as additional controls) was randomly shuffled 10,000 times. Full matrix permutation is possible because all DS values are normalized by EGFP control traffic efficiency mean and SEM values. For each random matrix, each siRNA random $\Sigma|<DS_i>|$ was computed, generating distributions according to the null hypothesis that siRNAs have no effect. Null hypotheses were rejected when the fraction of random values greater than or equal to the observed $\Sigma|<DS_i>|$ was lower than 0.05.

RESULTS

Network Connectivity between CFTR Interactors Potentially Modulating Stability at the PM

Studies from our team have identified five sets of CFTR interactors through three parallel approaches (under consideration elsewhere): a) proteins selectively interacting at the PM with either wt-CFTR (set 1: wt) or rescued F508del-CFTR (set 2: rescued), b) protein complexes involved in cAMP-mediated CFTR stabilization (set 3: EPAC), and c) adaptor proteins binding to tyrosine-phosphorylated (set 4: pep-py) or un-phosphorylated (set 5: pep-wt) CFTR peptide (unpublished data). All these complementary approaches were designed to identify potential modulators of CFTR PM stability through different pathways. We hypothesized that proteins simultaneously interacting with these different pathways may also have a significant influence on CFTR stability at the PM.

First, we mapped these sets of interactors on a human protein physical interaction network and explored how strongly they were connected through this network (**Table 2**). To evaluate the statistical significance of network connectivity, we had to consider that these interactor sets were selected by their presence in immunoprecipitated CFTR complexes. Therefore, we generated 500 random sets of interactors (for each of the five observed sets), with the same number of proteins at each observed network distance from CFTR. For each connectivity property (number of overlapping proteins between sets, number of direct interactions between set members, and number of common direct neighbors between sets), we recorded the average value across the 500 randomizations and a *p* value, which corresponds to the fraction of random set pairs showing a value higher than that observed with the experimental sets. The five sets have a low number of proteins in common, although 8 out of 10 pairs have a significantly higher overlap than expected by chance. The strong connectivity between these CFTR interactor sets is much more evident by looking at the numbers of direct physical interactions and common neighbors that are much higher than randomly expected and reach statistical significance for all set pairs. This strong connectivity supports our attempt to find new proteins simultaneously interacting with multiple CFTR PM stability-related pathways.

Specific Common Neighbors of CFTR Interactor Sets

We have identified 4411 proteins that have direct physical interactions with proteins in at least two CFTR interactor sets (2,420, 1,161, and 443 with direct neighbors in at least 3, 4, and 5 sets, respectively). However, to increase the likelihood that perturbations in one of these proteins will have an effect in these

TABLE 2 | Overlap, direct interactions, and common neighbors between CFTR interactor sets.

CFTR interactor sets		Overlap		Direct interactions		Common neighbors	
		Observed (expected)	*p*	Observed (expected)	*p*	Observed (expected)	*p*
wt	rescued	7 (1.2)	<0.002	183 (48.1)	<0.002	2,092 (1,244)	<0.002
	EPAC	3 (0.4)	0.004	54 (16.9)	<0.002	1,138 (692)	0.006
	pep-py	2 (0.3)	0.024	82 (15.3)	<0.002	1,535 (661)	<0.002
	pep-wt	0 (0.2)	0.547	28 (5.6)	<0.002	888 (310)	0.002
Rescued	EPAC	8 (1.1)	<0.002	164 (44.8)	<0.002	1,818 (1,191)	0.006
	pep-py	6 (0.9)	0.002	359 (41.8)	<0.002	2,300 (1,153)	<0.002
	pep-wt	3 (0.3)	0.002	113 (15.3)	<0.002	1,364 (515)	<0.002
EPAC	pep-py	1 (0.2)	0.117	57 (14.0)	<0.002	1,140 (636)	0.002
	pep-wt	2 (0.1)	0.002	23 (5.2)	<0.002	758 (301)	0.008
pep-py	pep-wt	4 (0.1)	<0.002	94 (5.0)	<0.002	1,473 (294)	<0.002

specific neighbor pathways and, consequently, on CFTR PM stability, we should require that these proteins are enriched in direct interactions with these CFTR interactor sets. With that aim, we performed a hypergeometric test to determine if these proteins are enriched in interactions with each of the five sets. As we performed this test to all proteins with neighbors in each of the five interactor sets, choosing a trivial *p* value cutoff may be associated with many false positives. To avoid this multiple testing problem, we selected for each set of CFTR interactors the 5% proteins with the lowest hypergeometric *p* values as specific neighbors of that set, and additionally required the selected proteins to have at least three interactions with two or more CFTR interactor sets. This strategy resulted in the selection of 478 proteins. We ranked these proteins by summing the ranks of their neighbor enrichment *p* values for all sets, discarding sets where the protein was not in the top 5% of significantly enriched proteins (protein list and associated statistics are available in **Supplementary Table 1** and the selection process is depicted in **Figure 1**).

Important Mediators of CFTR Interaction With EPAC, NHERF1, EZRIN, and SYK

The CFTR interactor sets were experimentally detected under conditions that modulate CFTR PM stability through the influence of factors such as EPAC, NHERF1, EZRIN, and SYK, previously known to be involved in the stabilization process (Mendes et al., 2011; Loureiro et al., 2015; Lobo et al., 2016). To uncover novel proteins that most likely influence CFTR PM stability, we aimed to quantify the contribution of a given protein to interfere (either positively or negatively) with the formation of complexes including CFTR and one of the referred stability factors.

For this purpose, we developed a bridge score that measures the fraction of short length paths (with two to four interactions) connecting two proteins of interest (CFTR and the stability factor) that are mediated by a candidate protein. Mediation of shorter paths is given a higher weight/score. The perturbation of proteins with high bridge scores will disrupt a higher fraction of short network paths linking the two proteins of interest. If the interactions in these paths stabilize or facilitate the assembly of the complex containing the two proteins of interest, the bridging protein may have a positive effect on the functional interaction between the two proteins. Reversely, if the interactions in the short paths compete with the formation of the complex, the bridging protein may negatively affect the functional interaction between the two proteins.

To compute the bridge score, we assembled a connected subnetwork containing CFTR, the four stability factors, and the common neighbors identified in the previous section (process described in **Figure 1**). The resulting subnetwork contained 482 proteins and 16,481 interactions. We considered a protein to be an important mediator between CFTR and one stability factor if its bridge score was in the top 25% of all scores with the corresponding stability factor. This procedure allowed the identification of 194 strong mediator proteins (**Supplementary Table 1**). Gene ontology analysis (**Supplementary Table 2**) showed that the two biological processes most significantly enriched within these 194 proteins were "protein localization to organelle" (GO:0033365, 54 proteins) and "regulation of protein modification process" (GO:0031399, 71 proteins), which supports their hypothetical role in the modulation of CFTR PM stability.

Hit Selection

To assess the impact of protein networks in CFTR trafficking, we selected hits for validation from the list of 478 proteins significantly enriched in neighbors from at least two CFTR interactor sets.

From the list of specific common neighbors of CFTR interactors, we started by excluding any proteins detected in the three initial experimentally derived interactor sets. Then, we focused on the top 25% of the list (proteins with higher neighbor enrichment rank sum as defined above). Then, we used data from Human Protein Atlas and excluded proteins with low expression in the "bronchus" (keeping thus only those with medium, high, or n/a protein expression). As we were interested in assessing CFTR PM stability, we then excluded proteins with annotation as ribosomal proteins/elongation or transcription factors/HNRNP/RNA processing/nuclear proteins. The next step was to exclude proteins already detected as CFTR interactors in previous studies (Wang et al., 2006; Pankow et al., 2015). This refined our list from 478 to 44 proteins. Finally, we used literature mining and refined this list to 25%, selecting 11 possible hits for validating the proteins listed in **Table 3**.

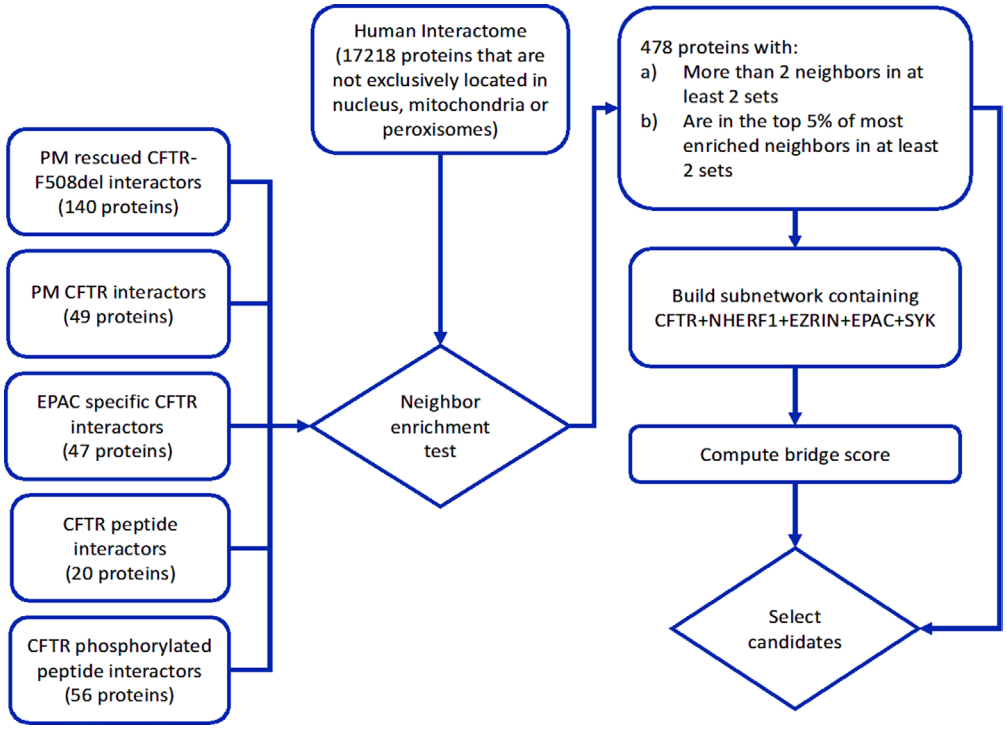

FIGURE 1 | Diagram of the network analysis procedures. CFTR interactor sets were mapped on a human protein physical interaction network, filtered for protein subcellular location to guarantee plausible interactions with CFTR. Proteins in the network were submitted to a neighbor enrichment test to select specific common neighbors of the CFTR interactor sets. A subnetwork containing the selected specific neighbors, CFTR, and related stability factors (EPAC, NHERF1, EZRIN, and SYK) was assembled and a bridge score was computed to identify strong network mediators of CFTR interaction with the referred stability factors. The ranking of neighbor specificity and bridge score were used to select candidates for experimental validation of CFTR PM stability modulation.

Assessment of CFTR Trafficking Under Knockdown of Selected Hits

We ordered two different siRNAs for each of the putative hit proteins from Dharmacon's siGenome library (see Materials and methods). We then used qPCR to assess the knockdown efficiency of the targeted transcript by each siRNA in CFBE cells (**Figure S1**). We verified that most siRNA pairs exhibited considerable differences in their ability to knock down the abundance of the mRNAs coding for the targeted proteins. Notwithstanding, we observed that the siRNAs GABARAP_1, GABARAPL2_1, IQGAP1_1, LRRK2_2, NF2_2, NOS2_2, and SMURF1_2 were able to significantly ($p < 0.05$) downregulate the hit mRNAs that they targeted (**Figure S1**), and these were chosen for further analysis on CFTR trafficking (wt-, F508del-, and F508del-CFTR rescued with VX-809). We used CFBE cell lines stably expressing a double-tagged (mCherry and FLAG) version of CFTR under the control of a doxycycline-inducible promoter so that CFTR expression could be induced 24 h after transfection with siRNA and immunofluorescence be performed at 24 h post-induction. CFTR at the PM is monitored by the Flag (Cy5) signal and total CFTR by mCherry fluorescence. CFTR trafficking is given by the ratio of PM CFTR to total CFTR (Botelho et al., 2015).

For this assay, cells were also transfected with two previously validated siRNAs targeting CFTR and COPBI (Botelho et al. 2015), as controls of the transfection efficiency and trafficking inhibition, respectively.

Results showed that the different proteins affected differently the three CFTR conditions tested (**Figure 2**). **Table 4** summarizes the top seven siRNAs that, when transfected into the three cell lines, promoted the largest increases in CFTR trafficking efficiency.

Altogether, we observed that siRNAs GABARAP_1, NOS2_2, and SMURF1_2 elicited consistent increments in the trafficking and overall PM levels of VX-809-rescued F508del-, wt-, and untreated F508del-CFTR, respectively (**Figure 2**), prompting us to analyze their impact on overall CFTR function in CFBE cells.

Knockdown of GABARAP and NOS2 Modulates wt- and VX-809 Rescued F508del-CFTR Function in Opposite Ways

To test the effect of knocking down the expression of the three selected candidates on CFTR function, we used a widely used and previously validated functional assay, based on the ability of iodide to quench the fluorescence of the halide-sensitive YFP-F46L/H148Q/I152L mutant (HS-YFP) (Loureiro et al., 2015; Matos et al., 2018). As shown in **Figure 3**, knockdown of either SMURF1, NOS2, or GABARAP had no apparent

TABLE 3 | Hits selected for validation.

UniProt identifier	Gene	Protein	Specific neighbor of	Strong mediator of CFTR with
Q9H0R8	GABARAPL1	Gamma-aminobutyric acid receptor-associated protein-like 1 (Early estrogen-regulated protein) (GABA(A) receptor-associated protein-like 1) (Glandular epithelial cell protein 1) (GEC-1)	All sets	EZRIN, NHERF1, SYK
O95166	GABARAP	Gamma-aminobutyric acid receptor-associated protein (GABA(A) receptor-associated protein) (MM46)	All sets	NHERF1
P60520	GABARAPL2	Gamma-aminobutyric acid receptor-associated protein-like 2 (Early estrogen-regulated protein) (GABA(A) receptor-associated protein-like 2) (Glandular epithelial cell protein 2)	All sets	EPAC, EZRIN, NHERF1, SYK
P35228	NOS2	Nitric oxide synthase, inducible (EC 1.14.13.39) (Hepatocyte NOS) (HEP-NOS) (Inducible NO synthase) (Inducible NOS) (iNOS) (NOS type II) (Peptidyl-cysteine *S*-nitrosylase NOS2)	All sets	NHERF1
Q5S007	LRRK2	Leucine-rich repeat serine/threonine-protein kinase 2 (EC 2.7.11.1) (Dardarin)	All sets	EPAC, EZRIN, NHERF1, SYK
P35240	NF2	Merlin (Moesin-ezrin-radixin-like protein) (Neurofibromin-2) (Schwannomerlin) (Schwannomin)	All sets	EZRIN, NHERF1
Q13418	ILK	Integrin-linked protein kinase (EC 2.7.11.1) (59 kDa serine/threonine-protein kinase) (ILK-1) (ILK-2) (p59ILK)	All sets except pep-wt	
Q9HCE7	SMURF1	E3 ubiquitin-protein ligase SMURF1 (hSMURF1) (EC 2.3.2.26) (HECT-type E3 ubiquitin transferase SMURF1) (SMAD ubiquitination regulatory factor 1) (SMAD-specific E3 ubiquitin-protein ligase 1)	All sets except wt	NHERF1, SYK
Q8TF42	UBASH3B	Ubiquitin-associated and SH3 domain-containing protein B (EC 3.1.3.48) (Cbl-interacting protein p70) (Suppressor of T-cell receptor signaling 1) (STS-1) (T-cell ubiquitin ligand 2) (TULA-2) (Tyrosine-protein phosphatase STS1/TULA2)	All sets except wt	SYK
Q13618	CUL3	Cullin-3 (CUL-3)	All sets	EZRIN, NHERF1, SYK
P46940	IQGAP1	Ras GTPase-activating-like protein IQGAP1 (p195)	All sets except pep-py	EZRIN, NHERF1, SYK

effect on HS-YFP fluorescence in untreated F508del-CFTR cells, compared to control cells (siLUC). In contrast, following VX-809 treatment of cells to allow some F508del-CFTR protein to reach the PM, the downregulation of GABARAP produced a moderate but clear effect on HS-YFP quenching (**Figure 3A**), corresponding to a significant ($p < 0.05$) 1.4-fold increase in the initial iodide influx rate, as calculated from the exponential decay fit of quantified fluorescence values (**Figure 3A**). Interestingly, whereas the downregulation of either SMURF1 or NOS2 appeared to have no significant effect on rescued F508del-CFTR, the knockdown of NOS2 produced an apparent 40% decrease in influx rate, when compared to control (siLUC) cells (**Figure 3B**). Notably, similar and statistically significant opposite effects were observed upon the downregulation of GABARAP and NOS2 in wt-CFTR cells (**Figure 3A** and **C**). Moreover, despite showing no observable consequence in the trafficking assay, the increment produced by GABARAP knockdown in these cells was clearly pronounced, triggering a 2.9-fold faster CFTR response, even after endogenous cAMP reduction by pretreatment of the cells with 1 μM indomethacin, prior to stimulation with 5 μM Fsk. Also noteworthy, the knockdown of SMURF1, which failed to produce a clear increase in wt-CFTR PM signal in the trafficking assay, produced a significant ($p < 0.01$) over twofold stimulation of the iodide influx rate in these cells (**Figure 3C**). Overall, interference with either of the three candidate proteins interfered with functional activity of wt-CFTR, but only GABARAP and NOS2 affected the clinically relevant rescued F508delCFTR, producing opposite functional outputs.

GABARAP Knockdown Increases the PM Stability of VX809-Rescued F508del-CFTR

Because the absolute increment of VX-809-rescued F508del-CFTR at the cell surface exceeded that computed for its trafficking efficiency upon downregulation of GABARAP, we asked whether the observed increase in both CFTR PM abundance and ion transport would rather reflect an improved stability of the rescued channels at the PM. To test this, we used the previously described thermal shift assay (Loureiro et al., 2015). This assay employs the mCherry-Flag-F508del-CFTR CFBE cell line used for the trafficking assay but with a different experimental procedure. First, F508del-CFTR is rescued with VX-809 and then stabilized at the cell surface by incubating the cells at 30°C for 24 h. Next, cells are returned to 37°C for 4 h, which destabilizes the rescued mutant channels causing their endocytic removal from the cell surface, unless the tested co-treatment procedure is able to delay or prevent CFTR internalization. Cells are then stained as before and analyzed by confocal microscopy. Images of the cell surface are used to quantify the fluorescence signal corresponding to the amount of CFTR at the membrane (**Figure 4A**; see Materials and methods). We used treatment with HGF as a positive control, since this growth factor was previously shown to induce the retention of VX-809-rescued F508del CFTR at the cell surface (Moniz et al., 2013; Matos et al., 2018). Indeed, co-treatment with HGF not only increased the overall PM abundance of CFTR at 30°C, as it was able to promote the surface retention of ~80% of the rescued protein, upon switching to 37°C (**Figure 4B** and **C**), corresponding to ~70% of the wt protein at this temperature

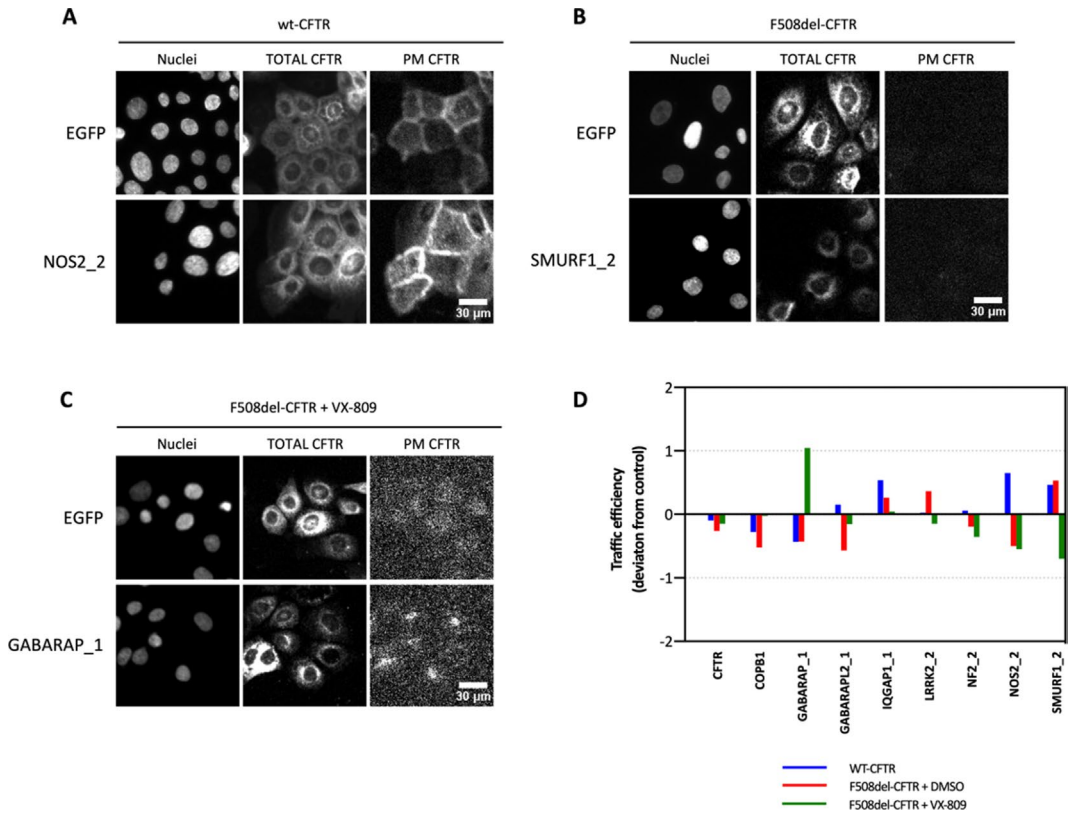

FIGURE 2 | CFTR trafficking upon knock-down of the possible hits. CFBE cell lines expressing mCherry-Flag-wt-, mCherry-Flag-F508del-, and mCherry-Flag-F508del-CFTR treated with VX-809 (3 μM for 48 h) under the control of a doxycycline-inducible promoter were transfected with siRNAs targeting CUL3, GABARAP, GABARAPL1, GABARAPL2, ILK, IQGAP1, LRRK2, NF2, NOS2, SMURF1, and UBASH3B. NT (non-treated cells) and COPB1 and EGFP (no-target siRNA) siRNA were used as controls. **(A–C)** Representative images for the three cell lines and one target for each cell line. PM CFTR image contrast was optimized after quantification to allow visualization of detected signal differences. **(D)** The CFTR traffic efficiency is obtained with the ratio PM/total CFTR with PM CFTR assessed by Flag staining (Cy5 fluorescence) and the amount of total CFTR with the mCherry signal. Data are shown as the deviation score as detailed in Formula 2, where positive values correspond to traffic efficiencies larger than the negative control (siRNA against EGFP) and negative values correspond to traffic efficiencies lower than the negative control, $n = 3$.

(**Figure 4C**). Importantly, the knockdown of GABARAP also partially prevented the internalization of rescued F508del-CFTR (**Figure 4B** and **C**). Albeit having a more moderate effect than HGF, the absence of GABARAP caused the retention of ~46% of the rescued protein at the cell surface at 37°C (~25% of wt-CFTR), which when compared to the ~30% control cells (siLUC; ~17% of wt-CFTR) reflects a ~1.5-fold increase in surface protein stability, in agreement with the observed extent of functional rescue.

DISCUSSION

In this work, we identified novel candidate proteins that are involved in the regulation of CFTR membrane traffic and/or its retention at the PM. The results were obtained based on experimental data from independent proteomic studies conducted in the host lab, namely, on PM CFTR levels following phosphorylation by SYK or the activation of EPAC1- or RAC1-mediated signaling. Subsequently,

TABLE 4 | Deviation score (DS)-based analysis of CFTR traffic efficiency for wt- or F508del-CFTR (incubated with either DMSO or VX-809) following the siRNA-mediated hit protein knockdown.

siRNA	$\langle DS_{wt} \rangle$	$\langle DS_{F508del} \rangle$	$\langle DS_{F508del+VX-809} \rangle$	$\Sigma\lvert\langle DS_i \rangle\rvert$	*p*
GABARAP_1	–0.433	–0.431	1.049	1.912	**0.013**
GABARAPL2_1	0.151	–0.570	–0.153	0.875	0.449
IQGAP1_1	0.536	0.264	0.044	0.844	0.479
LRRK2_2	0.031	0.364	–0.146	0.540	0.794
NF2_1	–0.701	0.319	–0.761	1.781	**0.023**
NOS2_2	0.650	–0.501	–0.549	1.701	**0.031**
SMURF1_2	0.463	0.533	–0.698	1.693	**0.032**

DS_i (i corresponding to wt-, F508del-, or F508del-CFTR+ VX-809) informs on the siRNA capacity to influence the traffic efficiency of a particular CFTR type. The sum of the three DS mean absolute values ($\Sigma\lvert\langle DS_i \rangle\rvert$) constitutes a global measure of the siRNA effect. Statistical significance (p) of these effects were calculated using a random permutation test (see Materials and Methods for details). Values highlighted in bold when significant.

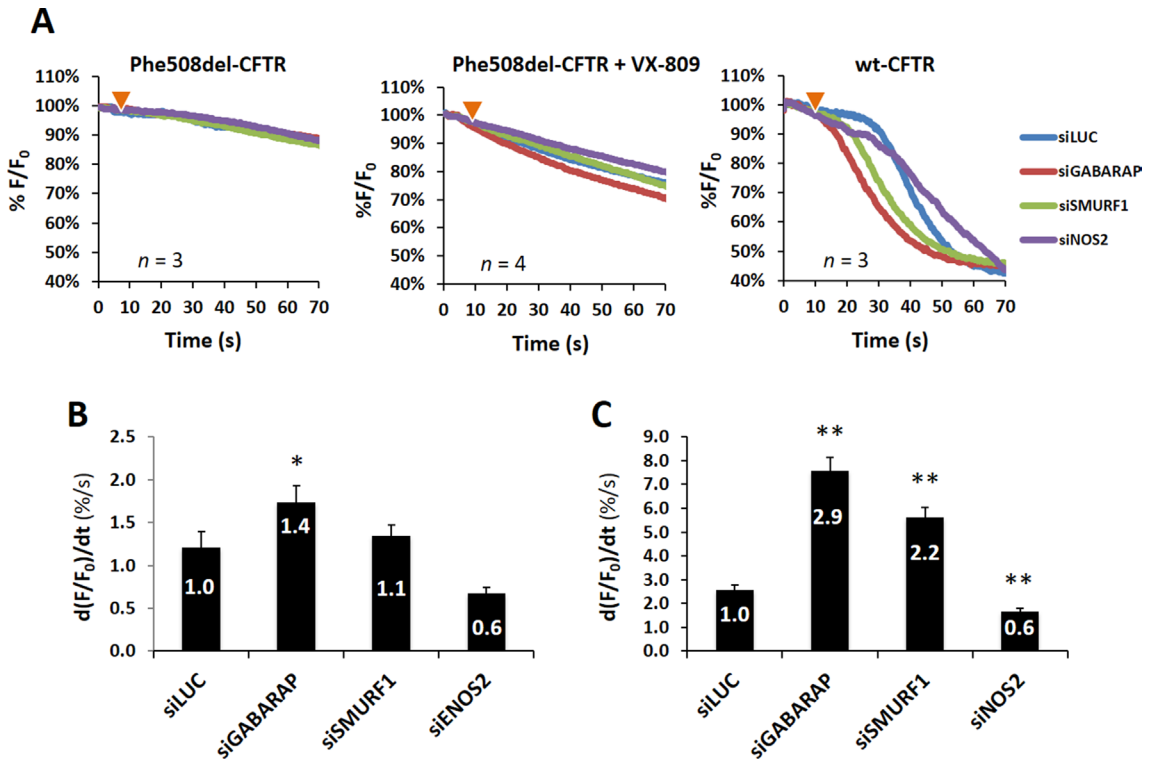

FIGURE 3 | Knockdown of selected candidate proteins influences wt- and F508del-CFTR function. **(A)** Representative fluorescence decay curves of the iodide influx assay. Wt- or F508del-CFBE cells stably expressing the HS-YFP sensor were transfected and treated for 48 h, as indicated. Fluorescence microscopy images were acquired continuously, first for 10 s (baseline) and then for 60 s after the rapid (≤1 s) addition of isosmotic buffer, containing 100 mM iodide together with either 5 μM Fsk (wt-CFTR cells) or 5 μM Fsk plus 20 μM Gen (F508del-CFTR cells). **(B)** Iodide influx rates from VX-809-treated (3 μM, 48 h) F508del-CFTR cells, transfected with the indicated siRNAs. The average fluorescence decay was fitted to an exponential decay function to derive the maximal slope [$d(F/F_0)/dt$, at $t = 0$] that is proportional to the initial influx of I^- into the cells. **(C)** Iodide influx rates, calculated as in **(B)**, for wt-CFTR CFBE cells, transfected as indicated. Data are means ± SEM of three independent assays. $^*p < 0.05$; $^{**}p < 0.01$.

the resulting interactomes were explored by computational network biology to identify novel protein nodes, never described before to interact with or regulate CFTR.

Recently, several authors have proposed network analysis methods to find novel proteins in common (or mediating the interactions) between two related network modules (Silberberg et al., 2017; Garcia-Vaquero et al., 2018). These candidate proteins may contribute to the common phenotypes associated with both modules. Here, we considered the different sets of CFTR interactors as network modules that have in common their potential role in regulating CFTR stability at the PM. To seek for novel proteins in common between these modules, we selected proteins specifically enriched in direct neighbors from at least two of the CFTR interactor sets. An analogous strategy has been successfully employed to expand the composition of disease-associated network modules (Ghiassian et al., 2015). This method requires candidates to have known direct interactions with module members, so it is limited by the completeness and accuracy of the accessed interaction databases. Our analysis is restricted to physical protein interactions, which will also limit our ability to find candidates regulating CFTR stability through indirect mechanisms such as expression regulation.

Besides the enrichment in protein localization and modification processes, the list of candidate proteins identified 12 (AHSA1, BAG3, COPS5, HDAC6, HSP90AB1, HSP90B1, HSPA5, LGLALS3BP, PABPC1, RACK1, USP19, and YBX1) out of 25 recently reviewed putative CFTR-interacting targets for restoring CFTR biogenesis and function identified through proteomic approaches (Lim et al., 2017). Both observations support the validity of our approach.

Among the novel protein nodes identified, the proteins encoded by the *GABARAP*, *NOS2*, and *SMURF1* genes were functionally validated in the physiologically relevant bronchio-epithelial cell line CFBE. For this, the expression of the endogenous proteins was depleted by RNA interference and the effect on PM localization and function of CFTR was analyzed.

NOS2 encodes the inducible isoform of nitric oxide synthase (NOS), which is expressed maximally following an inflammatory stimulus and produces relatively large micromolar quantities of NO. While macrophages and neutrophils express higher levels of iNOS in the lung, it can also be expressed by bronchial epithelial cells. NOS2 expression in these cells is upregulated by lipopolysaccharide (LPS) and by proinflammatory cytokines, such as interleukin-1, tumor necrosis factor alpha, and gamma interferon (Ermert et al., 2002). Expression of iNOS appears to be nearly absent in bronchial cells from CF patients (Meng et al., 1998; Thomas et al., 2000; Dotsch et al., 2002) and may be caused by abnormalities in the signaling system that normally causes its induction, such as cytokine receptors, second messengers, or transcription factors (Meng et al., 1998; Satitsri et al., 2016). Given the apparent negative

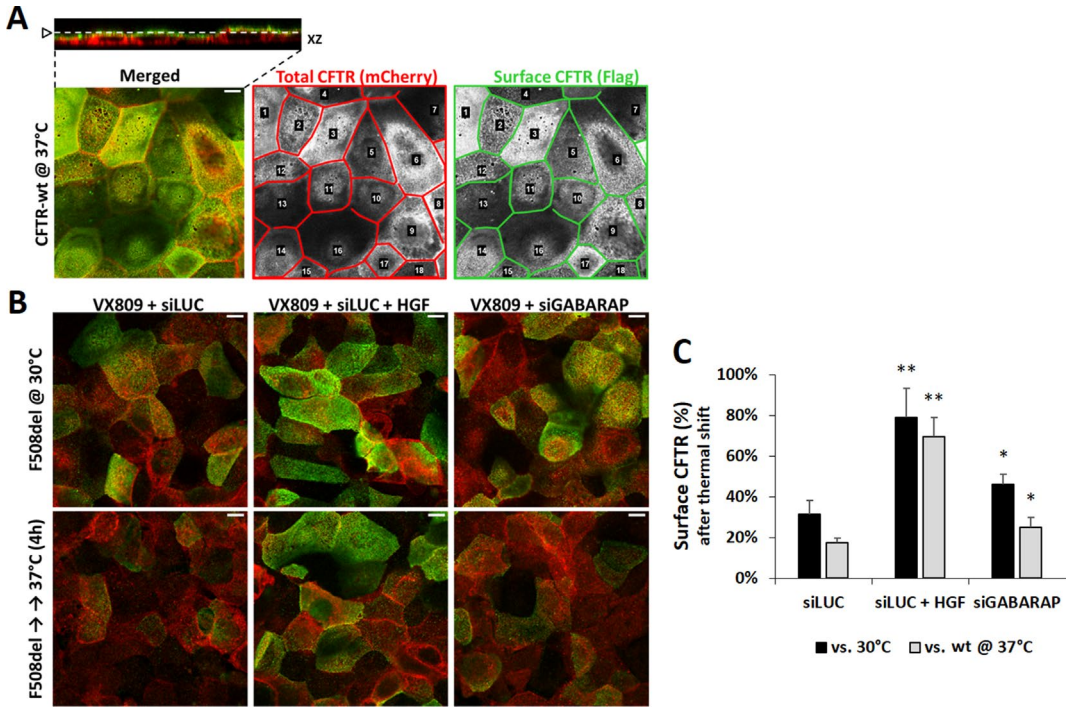

FIGURE 4 | GABARAP downregulation increases rescued F508del-CFTR PM abundance. **(A)** Confocal images of Dox-induced, mCherry-Flag-wt-CFTR expressing CFBE cells. Images are of double-labeled non-permeabilized cells, where mCherry fluorescence is proportional to the total amount of CFTR and Alexa Fluor 488 fluorescence is proportional to the amount of CFTR present at the cell surface. A 0.2-μm Z-stack of 1-Airy confocal images was acquired (top left inset) to select the best *XY* plane to analyze the CFTR protein at the cell surface. ImageJ software (NIH) was used to delimit each single cell as individual ROIs (regions of interest) to allow measurement of mCherry (red lined lattice) and Alexa488 (green lined lattice) fluorescence signal intensities. **(B)** Confocal images, obtained as in (A) of Dox-induced, VX-809-treated (3 μM, 48 h) mCherry-Flag-F508del-CFTR CFBE cells, transfected with either siLUC or siGABARAP and co-treated with 50 ng/ml HGF, when indicated. Cells were first pre-incubated for 24 h at 30°C to stabilize PM rescued F508del-CFTR and then moved to 37°C for 4 h to allow destabilization and internalization of the recued protein, unless otherwise prevented by the treatment conditions. White bars correspond to 10 μm. **(C)** Analysis of single-cell fluorescence signals in three independent experiments. Data presented refer to mean values ±SEM of three independent assays. CFTR at the surface was estimated by the ratio of Flag/mCherry signals in all conditions and expressed as percentage of surface F508del-CFTR at 30°C (siLUC, black bars) or surface wt-CFTR at 37°C (gray bars). $^{*}p < 0.05$; $^{**}p < 0.01$.

functional effect on both wt and VX-809-rescued F508del-CFTR observed here upon iNOS downregulation, our results suggest that, besides an improved resistance to microbial infection, any therapeutic approach that increased iNOS production in the airways might further benefit CF patients by stimulating residual CFTR function. This has, however, to be further investigated since, in a previous study, micromolar concentrations of NO were shown to inhibit forskolin-stimulated cAMP production by adenylyl cyclase and cAMP-dependent Cl^- transport and fluid secretion mediated by CFTR (Spirli et al., 2003).

SMURF1 functions as an E3 ubiquitin ligase protein and has been shown previously to regulate endocytosis of other membrane proteins such as the bone morphogenetic protein type II receptor (BMPR), related to pulmonary arterial hypertension. Blockage of SMURF1 ubiquitin ligase activity increased cell surface expression of BMPR (Murakami and Etlinger, 2019). Here, we observed that depletion of SMURF1 produced a twofold stimulation of the iodide influx rate in CFTR wt cells but did not promote any significant rescue of F508del-CFTR to the PM. Moreover, while endogenous cAMP depletion by pretreatment with indomethacin delayed the response to Fsk in control wt-CFTR CFBE cells, this effect was much less pronounced upon SMURF1 knockdown. This is suggestive that the knockdown of these proteins can have an effect not only in CFTR's PM abundance but also on its gating efficiency. Such an effect might nevertheless have therapeutic potential for class III to V CFTR mutations.

Similar effects were also observed upon knockdown of the GABA receptor-associated protein GABARAP, which belongs to the Atg8 family of ubiquitin-like proteins that are involved in the regulation of autophagy. GABARAP proteins associate with the membrane of the autophagosome through conjugation of phosphatidylethanolamine (Ichimura et al., 2000) and are primary drivers of autophagy working upstream of LC3 proteins and operate mainly at the late stages of autophagy driving the fusion of autophagosomes to lysosomes (Nguyen et al., 2016). Our results show that knockdown of GABARAP increased the temperature-dependent stability and function at the PM of F508del-CFTR after correction with VX-809. Furthermore, depletion of GABARAP also increased wt-CFTR function and, similar to SMURF1 knockdown, its response to Fsk stimulation after pretreatment with indomethacin. Since we observed no detectable impact of GABARAP downregulation on wt-CFTR traffic, it is possible that GABARAP may function by stabilizing the molecular machinery required to anchor and activate CFTR at the PM, thus increasing its

gating efficiency. While the high levels of wt-CFTR at the surface may have led to an underestimation of fluorescence signals at the cell surface, the PM stabilizing effects observed with VX-809-rescued F508del-CFTR seem to support a functional potentiation hypothesis. In this case, interference with GABARAP activity may also prove beneficial for class III to class V CFTR mutations.

The identification of novel regulators of CFTR levels at the PM has important biomedical implications. The pharmacological rescue of mutant CFTR folding or gating improves symptoms in CF patients (Zhang et al., 2016); however, a truly relevant clinical benefit appears to require the targeting of additional cellular processes: the trafficking of CFTR as well as its retention at the PM (Farinha and Matos, 2016). The proteins identified here suggest that the cellular processes of membrane traffic, protein stabilization at the PM, and NO signaling are suitable targets for novel therapeutic approaches in CF.

Besides providing new insights into CFTR biology and suggesting possible therapeutic targets in CF, the network biology approach delineated in this work provides a framework applicable to other human diseases related to the traffic and function of PM transport proteins.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

CL, JS, and AM conducted the experiments. PJ, CF, and PM designed the experimental studies. FP provided the network analysis. PJ, CF, PM, and FP analyzed the data and wrote the manuscript.

FUNDING

This work was supported by FCT, Portugal, through center grant UID/MULTI/04046/2019 to BioISI and the BioSys PhD program PD65-2012 (fellowships SFRH/BD/52488/2014, SFRH/BD/106084/2015, and SFRH/BD/52490/2014 to CL, JS, and AM, respectively).

ACKNOWLEDGMENTS

We would like to acknowledge technical support by the high-throughput imaging facility of BioISI and thank Prof. Margarida Amaral for providing the CFBE cells expressing mCherry-Flag-CFTR.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00619/full#supplementary-material

FIGURE S1 | qPCR assessment of siRNA efficiency in CFBE cells for the targeted transcript, $^*p < 0.05$.

TABLE S1 | Specific common neighbours of CFTR interactor sets.

TABLE S2 | Enriched biological processes in the list of strong mediator proteins.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

MINI REVIEW
published: 07 December 2018
doi: 10.3389/fphar.2018.01429

Human Primary Epithelial Cell Models: Promising Tools in the Era of Cystic Fibrosis Personalized Medicine

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Edited by:
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *10 September 2018*
Accepted: *19 November 2018*
Published: *07 December 2018*

Citation:
Awatade NT, Wong SL, Hewson CK, Fawcett LK, Kicic A, Jaffe A and Waters SA (2018) Human Primary Epithelial Cell Models: Promising Tools in the Era of Cystic Fibrosis Personalized Medicine. Front. Pharmacol. 9:1429. doi: 10.3389/fphar.2018.01429

Cystic fibrosis (CF) is an inherited disorder where individual disease etiology and response to therapeutic intervention is impacted by CF transmembrane regulator (CFTR) mutations and other genetic modifiers. CFTR regulates multiple mechanisms in a diverse range of epithelial tissues. In this Review, we consolidate the latest updates in the development of primary epithelial cellular model systems relevant for CF. We discuss conventional two-dimensional (2-D) airway epithelial cell cultures, the backbone of *in vitro* cellular models to date, as well as improved expansion protocols to overcome finite supply of the cellular source. We highlight a range of strategies for establishment of three dimensional (3-D) airway and intestinal organoid models and evaluate the limitations and potential improvements in each system, focusing on their application in CF. The *in vitro* CFTR functional assays in patient-derived organoids allow for preclinical pharmacotherapy screening to identify responsive patients. It is likely that organoids will be an invaluable preclinical tool to unravel disease mechanisms, design novel treatments, and enable clinicians to provide personalized management for patients with CF.

Keywords: cystic fibrosis, organoid, personalized medicine, CFTR, drug development, sweat chloride, CFTR modulator

INTRODUCTION

Cystic Fibrosis (CF; OMIM 219700) is the most common life-limiting, autosomal recessive monogenic disease in Caucasian populations (Riordan, 2008). It is caused by mutations in the gene encoding CF transmembrane conductance regulator (CFTR), an anion channel essential for regulating trans-epithelial secretion of chloride (Cl^-) and bicarbonate (HCO_3^-). The loss of CFTR function leads to abnormalities at the mucosal surfaces in multiple exocrine organs including the lungs, pancreas, liver, and intestine. Notably however, progressive lung disease and respiratory failure are the major cause of morbidity and mortality for most patients (Ratjen et al., 2015). While

the regulatory role of CFTR in ion and fluid transport is widely known, the exact mechanism of its defect in the development of CF remains debatable. Two competing hypotheses attempt to explain CF pathogenesis; (1) the "high salt" hypothesis postulates that diminished trans-epithelial Cl^- conductance associated with CFTR dysfunction results in increased retention of salt at the mucosal surface, which negates the activity of antimicrobial peptides present in the epithelial apical secretions (Zabner et al., 1998; Wine, 1999); (2) the more favorable "low volume" hypothesis suggests the lack of epithelial sodium channel (ENaC) inhibition in the absence of CFTR function causes sodium (Na^+) hyper-absorption, with accompanying intracellular flux of Cl^- and water (Matsui et al., 1998; Wine, 1999). Mucus becomes dehydrated, viscous and sticky which then leads to decreased airway surface liquid (ASL) height, as well as mucus stasis and obstruction (Riordan, 2008). Irrespective of the mechanism of action, impaired mucociliary clearance in the lung and the innate inability to eradicate inhaled pathological agents are hallmarks of CF. Recurrent cycles of lung infection and exacerbations result in chronic inflammatory responses which then lead to structural lung damage, loss of lung function, and ultimately respiratory insufficiency (Ratjen et al., 2015).

Major advances in symptom management have been instrumental in delaying disease progression in CF. Administration of enzyme replacement therapy, nutritional support, mucolytic agents airway clearance techniques, and antibiotics for bacterial lung infection, along with early detection of disease (newborn screening, pathogen surveillance) has dramatically improved life expectancy in CF patients over the last 4 decades. The median age of death is now above 40 years (Keogh et al., 2018). Importantly, a class of small molecule compounds termed CFTR modulators were recently discovered. These compounds directly correct defective CFTR protein in CF patients and have now transformed the therapeutic landscape of CF in a precision-based fashion. The first part of this review outlines the current developments in CFTR modulators addressing the different dysfunctional CFTR mutation class and highlights the need for individualized strategies to restore their function.

The second part of this review discusses the evolving use of different epithelial cell models and reporter assays for assessing clinical response to CFTR-directed drugs on a personalized basis, and highlights the strengths and limitations of each application. This review also elaborates on the breakthrough discoveries of long-term cultures of patient-derived 3-dimentional (3-D) airway and rectal organoids. We also discuss their emerging use as *ex vivo* biomarkers and preclinical predictive tools for the disease.

Abbreviations: 2-D, 2-Dimensional; 3-D, 3-Dimensional; ALI, Air-liquid interface; ASL, Airway surface liquid; BAL, Bronchoalveolar lavage; BEGM, Bronchial epithelial growth media; BMI, Body mass index; BMP, Bone morphogenic protein; CaCC, Calcium-activated chloride channels; CF, Cystic Fibrosis; CFTR, Cystic Fibrosis transmembrane conductance regulator; Cl^-, Chloride; CRCs, Conditionally reprogrammed cells; DMSO, Dimethyl sulfoxide; ECM, Extracellular matrix; ENaC, Epithelial sodium channel; FEV1, Forced expiratory volume—one second; FIS, Forskolin-induced swelling; FRT, Fischer rat thyroid; HAE, Human airway epithelial; HBE, Human bronchial epithelial; HCO_3^-, Bicarbonate; HNE, Human nasal epithelial; HTS, High-throughput screening; ICM, Intestinal current measurement; Na^+, Sodium; P#, Passage #; PTC, Premature termination codon; ROCK, Rho-associated protein kinase; TEER, Trans-epithelial electrical resistance; TGF-β, Transforming growth factor-beta.

CURRENT CFTR-BASED THERAPY WITH CFTR MODULATORS

More than 2,000 CFTR mutations have been identified so far and at least 336 of these are reported to lead to symptoms characteristic of CF (Cystic Fibrosis Mutations Database report 31 August 2018 (US CF Foundation, 2011). It is thus perceivable that a multi-pronged approach is required to target the different defective mechanisms that each CFTR mutation confers. For the past 15 years, high-throughput screening (HTS) has accelerated the process of drug discovery. To identify candidate CFTR modulators, hundreds of thousands of chemical compounds with diverse structures were screened and the potential of a compound to rescue or activate CFTR was tested in cellular-based assays (Pedemonte et al., 2005; Van Goor et al., 2006; Sutanto et al., 2018). The promising compounds, called "hits," underwent further medicinal and chemical optimization to improve potency and minimize potential off-target activities of the compounds. This process has led to successful identification of multiple compounds, some of which have moved forward to human clinical trials.

The different approaches targeted toward correcting each CFTR mutation class as well as the compounds currently tested in clinical trials are summarized in **Table 1**. CFTR-modulating compounds are classified into five main groups: read-through agents, correctors, potentiators, stabilizers, and amplifiers. Of these, two classes of modulators (potentiators and correctors) have gained regulatory approval to treat CF patients with specific CFTR mutations.

Potentiators

Potentiators were developed for CFTR mutant proteins that are expressed at the apical membrane of epithelial cells but are functionally impaired. HTS performed by Vertex pharmaceuticals involving 280,000 small molecule compounds led to the discovery of the potentiator VX-770 (Generic name: Ivacaftor; Trade name: Kalydeco) (Van Goor et al., 2010). Ivacaftor corrects the gating impairment of Class III mutations. Its use has now been extended beyond the most common Class III G551D-CFTR mutation, to additional Class III mutations, as well as CFTR mutations with conductance (Class IV−R117H) or biosynthesis (Class V−3,849 + 10 kb C>T) defects. In clinical studies of participants with G551D-CFTR mutations, the impact of Ivacaftor on CFTR was evidenced by normalization of sweat Cl^-, improved lung function (10% mean increase in FEV_1), reduced episodes of pulmonary exacerbations and improved body mass index (BMI) (Accurso et al., 2010; Ramsey et al., 2011; Davies et al., 2013).

Correctors

The potentiators' mode of action does not benefit the majority of the CF population who have the Class II F508del-CFTR mutation, as F508del-CFTR is degraded while transitioning

TABLE 1 | CFTR mutation class and current modulator therapeutic approach.

Mutation class	Molecular defect	Type of mutation	Therapeutic approach	Approved or drugs in clinical trial	Mutation examples
IA~	No mRNA synthesis	Large deletions	Bypass therapies (Activate alternative Cl^- channels)	–	Dele2, 3(21 kb) 1717-1G->A 621+1G->T
IB~	Protein synthesis	Nonsense (PTC), Frame-shift, Splicing	Read-through compounds	Phase 2: QBW276, SPX-101 Phase 1: AZD5634, BI443651	G542X, W1282X
II	Trafficking	Missense	Correctors and potentiators	Approved: Orkambi, Symdeko Phase 3: VX-445[#], VX-659[#] Phase 2: VX-152[#], VX-440[#], GLPG2222, GLPG2737, FDL169 Phase 1: PTI-801	F508del, R560T, A561E
III	Channel Gating	Missense	Potentiators	Approved: Ivacaftor Phase 2: VX-561, QBW251, GLPG1837, GLPG2451, GLPG3067 Phase 1: PTI-808	G551D, S1251N, G178R
IV	Conductance	Missense	Potentiators	Approved: Ivacaftor	R334W, R347P, R117H
V	Protein synthesis	Missense, Alternative splicing	Correctors, Potentiators, Antisense Oligonucleotides	Approved: Ivacaftor	3849+10kbC>T, 3272-26A>G, 2789+5G>A
VI	Reduced CFTR stability at PM	Missense, Frameshift	Stabilizers	–	120del23, N287Y, Q1412X

Compounds starting with VX are developed by Vertex Pharmaceuticals, GLPG by Galapagos NV and AbbVie, FDL, Flatley discovery lab; QBW, Novartis pharmaceuticals; PTI, Proteostasis therapeutics; SPX, Spyryx biosciences; AZD, AstraZeneca; BI, Boehringer ingelheim; Orkambi, VX-809+VX-770; Symdeko, VX-661+VX-770; Ivacaftor, VX-770; [#]Triple combination therapy with VX-661 and VX-770. PTC, Premature termination codons; PM, Plasma membrane; ~, classification is based on (Marson et al., 2016).

through the endoplasmic reticulum, with very little or no mutant protein reaching the apical membrane of epithelial cell. Effective rescue of the F508del mutation thus requires chaperones that can repair defective protein folding and rescue trafficking of the mature CFTR to the plasma membrane. VX-809 (Generic name: Lumacaftor; available as Lumacaftor-Ivacaftor combination therapy—Trade name Orkambi) restored F508del-CFTR channel activity to 15% of wild-type CFTR in *in vitro* preclinical testing performed in primary airway epithelial cells (Van Goor et al., 2012). This result led to Lumacaftor monotherapy clinical trials in CF patients homozygous for the F508del-CFTR mutation, where a significant improvement in sweat Cl^- concentrations were observed but lung function remained unchanged (Clancy et al., 2017). Considering that F508del channel gating defect, administration of Lumacaftor-Ivacaftor combination therapy was proposed as a solution that may augment correction of CFTR function to clinically significant levels.

Combination Therapy

Lumacaftor-Ivacaftor combination therapy increased CFTR activity at the plasma membrane *in vitro* (Van Goor et al., 2012). However, results from phase III trials in children and adults homozygous for the F508del-CFTR mutation showed that the combination therapy failed to produce the magnitude of clinical improvements observed with Ivacaftor. While a reduction in pulmonary exacerbations and improved BMI was observed, there was only a modest improvement in lung function (2–3%) (Boyle et al., 2014; Wainwright et al., 2015). In addition, CF patients receiving Lumacaftor-Ivacaftor combination therapy reported unwanted side effects such as dyspnoea, liver damage, and bronchospasm. Lumacaftor is also associated with significant drug-drug interactions which alter its pharmacokinetic profile and potentially hamper its therapeutic efficacy (Talamo Guevara and McColley, 2017). Tezacaftor is a new CFTR corrector with an improved pharmacokinetic profile, longer half-life and less drug-drug interactions compared to Lumacaftor. It has recently been approved as a combination therapy with Ivacaftor (Trade name: Symdeko/Symveki) for the treatment of patients homozygous for F508del mutation by the Food and Drug Administration (FDA) and European Medicines Agency. Phase III clinical trials data showed that improvement in lung function with Tezacaftor-Ivacaftor combination therapy was generally comparable or better than those observed in patients treated with Lumacaftor-Ivacaftor combination therapy (Rowe et al., 2017; Taylor-Cousar et al., 2017). It is notable however that while overall benefit was demonstrated, individual patient responses have been heterogeneous in the clinical trials of both Lumacaftor-Ivacaftor and Tezacaftor-Ivacaftor.

Theratyping

It is now known that patients display a spectrum of responses to CFTR-modulator drugs despite having the same CFTR mutation variant (Wainwright et al., 2015; Donaldson et al., 2018). This suggests that although the current classification system may be an important indicator for prognosis and disease severity in CF, it is inadequate for predicting how individual patients respond to therapy. Indeed, *in vitro* experimental studies have shown that contrary to findings in the Class II F508del-CFTR mutation, other Class II processing mutations such as N1303K, R560S, and G85E could not be rescued by Lumacaftor treatment (Awatade et al., 2015, 2018; Dekkers et al., 2016b; Lopes-Pacheco et al.,

2017). These results suggest that the underlying pathomechanism of each CFTR mutation is distinct and that individualized strategies to restore their function may be warranted.

To address this, CFTR mutations have been classified according to their response to modulator compounds. This approach termed "theratyping," groups together patients who harbor different CFTR mutations but respond to the same CFTR-directed compounds. It is clear that treatment regimens of the future will need to take into consideration the individual's genetic makeup and not just their CFTR mutations. A personalized approach will optimize patient's clinical outcomes by accounting for the specific genetic mutations of the individual patient.

TOWARD PERSONALIZED MODULATOR THERAPY

One of the major hurdles to the development of novel treatment regimens in CF is the bench-to-bedside translation of scientific knowledge. Many drugs that perform well in a laboratory setting fail to advance in clinical trials, largely due to inappropriate selection of *in vivo* and *in vitro* models for HTS. In the context of CF, animal models and immortalized epithelial cell lines do not fully portray patient-specific disease phenotypes. Animal models of CF have provided insights into disease pathophysiology. However, their generation is time consuming, expensive, and more importantly they poorly represent the full repertoire of disease manifestations in individual patients. For instance, mouse models of CF do not have lung disease and bacterial infections, attributed to the compensatory effect of a secondary Cl^- ion channel (Lavelle et al., 2016). On the other hand, immortalized epithelial cell lines derived from primary patient material have contributed tremendously to CF research, but not without limitations. Their generation from primary patient material is very inefficient. It involves extensive adaptation and selection to *in vitro* 2-D culture conditions, as only rare clones are able to expand and be maintained over many passages. Furthermore, these cell lines may have undergone substantial genetic changes and no longer retain features of the original parental cells. Drug development pipeline for new CFTR-directed compounds have relied heavily on unpolarized Fischer rat thyroid (FRT) epithelial cell lines. It is thus not surprising that this model has a higher propensity of false-positive and false-negative "hits" compared to that performed in primary human bronchial epithelial (HBE) cells.

PATIENT-DERIVED ORGANOTYPIC CELLULAR MODELS FOR PERSONALIZED MEDICINE

Although CF is a monogenic disease, the diverse mutation variants identified within the CFTR gene as well as the presence of modifier genes (known and unknown), warrants adoption of new technologies to extend research capabilities. There is a clear unmet need for a representative library of patient-specific epithelial cell models for disease modeling, preclinical testing of drug response, and biobanking for future drug discovery. The cell models can be derived from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or tissue-resident adult stem cells. Whereas, ethical concerns pertaining the source of human ESC, limit their use in research, generation of CFTR gene corrected iPSC lines enable disease modeling, drug discovery and toxicology studies [reviewed in (Pollard and Pollard, 2018)]. In this Review, we focus on airway and intestine epithelium models derived from adult stem cells.

Human Airway Epithelial (HAE) Cells

The pulmonary epithelium is divided into three regions; upper (nasal and oral cavities), lower (trachea and primary bronchi), and distal small airway epithelia (alveolar). Persistent inflammation, bacterial colonization, and airway structural changes in CF occur in the lower respiratory tract. Primary HBE cells are therefore the gold standard for studying CF disease pathogenesis and evaluating CFTR functional response (Van Goor et al., 2006; Awatade et al., 2015). HBE cells can be isolated from biopsy samples, lung explants and cadavers. Explanted lung and post-mortem samples from individuals with CF provide high cell yield. However, the extensive damage to the tissue, particularly the epithelial cell layer, plus the presence of chronic microbial colonization present technical challenges to establishing successful *ex vivo* cultures. Acquisition of airway biopsies or brushings involves highly invasive procedures, while bronchoalveolar lavage (BAL) fluid and induced sputum samples usually do not provide sufficient cell counts to initiate culture. Therefore, supply of CF patient-derived HBE cells are often limited and hard to come by.

Human nasal epithelial (HNE) cells are increasingly used as surrogates for the lower airway epithelium in CF research (de Courcey et al., 2012; Brewington et al., 2018a). HNE cells demonstrate many characteristics common to HBE cells including the ability to form polarized, pseudostratified epithelium mimicking *in vivo* airways and the expression of ion channels such as CFTR. Their advantage is the lack of need for invasive procurement (Clarke et al., 2013). HNE cells are grown using the same culture media and protocol as for HBE cells.

Human Airway Epithelial (HAE) Cell Culture Models

Primary human airway epithelial cells are conventionally cultured as monolayers (2-D cultures). Expansion of epithelial cells is often necessary in the initial passages for biobanking purposes and to generate enough cell numbers needed to differentiate cells under air-liquid interface (ALI) conditions. HBE cells are most "*in vivo*" like when fully differentiated; they display a striking phenotypic resemblance to the lower airway epithelium. They form pseudostratified epithelium with mucociliary differentiation indicated by the presence of functional beating cilia and mucus secretion. They also exhibit characteristic epithelial barrier functions, including expression of cell junctional proteins and the development of robust trans-epithelial electrical resistance (TEER) values (Berube et al., 2010). The polarized, differentiated phenotype is critical for *in vitro* measurement of CFTR function as the protein is primarily expressed at the apical surface of ciliated cells. While ALI cultures accurately represent *in vivo* phenotypes, their wider use

is deterred by limited propagation of cells in culture, attributed to squamous transformation and cellular senescence (Gentzsch et al., 2017). To date, three expansion methods have been used to extend the lifespan of cells and delay cellular senescence beyond that of the standard cultures.

Improved Cell Expansion Culture Methods

Conditionally reprogrammed cells (CRCs) are generated by co-culturing patient-derived airway epithelial cells with irradiated fibroblast feeder cells. Specialized conditioned media (termed F-Media) which contains Rho-associated protein kinase (ROCK) inhibitor, promotes serial passages of airway epithelial cells and enhances population doubling without compromising the characteristic epithelial cell morphology. Both ROCK inhibitor and the feeder layer are essential in maintaining the stem cell-like phenotype evident in CRCs (Reynolds et al., 2016; Martinovich et al., 2017). Their removal results in differentiated cell lines, with intact barrier function and the ability to polarize and form mucociliary epithelium. CFTR-mediated Cl^- transport in these cells is also preserved.

A modified CRCs protocol using BEGM, ROCK inhibitor and an irradiated feeder layer cultured in reduced oxygen concentration (2%) demonstrated some advantage over the standard CRCs. The modified CRCs could support airway epithelial cell growth up to passage 10 with robust formation of pseudostratified epithelium at the extended passage, although a modest decrease in CFTR-dependent Cl^- transport was observed. Meanwhile, reduced numbers of ciliated cells and goblet cells were observed in standard CRCs cultures at P10 and robust TEER could not be established (Peters-Hall et al., 2018).

A feeder-free culture protocol that relies on disruption of SMAD signaling pathway through inhibition of dual ligands, transforming growth factor-beta (TGF-β) and bone morphogenic protein (BMP), showed airway epithelial cells could be expanded up to 18–25 passages without loss of proliferative potential (Mou et al., 2016). Combined TGF-β/BMP inhibition led to basal cell hyperplasia with hyper-proliferative p63 cells (basal cell marker) and produced homogenous, tightly packed cells, resembling stem cell morphology. Epithelial cells expanded using dual SMAD inhibition method can undergo mucociliary differentiation up to P12 with preserved TEER although Na^+ currents and Cl^- conductance declined fairly rapidly after serial passage 6 (Mou et al., 2016).

Three-Dimensional Airway Spheroids and Organoids

Two-D cultures lack a third dimension, the scaffolding extracellular matrix (ECM), which establishes intercellular cues or network in the *in vivo* airway epithelium. Therefore, 3-D cell cultures are a major area of development, where cells are cultured in a matrix (such as matrigel) or are cultured in such a manner that they develop ECM-like scaffolds between them, thus mimicking the *in vivo* phenotype more faithfully. While cell-derived ECMs such as Matrigel have been most commonly used for development of organoids, their undefined components introduce inconsistency in replicating the native 3-D culture environment (Czerwinski and Spence, 2017). To overcome this challenge, new biomaterial systems, such as polymers and hydrogels are being developed [reviewed in (Dye et al., 2017)].

Different methods for generating organoids (or spheroids) from human lung cells have been described to date, each producing organoids with distinct definitive structure and cellular compositions. Barkauskas and colleagues have elegantly reviewed the different lung organoids (airway organoids inclusive) established from varying lung progenitor cell populations, including basal cells in the proximal airways, secretory club Clara cells in bronchioles and alveolar type II cells in the alveoli, as well as those from embryonic and iPSCs (Barkauskas et al., 2017). Given CFTR is primarily expressed at the apical surface of ciliated cells and recently discovered in the pulmonary ionocytes in tracheal epithelium (Montoro et al., 2018; Plasschaert et al., 2018), only airway organoids displaying proximal differentiation are discussed here in light of their relevance for measuring CFTR functional response (**Table 2**).

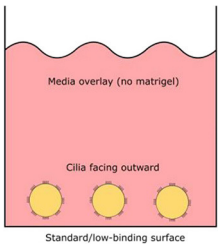
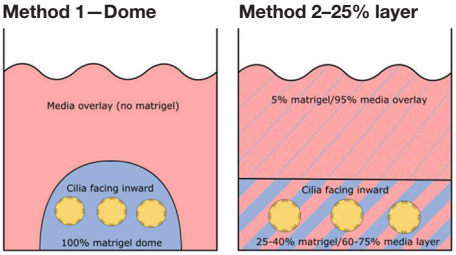
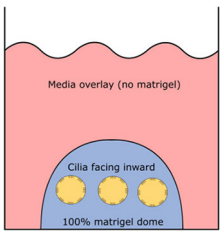
Three-Dimensional Airway Explant Spheroid (ECM—Free)

Airway spheroid cultures were first described in 1991 when nasal epithelial multicellular spheroids were generated from non-dissociated nasal epithelial sheets (Bridges et al., 1991). These explant spheroids were maintained in culture media (no matrigel) and were reported to form rapidly, within 2–5 days. Explant spheroids have an apical-membrane-out orientation; apical ciliated cells face the media bath and the basolateral membrane lines the fluid-filled lumen (Bridges et al., 1991). Pedersen and colleagues later found amiloride-sensitive Na^+ transport drives changes in the lumen size, suggesting nasal spheroids may be a useful model for ion/fluid transport studies and for investigating diseases associated with defective ion channels such as cystic fibrosis (Pedersen et al., 1999). Nasal spheroids were also responsive to forskolin stimulation (Guimbellot et al., 2017). As the CFTR-expressing apical side of nasal spheroids face outward, forskolin causes the outflow of fluid from the lumen to the exterior bath and the spheroids to shrink in size. This response was blunted in spheroids established from CF patients but could be partially rescued by CFTR modulators (Guimbellot et al., 2017). Notably, successful spheroids have also been established from bronchial brushings, although CFTR function was not tested in the study (Deslee et al., 2007). Interestingly, no evidence of cell proliferation was observed throughout the nasal spheroid formation process and the spheroids plateaued in size once fully formed. This suggests that the spheroids are self-organizing aggregates of terminally differentiated epithelial cells only (Castillon et al., 2002). While spheroids may be ready for *ex vivo* CFTR measurement rapidly, they are short-lived, lasting for up to 12 weeks only and biobanking for future drug screening is not possible (Guimbellot et al., 2017).

Three-Dimensional Spheroid From Airway Basal Progenitor Cells

Airway spheroids, both bronchial and nasal can also be derived from primary airway basal cells embedded in the

TABLE 2 | Comparison of three-dimensional organotypic airway epithelial culture methods.

	Respiratory—nasal and bronchial		
	Airway explant spheroid	**Airway basal cell spheroid**	**Long term expanding airway organoid**
Cultures		**Method 1—Dome** **Method 2–25% layer** 	
Cellular source	Freshly isolated non-dissociated airway epithelial sheets	Airway basal progenitor cells expanded as traditional primary cultures or conditionally reprogrammed cultures	Lung biopsies and cells from BAL fluid
Media	Standard, non-differentiating culture media e.g., BEGM	Differentiation media—same as those used for 2D ALI cultures	Media containing biochemical cues for self-renewal. Content: Advanced DMEM/F12 R-Spondin 1 Noggin FGF 7 FGF 10 A83-01 Y-27632 SB202190 B27 supplement N-Acetylcysteine Nicotinamide Glutamax
Require ECM (matrigel)	No	Yes	Yes
Constitute differentiated epithelium structure	Yes (*ex vivo*—already differentiated)	Yes (after 14–21 days culture)	Yes (duration to form not reported)
Orientation of apical ciliated side	Facing outwards (media)	Facing inwards (lumen)	Facing inwards (lumen)
Use of cultures	End-point experiments	End-point experiments	Can be passaged for on-going cultures (reported up to P18)
CFTR functional assay	Yes—spheroids shrink when CFTR is activated	Yes—spheroids swell when CFTR is activated	Yes—spheroids swell when CFTR is activated
Cryopreservation	No	No	Not reported
References	Bridges et al., 1991 Pedersen et al., 1999 Deslee et al., 2007 Guimbellot et al., 2017	Danahay et al., 2015 Hild and Jaffe, 2016 Brewington et al., 2018c	Sachs et al., 2018 Zhou et al., 2018

2D, Two-dimensional; ALI, Air-liquid interphase; BAL, Bronchoalveolar lavage; BEGM, Bronchial epithelial cell growth media; ECM, Extracellular matrix; FGF, Fibroblast growth factor; TGF-β, Transforming growth factor-beta.

basement membrane matrix matrigel (Hild and Jaffe, 2016; Brewington et al., 2018c). Matrigel is fundamental for the formation of intact spheroids, with a lumen surrounded by a slightly thickened wall upon maturity (7–14 days) and a complete cell-apex-in morphology (Danahay et al., 2015; Brewington et al., 2018c). Different culture protocols using varying concentrations of matrigel have been reported; (1) cells are resuspended in 100% matrigel and seeded as spherical drops, then overlaid with ALI differentiation media; (2) cells are resuspended in 5% matrigel with ALI differentiation media and then layered over a denser 25–40% matrigel base layer which the cells sink into (Hild and Jaffe, 2016; Tan et al., 2017; Brewington et al., 2018c). Method 1 (100% matrigel) was reported to yield the best success, as the sinking method could result in formation of some spheroids with cell-apex-out morphology and disorganized cellular aggregates (Brewington et al., 2018b), likely due to incomplete embedding of cells in the denser matrigel base layer e.g., growing on top of

TABLE 3 | Comparison of human cell models relevant for CF therapeutic application.

Cell models	Established immortalized cell lines	Pulmonary				Gut		
		HBE		Nasal		Intestinal		
		Bronchial ALI	Bronchial organoid	Nasal ALI	Nasal organoid	Rectal biopsies	Rectal organoid	Organoid 2-D monolayer
Patient-specific	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Tissue source	-	Lung explants Bronchial brushing/biopsies Bronchoalveolar lavage fluid		Nasal brushing/curettage		Rectal biopsies		
Invasiveness of sample acquisition	-	+++		+		++		
Easy-to-culture	- Flexible - Easy to manipulate	- Limited expansion and differentiation potential (decreases as a function of passage) - ALI cultures take 4 weeks to form pseudostratified epithelium -No standardized protocol available for airway organoids				- Long term expanding cultures - Long term expanding cultures -Established and standardized protocol for organoid -Monolayer in development phase		
Phenocopies CF lung	No (prone to artifact)	Yes (gold standard)	Yes	Yes	Yes	No	No	No
Phenocopies CF intestine	No	No	No	No	No	Yes	Yes	Yes
CFTR functional readout	- Ussing - Halide assay	- Ussing	- FIS	- Ussing	- FIS	ICM	- FIS/SLA	Ussing
Assay dynamic range	Large	Moderate	Moderate	Small	Small	Large	Large	Large
Biochemical/physiological readout	- CFTR western blotting	- CFTR western blotting - ASL -CBF	- CFTR western blotting	- CFTR western blotting	- CFTR western blotting		- CFTR western blotting	
Drug screening	- High-throughput - Hit-to-lead drug development	- Mid-throughput - Checkpoint before drugs enter clinical trial/use	Exploratory	Mid-throughput	Exploratory	Low-throughput	Mid-throughput	Exploratory
Predictive value	++	+++	Exploratory	Exploratory	Exploratory	++	++	Exploratory

+++, High; ++, Moderate; +, Low; 2-D, 2-Dimensional; ALI, Air-liquid interphase; ASL, Airway surface liquid; CBF, Cilia beating frequency; FIS, Forskolin-induced swelling; HBE, Human bronchial epithelial; ICM, Intestinal current measurement; SLA, Steady-state lumen area.

or at the interface of the denser matrigel base layer. For certain downstream applications such as live imaging (e.g., forskolin-induced swelling—see section The Cell Model of the Future), the sinking method confers an advantage because spheroids are suspended in the same plane and permit HTS. We also note that successful differentiated, pseudostratified spheroid formation has been reported from airway basal cells maintained in the standard BEGM media and those derived from conditionally reprogrammed culture (Hild and Jaffe, 2016; Brewington et al., 2018c). Airway basal cell spheroids have no self-renewal capacity and are used for end-point applications.

Three-Dimensional Airway Organoid—Long-Term Expanding

Long term expanding human airway organoids were first reported by Sachs et al. (Sachs et al., 2018). These organoids were established from lung biopsies or cells isolated from BAL fluid and then cultured in media containing biochemical cues for self-renewal such as R-spondin, Noggin, fibroblast growth factor (FGF) and TGF-β inhibitors. The established organoids comprised of a polarized, pseudostratified airway epithelium containing basal cells, ciliated cells, mucus-producing goblet cells, and secretory club (Clara) cells. The airway organoids are amenable to passaging by mechanical disruption every other

week for at least a year, with no loss in proliferation reported up to P18. In addition, single cell suspensions dissociated from the indefinitely expanding airway organoids yield an improved 2-D ALI culture (Zhou et al., 2018). These cultures displayed full ALI differentiation by day 12–14 as opposed to 21–28 days for conventional cultures grown from 2-D basal cells (Zhou et al., 2018). Together, these findings suggest the possibility of expanding isolated primary lung epithelial cells in 3-D, given cells maintained under conditionally reprogrammed and dual SMAD inhibition culture conditions do not expand beyond P10 without reaching senescence (Mou et al., 2016; Gentzsch et al., 2017). The indefinite expansion of airway organoids also means in theory the availability of "endless" amounts of ready-to-use airway epithelial cells.

Human Intestinal Epithelial Cells

While work in CF research has primarily focused on primary airway epithelial cells (the gold standard), patient-derived intestinal epithelial cells also present an invaluable resource in characterizing the relationship between the CFTR gene mutation and the disease phenotype. More recently, they play an important role in the development of HTS strategies to elucidate novel drugs for the treatment of CF.

Intestinal Organoids

Compared to human airway tissue, colon tissue damage in CF patients is minimal and the rectal epithelium is accessible in a less invasive manner. The abundant expression of CFTR in the distal colon makes rectal biopsies an attractive cellular source for interrogating CFTR function (Hug et al., 2011). Assessment of intestinal current measurement (ICM) as a readout for CFTR activity has provided strong evidence for the diagnostic and prognostic utility of rectal biopsies (De Jonge et al., 2004; Hirtz et al., 2004; Mall et al., 2004; De Boeck et al., 2006; Taylor et al., 2009; Derichs et al., 2010; Sousa et al., 2012; Clancy et al., 2013; Cohen-Cymberknoh et al., 2013). ICM can be applied to biopsies collected from CF patients which are subsequently exposed to CFTR modulators, to assess the efficacy of treatment in a personalized manner. One concern is the possibility of reduced penetration of modulator drug into the biopsy tissues under *ex vivo* conditions. Other limitations to the use of rectal biopsies include the small number of biopsies collected (4–8 biopsies) and the need for all biopsies to be tested on collection day i.e., the biopsies cannot be preserved for further testing.

Emergence of intestinal organoids (or mini-guts) overcome these limitations by extending the use of rectal biopsies in cultures. Intestinal organoids can be grown from crypts isolated from freshly isolated rectal biopsies. Crypts are rich in $Lgr5^+$ stem cells which grow and differentiate into self-organizing, multicellular structures (Sato et al., 2009; Jung et al., 2011). These organoids contain all of the distinct intestinal cell types present in the *in vivo* epithelium (Leushacke and Barker, 2014). The growth and differentiation of $Lgr5^+$ stem cells into eventual closed epithelial structures with an internal lumen requires a fine balance of growth factors (R-spondin, EGF, Noggin, Wnt-3a), inhibitors of TGF-β and BMP signaling and the basement membrane matrix (matrigel). Intestinal organoids can be indefinitely cultured and remain genetically and phenotypically stable upon repeated passaging and long term culture (Ikpa et al., 2014). Capitalizing on the high expression of CFTR in rectal tissues and rapidly expanding stem cells, they make an attractive model for assessment of CFTR functional response in pharmacologic testing. They provide the added advantage to test combination of modulators without established safety profiles as part of pre-clinical evaluation in CF patients with rare CFTR genotype.

FORSKOLIN-INDUCED SWELLING ASSAY AS A PROXY FOR CFTR FUNCTION

The primary functional assay to assess CFTR activity in organoids, the forskolin-induced swelling (FIS) assay is CFTR dependent. This approach does not measure the net transepithelial ionic transport. Rather, forskolin is used to stimulate intracellular cAMP production which then activates CFTR at the plasma membrane. CFTR activation drives chloride and fluid flux to the organoid lumen (apical membrane facing inwards), causing rapid swelling of organoids with functional CFTR or those derived from non-CF subjects. This swelling effect is significantly reduced or absent in organoids derived from CF subjects who exhibit differing rates of FIS with different classes of CFTR mutations and also between individuals with the same CFTR mutation (Dekkers et al., 2013). There is accumulating evidence that the CFTR modulator-corrected FIS response is predictive of patient-specific clinical response, with close correlation observed between rescued swelling and improvement in lung function (measured by FEV1) and sweat chloride concentration (Dekkers et al., 2016a; Guimbellot et al., 2017; Brewington et al., 2018c). Intestinal organoids can be disrupted to single cells to generate 2-D-monolayers on porous membranes for electrophysiological studies. FIS was shown to positively correlate with forskolin-induced current in subject-matched organoid-derived monolayers, supporting the notion that CFTR-dependent fluid secretion in rectal organoids reflects CFTR-dependent ion transport (Zomer-van Ommen et al., 2018)

The FIS assay has been adapted for use in airway organoids but is much less well-characterized compared to rectal organoids (Guimbellot et al., 2017; Brewington et al., 2018c; McCarthy et al., 2018). Studies performed thus far, have involved cultures from small datasets of patients. Given the lack of standardized culture protocol for airway organoids, forskolin induces either swelling or shrinking of airway organoid depending on the orientation of CFTR-expressing apical epithelium (swelling for apical membrane facing inwards organoids and vice versa). Similar to rectal organoids, forskolin-induced changes in cross sectional area is dependent on the severity of CFTR genotypes, and could be altered with CFTR modulators. However, the changes are far smaller given the lower expression of CFTR in the airways compare to the intestine. It is noteworthy that only a single concentration of forskolin (10 μM) has been

interrogated in airway organoids FIS, while a total of eight concentrations (0.008–5 μM) were used in rectal organoids (Dekkers et al., 2016a; Guimbellot et al., 2017; Brewington et al., 2018c). To detect CFTR rescue with adequate resolution and sensitivity, higher-powered objectives are used resulting in lower throughput application compared to rectal (Dekkers et al., 2016a; Guimbellot et al., 2017; Brewington et al., 2018c). Establishment of a broader assay dynamic range may facilitate higher throughput use of FIS in airway organoids. Although preliminary, favorable correlation between *in vitro* FIS response to clinical response FEV1, sweat chloride concentration and BMI have been reported (Brewington et al., 2018c; McCarthy et al., 2018).

THE CELL MODEL OF THE FUTURE

In this Review, we described the rapidly developing field of organoid models specific to CF. Organoids, with their close resemblance to the human organs most affected by CF disease, hold great appeal for translational research. The capacity to adapt these models for assays such as FIS is important for theratyping and for conferring decisions on personalized CFTR pharmacotherapy. This provides an almost immediate application of *in vitro* research findings in the clinical setting. Nonetheless, it is apparent that each model has respective strengths and limitations (**Table 3**). The question as to which model has the essential features for precision medicine in CF and best predicts the long-term clinical benefits of a drug remains. Is it sufficient to use just one model and if so which cell model is best?

Intestinal organoids are the most developed so far amongst the 3-D model systems and seemingly an easier model to establish. But how representative is the intestinal epithelium to its respiratory counterpart? There are clear physiological differences between the airway mucosal surface and the gut. First, alternative ion channels critical for solute and water transport in the airways such as ENaC and calcium-activated chloride channels (CaCC), are either absent or present in negligible amounts in the gut to be functional (Rajendran et al., 2018). A modified swelling assay showed airway but not rectal organoids swell upon addition of E_{act}, an activator of the alternative chloride channel TMEM16A (Sachs et al., 2018). Second, there is no mucociliary clearance in the gastrointestinal tract. These differences highlight the need for organoids of bronchial or nasal epithelial origin to provide a closer resemblance to the *in vivo* airways. It then seems logical that one will need to choose the most appropriate 3-D cell culture model for each specific application.

AUTHOR CONTRIBUTIONS

SAW, NTA, and SLW wrote the manuscript with critical input from CKH, LKF, AK and AJ.

FUNDING

SAW, NTA, and SLW are supported by Sydney Children's Hospitals Foundation (SCHF). AK holds a Rothwell Family Fellowship.

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Conflict of Interest Statement: AK is a scientific advisor for Unizyme Laboratories A/S.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

PROTOCOLS
published: 26 October 2018
doi: 10.3389/fphar.2018.01176

In vitro Methods for the Development and Analysis of Human Primary Airway Epithelia

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Cystic fibrosis (CF) is a chronic disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, which encodes for a channel expressed at the apical surface of epithelial tissues. Defective chloride and bicarbonate secretion, arising from CFTR mutations, cause a multi-organ disease. In the airways, impaired ion transport results in a thick mucus, dehydration of the periciliar region and bacterial infections. Over the last years, basic research has sustained a great effort to identify therapies that are able to correct defective CFTR. For this purpose, *in vitro* cell models have played a key role in the study of mechanisms of the disease and to assess CFTR modulator therapies. Cultures of human primary bronchial epithelia are considered a physiologically relevant disease model due to their ability to maintain most of the morphological and functional characteristics of the airway epithelium *in vivo*. Despite their value, these cells are limited by the availability of human lung tissue and by the complexity of the culture procedure. However, primary human nasal cells can be considered as an alternative model for the study of CF pathophysiology since they are easier to obtain and recapitulate the properties of bronchial cultures. Over the years, several groups have optimized a protocol with key steps to culture and fully amplify differentiated primary airway epithelia. Our approach provides epithelia monolayers grown on porous filters, characterized by high transepithelial electrical resistance and an electrical potential difference. These parameters are required to perform electrophysiological experiments devoted to the study of ion transport mechanisms in airway epithelia. The aim of this study was to describe different methods to expand and differentiate isolated cells into fully polarized monolayers of airway epithelium, in order to provide an optimized protocol to support physiopathology analysis and to evaluate therapeutic strategies.

Keywords: cystic fibrosis, bronchus, airway epithelial cells culture, short-circuit current recording, transepithelial electrical resistance measurement, periciliary mucus properties

OPEN ACCESS

Edited by:
Miquéias Lopes-Pacheco, Universidade de Lisboa, Portugal

Reviewed by:
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Guido Veit, McGill University, Canada

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *01 August 2018*
Accepted: *28 September 2018*
Published: *26 October 2018*

Citation:
Gianotti A, Delpiano L and Caci E (2018) In vitro Methods for the Development and Analysis of Human Primary Airway Epithelia. Front. Pharmacol. 9:1176. doi: 10.3389/fphar.2018.01176

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene coding for the protein cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a chloride channel expressed at the apical surface of secretory epithelia where it plays an important role in salt and fluid homeostasis (Saint-Criq and Gray, 2017).

The consequences of mutated CFTR are most important in the airways. The resulting defective chloride secretion, coupled to enhanced sodium absorption, impairs normal mucociliary

clearance due to dehydration of the airway surface and consequently causes uncontrolled inflammation and chronic bacterial infections (Castellani and Assael, 2017).

Recently, bicarbonate has emerged as another important anion that contributes to the genesis of CF lung disease due to its role in mucus release (Quinton, 2008; Borowitz, 2015).

Over the last years, basic research has sustained a great effort to define a strategy to identify novel therapies that correct the basic defects responsible for CFTR loss-of-function.

Studies have demonstrated that pharmacological rescue of the CFTR protein, in the presence of small molecules called correctors and potentiators, can repair folding and gating defects (Pedemonte et al., 2005; Okiyoneda et al., 2013; Van Goor et al., 2009, 2011).

The most successful compounds, ivacaftor and lumacaftor developed by Vertex Pharmaceuticals company, have been approved for treatment of CF patients (Wainwright et al., 2015).

Drug discovery is supported by *in vitro* cell models that offer the opportunity to study epithelium physiology, the alterations caused by mutated CFTR, and the efficacy of therapies that aim to correct basic CF defects.

In vitro cell models for airway epithelium studies have been mostly based on primary human bronchial epithelial cells (hereafter termed HBEC), obtained from CF lung resection.

HBEC provide the ideal tool since they exhibit several of the morphological and functional defects of airway epithelia. Despite their value, it is difficult to acquire a large amount of cells and they can only be grown for 4–5 passages before reverting to a poorly differentiated phenotype.

Primary nasal epithelial cells (hereafter termed HNEC) have been recently proposed as an alternative method to HBEC culture.

HNEC are easy to collect by nasal brushing and recapitulate the properties of HBEC cultures (McDougall et al., 2008; Mosler et al., 2008). Furthermore, this model is very useful to predict the clinical treatment efficacy in patients (Pranke et al., 2017).

Several research groups have optimized protocols that allow isolation, expansion, and differentiation of primary HBEC and HNEC (Fulcher et al., 2005; Yaghi et al., 2010; Neuberger et al., 2011; Müller et al., 2013; Stokes et al., 2014).

Primary cells have a very limited proliferative capacity *in vitro* and it is possible to culture for at least five-six passages before noting a slowing down of cell growth. To overcome this problem, culturing of bronchial and nasal epithelial cells under CRC conditions, namely irradiated feeder cells and the RhoA kinase inhibitor Y, enhances cell growth and lifespan while preserving electrophysiological and morphological properties (Gentzsch et al., 2017).

The aim of this article was to present a detailed protocol, optimized in our laboratory, for culture and differentiation of airway epithelia. This method results in large-scale production of isolated HBEC and HNEC and fully differentiated epithelia that exhibit the morphological and functional defects of CF airways. Therefore, these cell models are very useful for improving our knowledge about physiopathology mechanisms involved in CF and to support therapeutics strategies.

Our approach is based on the isolation of airway cells from bronchi or nasal brushings obtained from CF and non-CF subjects. Then, isolated cells are cultured and expanded with a high proliferation rate using a proliferative serum-free medium. This step is followed by epithelial cell differentiation on permeable supports, whose ion transport properties can be evaluated with electrophysiological techniques (Galietta et al., 1998; Scudieri et al., 2012).

For this purpose, we review two powerful methods for ion transport measurements underlining the application, advantages, and limits. These include Ussing chamber and Trans-Epithelial Electrical Resistance (TEER) techniques (Li et al., 2004; Srinivasan et al., 2015).

Moreover, we describe a cell culture protocol to achieve a fully differentiated mucociliary airway epithelium to study the properties of periciliary mucus considering its important involvement in the CF pathology. Indeed, the reduction of fluid secretion in CF alters the composition of the airway surface fluid (ASL) and induces the production of mucus with rheological properties making it inadequate for normal physiology (Gianotti et al., 2016).

Finally, our culture protocol allows morphological and functional characteristics of the airway epithelium to be reproduced *in vivo,* supporting the study of CF physiopathology and therapeutic strategies.

MATERIALS AND EQUIPMENTS

Cell Culture

- Petri dishes, culture flasks, Pasteur pipette, physiological saline solution.
- Snapwell permeable support: 3801 (Corning, Tewksbury, MA, United States).
- HTS Transwell permeable support: 3379 (Corning).
- HBSS (hepes buffered saline solution): hepes (20 mM, Sigma #H4034, Saint Louis, MO, United States), sodium chloride (121.0 mM, Sigma #S9888), potassium chloride (2.7 mM, Sigma #P3911), glucose (9.4 mM, Sigma #G7528), sodium phosphate dibasic (7.2 mM, Sigma #S7907), phenol red (1:1000 of 0.5% stock solution, Sigma #P5530). Adjust pH to 7.4 and bring to volume; sterilize with a filter and store at +4°C.
- Protease solution: protease powder of *Streptomyces griseus* XIV (0.3 g, Sigma #P5147) dissolved in 130 ml HBSS and 30 ml Ham's F12 w/o L-glut. Prepare before using, sterilize with a filter and store at +4°C.
- Ca^{2+}/Mg^{2+} – free phosphate buffered saline (PBS).
- Ca^{2+}/Mg^{2+} – Dulbecco's phosphate buffer saline (D-PBS).
- Trypsin solution: Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS) containing 0.05% trypsin and 0.02% EDTA solution.
- Rat tail collagen solution: rat tail collagen (1 mg, Sigma #C7661) dissolved in 1 ml acetic acid 0.1 M.
- SMAD inhibitors cocktail: A 83-01 (1 μM, Sigma #SML0788), DMH1 (1 μM, Sigma #D8946).

- Rock inhibitor: Y-27632 2HCl (5 μM, Sigma #SCM075).
- Proliferative serum-free medium: See **Table 1** and **Supplementary Materials**. Sterilize with a filter and store at +4°C. This medium may be frozen. Protect from light with aluminum foil.
- Cryomedium: prepare a solution of fetal bovine serum (FBS) with 10% DMSO. Prepare before using, store at +4°C for no longer than 1 month.
- Medium with serum to neutralize trypsin: DMEM High glucose w/o L-glut, Ham's F12 w/o L-glut, FBS 10%, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM). Sterilize with a filter and store at +4°C.
- Differentiation serum-supplemented medium: See **Table 2** and **Supplementary Materials**. Sterilize with a filter and store at +4°C for no longer than 3 weeks. This medium may not be frozen, freezing seems to destroy the ability to induce differentiation and the appearance of electrical resistance. Protect from light with aluminum foil.

TABLE 1 | Proliferative serum-free medium.

Compound	Final concentration
LHC basal medium (Life Technology #12677-019)	500 ml
RPMI 1640	500 ml
Stock 4	5 ml/L
Stock 11	0.5 ml/L
Calcium stock	40.6 μM
Insulin stock	0.4325 μM
Transferrin stock	61.5 μM
EGF stock	0.403 nM
P/E stock	2.5 ml/L
RA/T3 stock	0.05 ml/L
Trace element solution	5 ml/L
HC stock	0.1 μM
Bovine pituitary extract (BPE) (Gibco #13028-014)	25 mg/L
Penicillin	100 U/ml
Streptomycin	100 μg/ml
L-glutamine	2 mM

TABLE 2 | Differentiation serum-supplemented medium.

Compound	Final concentration
DMEM High glucose w/o L-glut	500 ml
Ham's F12 w/o L-glut	500 ml
Insulin stock	0.4325 μM
Transferrin stock	61.5 μM
P/E stock	2.5 ml/L
RA/T3 stock	0.1 ml/L
HC stock	0.2 μM
Bovine pituitary extract (BPE) (Gibco #13028-014)	25 mg/L
New Zealand Serum	20 ml/L
Penicillin	100 U/ml
Streptomycin	100 μg/ml
L-glutamine	2 mM

- Differentiation serum-free medium: See **Table 3** and **Supplementary Materials**. Sterilize with a filter and store at +4°C for no longer than 3 weeks. This medium cannot be frozen, freezing seems to destroy ability to induce differentiation and the appearance of electrical resistance. Protect from light with aluminum foil.

Electrophysiological Equipment

- Epithelial voltohmmeter: EVOM2 [World precision Instrument (WPI), Sarasota, FL, United States].
- Voltage-clamp (DVD-1000 voltage/current clamp, WPI Instruments).
- PowerLab 4/25 (AD Instruments, Colorado Springs, CO, United States).
- Ag/AgCl electrodes: EK1 (WPI Instruments).
- Agar salt bridges (1 M potassium chloride in 2% agar).
- Ussing chambers (Vertical Diffusion Chamber, Warner Instruments, Hamden, CT, United States).
- Latex tubes for perfusion.
- Ringer's Solution for Ussing chamber: sodium chloride (126 mM, Sigma #S9888), potassium dihydrogen phosphate (0.38 mM, Sigma #NIST200B), potassium phosphate dibasic trihydrate (2.13 mM, Sigma #P9666), magnesium sulfate heptahydrate (1 mM, Sigma #M9397), calcium chloride dihydrate (1 mM, Sigma #C8106), glucose (10 mM, Sigma #G7528), sodium bicarbonate (24 mM, Sigma #S5761), phenol red (1:1000 of 0.5% stock solution, Sigma #P5530). Sterilize with a filter and store at +4°C.
- Coon's modified Ham's F-12 medium for TEER: hepes (20 mM), FBS 10%, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM). Sterilize with a filter and store at +4°C.
- Amiloride hydrochloride (Sigma #A7410), CPT-cAMP (Sigma #C3912), forskolin (Selleckchem #S2449, Munich, Germany), genistein (Sigma # G6649), lumacaftor (VX-809, Selleckchem #S1565), ivacaftor (VX-770, Selleckchem #S1144), CFTR-inh172 (Selleckchem

TABLE 3 | Differentiation serum-free medium.

Compound	Final concentration
DMEM High glucose w/o L-glut	500 ml
Ham's F12 w/o L-glut	500 ml
Insulin stock	0.87 μM
Transferrin stock	123 μM
EGF stock	0.806 nM
Epinephrine stock	2.73 μM
RA stock	0.099 μM
T3 stock	0.01 μM
HC stock	1.4 μM
Bovine pituitary extract (BPE) (Gibco #13028-014)	50 mg/L
BSA stock	22.58 μM
Penicillin	100 U/ml
Streptomycin	100 μg/ml
L-glutamine	2 mM

#S7139), UTP (Sigma #U6625), ATP (Sigma #A9187), PPQ-102 (Sigma #219677).

STEPWISE PROCEDURES

Primary HBEC and HNEC Culture

In this step, we describe a cell culture protocol which can provide, from a relatively little amount of sample, a large number of polarized bronchial and nasal epithelia useful for the study of airway physiopathology and predicting *in vivo* drug efficacy.

This method is based on two distinct phases:

1. Expansion of HBEC and HNEC using a proliferative serum-free medium. This culture medium allows the expansion of airway epithelial cells with a high proliferation rate and a low density growth for at least five passages before cell proliferation slows down. Cumulative population doubling level at fifth passage is approximately 33.
2. Differentiation of epithelia on permeable supports using a "air liquid interface" (ALI) condition:
   - serum-supplemented medium condition: cells differentiate into fully polarized monolayers of airway epithelium in order to perform electrophysiological experiments.
   - serum-free medium condition: cells form a fully differentiated mucociliary airway epithelium useful for the study of periciliary fluid properties of airways (Hajj et al., 2007).

Isolation and Expansion of Primary HBEC Derived From CF and Non-CF Donors Following Lung Transplantation

1. Dissect and wash the mainstem human bronchus, derived from CF and non-CF individuals undergoing lung transplant, with physiological saline solution. Transfer the sample into a saline solution at +4°C in the laboratory and process within 4 h.
2. To clean the bronchus of the mucus, wash the sample twice in a 50 ml conical tube containing physiological saline solution.
3. Place the bronchus overnight at +4°C in protease XIV solution in order to separate the epithelial cell layer from the underlying connective tissue.
4. Remove the bronchus gently from the protease XIV solution and place it in a 100-mm Petri-dish containing physiological saline solution. Collect epithelial cells by vigorously flushing the bronchial lumen with physiological saline solution (**Figure 1**).
5. Pellet the detached epithelial cells by centrifugation at 900 rpm for 10 min.
6. Resuspend the cells in 3 ml of trypsin solution, gently pipette five times using a 1-ml pipette tip and incubate at 37°C for 5 min.
7. Neutralize trypsin solution by adding an equal volume of medium with serum.

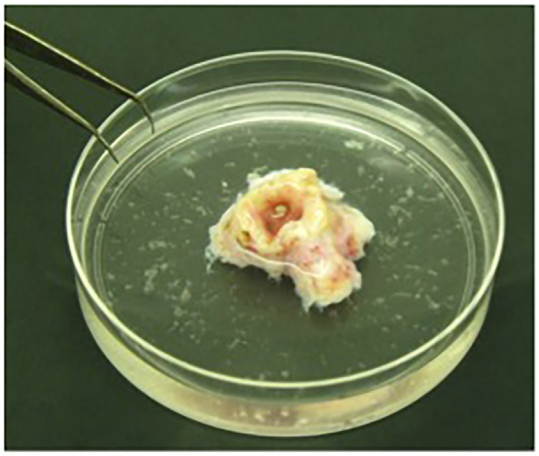

FIGURE 1 | Detached epithelial cells isolated from bronchus. Bronchus is placed in a 100-mm Petri-dish and epithelial cells are collected by flushing the bronchial lumen vigorously with physiological saline solution.

8. Add proliferative medium to bring the final volume to 35 ml.
9. Count the cells to determine the cell yield and then centrifuge at 900 rpm for 10 min.
10. Coat a T150 flask with 100 $\mu l/cm^2$ of rat tail collagen solution and place in an incubator at 37°C (5% CO_2) for at least 15 min or up to 6 h. Before use, carefully aspirate the collagen solution with a Pasteur pipette.
11. Resuspend the pellet in proliferative medium in order to seed approximately 8000 cells/cm^2 in the collagen-coated flask and incubate at 37°C (5% CO_2).
12. To expand the cell culture, replace the proliferative medium every 24 h until the cell density reaches 70% confluence.
13. When the bronchus was derived from a CF patient, treat the medium with antibiotics for the first 6 days to eradicate bacterial contamination. The mixture of antibiotics is designed based on the results of the antibiogram of the bacteria isolated from the patient's most recent expectorate. If the antibiogram is not available use the following cocktail: colistimethate sodium, piperacillin/tazobactam. Use a specific antibiotic cocktail at a 10X concentration for the first 48 h, then reduce every two days by 1:2 to minimize cell toxicity (**Table 4**).
    To overcome fungal contamination, treat the medium with 2.5 $\mu g/ml$ of amphotericin B for at least the first 7 days of culture.
14. When the cell density reaches 70% confluence, harvest HBEC for further expansion or freeze aliquots for future use.
15. To detach HBEC, remove the proliferative media and add 15 ml of warm $Ca^{2+}Mg^{2+}$-free phosphate buffer solution (PBS) and place at 37°C (5% CO_2) for 6 min.
16. Remove PBS and add 2.4 ml of trypsin solution and incubate at 37°C (5% CO_2) until detachment.

TABLE 4 | Antibiotic and antifungal dose concentrations suggested for CF cell culture treatment.

Antibiotic	Dose 10x	Antibiotics type	Indications
Tobramycin	8 μg/mL	Aminoglycoside	Gram-negative Bacteria
Amikacin	25 μg/mL	Aminoglycoside	Gram-negative Bacteria
Gentamicin	40 μg/mL	Aminoglycoside	Gram-negative Bacteria
Piperacillin-Tazobactam	50 μg/mL	Beta-lactamase inhibitors	Gram-positive Bacteria
Ticarcillin	250 μg/mL	Beta-lactamase inhibitors	Gram-positive Bacteria
Imipenem	2000 μg/mL	Beta-lactamase inhibitors	Gram-positive Bacteria
Meropenem	250 μg/mL	Beta-lactamase inhibitors	Gram-positive Bacteria
Ceftazidime	250 μg/mL	Beta-lactamase inhibitors	Gram-positive Bacteria
Oxazolidinone (Linezolid)	2 mg/mL	Inhibitor on the ribosomal 50S	Gram-positive Bacteria
Teicoplanin	60 μg/mL	Semisynthetic glycopeptide	Gram-positive Bacteria
Vancomycin	50 μg/mL	Glycopeptide	Gram-positive Bacteria
Ciprofloxacin	25 μg/mL	Fluoroquinolone	Gram-positive and negative Bacteria
Levofloxacin	100 μg/mL	Fluoroquinolone	Gram-positive and negative Bacteria
Trimethoprim + Sulfamethoxazole	25 μg/mL	Sulfonamides	Gram-positive and negative Bacteria
Colistimethate sodium	50 U/mL	Polymyxin	Multi-drug resistant Bacteria
Amphotericin B	25 μg/mL	Macrocyclic polyene	Antifungal

17. Neutralize the trypsin solution by adding an equal volume of medium with serum.
18. Add 20 ml PBS and gently swirl the flask to collect the cells.
19. Transfer the cell suspension to a 50 ml conical tube.
20. Count the cells to determine the cell yield. The expected yield for one T150 flask is ∼6 million cells.
21. Centrifuge at 1500 rpm for 5 min.
22. Resuspend the pellet in 10 ml of proliferative medium and split the cell culture between 3 collagen-coated T150 flasks for further expansion.
23. Replace the growth media every 24 h during the cell expansion phase.
24. Freeze cells every cell splitting in order to have a bio-bank for every passage.
25. For this, resuspend cell culture in cryoprotective-medium at a density of 2 million cells/ml. Place 1 ml of this culture into each cryovial and freeze in a cell freezing container at −80°C for at least 24 h before transferring to a liquid nitrogen tank for long-term storage.
26. To thaw frozen HBEC, place the cryovial in a 37°C water bath for 2 min and then transfer the cell culture into a 15 ml conical tube containing proliferative medium. Centrifuge at 1500 rpm for 5 min.
27. Coat 2 T75 flasks with 100 μl/cm^2 of rat tail collagen solution and place in an incubator at 37°C (5% CO_2) for at least 15 min or up to 6 h. Before use, aspire carefully the collagen solution with a Pasteur pipette.
28. Resuspend the pellet in 8 ml proliferative medium and split the culture in 2 collagen-coated T75 flasks already containing 8 ml growth medium/flask. Replace medium every 2 days and maintain culture as described above.

Isolation and Expansion of Primary HNEC Derived from CF and Non-CF Donors Following Nasal Brushing

1. Collect HNEC by nasal brushing of both nostrils. The nasal brushing is performed without local anesthesia after nasal washings with physiological saline solution, in order to remove the mucus (two washes a day in the previous week and a wash immediately before nasal cells collection).
2. Use a soft sterile interdental brush with 2.5–3 mm bristles scraping along the middle portion of the inferior turbinate with anterior-posterior rotational movements in each nostril, under direct vision.
3. Carefully monitor patient for vital and minor signs, comfort and pain.
4. Place brush in a 15 ml conical tube containing 9 ml of $Ca^{2+}Mg^{2+}$-D-PBS, transfer the sample to the laboratory and process within 4 h.
5. Place the sample in a 37°C water bath for 20 min and then centrifuge at 900 rpm for 5 min to detach all cells from brush.
6. Coat 1 T25 flask with 100 μl/cm^2 of rat tail collagen solution and place in incubator at 37°C (5% CO_2) for at least 15 min until 6 h. Before use, carefully aspirate the collagen solution with a Pasteur pipette.
7. Carefully remove the brush and resuspend the cell pellet in 6 ml of proliferative medium and seed it in the coated T25 flask. To improve growth rate and to extend the number of population doublings *in vitro*, the culture medium is supplemented with a SMAD and Rock inhibitors cocktail required for the conditional reprogramming and immortalization of human epithelial cells (Palechor-Ceron et al., 2013; Mou et al., 2016).
8. Maintain cultures as previously described for HBEC.

Generation of Differentiated Nasal and Bronchial Epithelia With Serum-Supplemented Medium

1. Culture and detach HBEC and HNEC on the fourth passage when the cell density reaches 90% confluence.

2. Count the cells to determine the cell yield. The expected yield for T75 and T150 flasks is ∼4 and 8 million cells, respectively.
3. Seed 500.000 cells/insert on Snapwell permeable supports in 500 μl proliferative medium. Add 2 ml proliferative medium on the basolateral side.
4. Alternately, seed 200.000 cells/insert on HTS Transwell permeable supports in 300 μl proliferative medium. Add 25 ml proliferative medium in the bottom well.
5. After 24 h, replace the proliferative medium with differentiation serum-supplemented medium. Add 500 μl and 2 ml of medium on the apical and basolateral sides, respectively, on a Snapwell insert.
6. Alternately, add 200 μl to the apical side and 25 ml in the bottom well of the HTS Transwell insert.
7. Replace medium daily on both sides of the permeable supports until the sixth day (liquid-liquid culture).
8. Check differentiation of cells into a tight epithelium by measuring transepithelial electrical resistance and potential difference with an epithelial voltohmmeter EVOM2.
9. Good values measured in Snapwell supports are ∼−40 mV and ∼−30 mV for CF and non-CF epithelia, respectively, and 1000–1700 Ω for both genotypes.
10. Subsequently, totally remove the apical medium (ALI condition) and replace medium every two days only in the basolateral side. This condition favors a fully differentiated epithelium.
11. Maintain HBEC and HNEC culture for 2–3 weeks.

Generation of Differentiated Nasal and Bronchial Epithelia With Serum-Free Medium

1. Culture and detach HBEC and HNEC on the fourth passage when cell density reaches 65% confluence.
2. Coat the Snapwell support with 200 μl of rat tail collagen solution and place in an incubator at 37°C (5% CO_2) for at least 15 min or up to 6 h. Before use, carefully aspirate the collagen solution with a Pasteur pipette.
3. Count and seed 150.000 cells/insert on Snapwell supports in 500 μl proliferative medium. Add 2 ml proliferative medium to the basolateral side.
4. After 24 h, replace the proliferative medium with fresh medium at both sides of the permeable support.
5. After 24 h, replace the proliferative medium with serum-free differentiation medium only in the basolateral side (ALI condition).
6. Replace medium every 2 days only on the basolateral side of the permeable support until the 15th day.
7. Check differentiation of cells into a tight epithelium by measuring transepithelial electrical resistance and potential difference with an epithelial voltohmmeter EVOM2.
8. Good values are ∼−8 for CF and ∼−5 for non-CF epithelia, respectively, and 600–800 Ω for both genotypes.
9. Maintain the ALI culture condition epithelia for 4–5 weeks.

HBEC and HNEC Monolayers Application

Well differentiated HBEC and HNEC epithelia are useful to support the following studies:

1. Electrophysiological experiments aimed at the study of ion transport mechanisms in airway epithelia.
2. Analysis of protein expression with immunofluorescence or Western Blot techniques.
3. Gene expression analysis by RT-qPCR, microarray or RNAseq.
4. Investigation of innate defense mechanisms and response to proinflammatory stimuli.

Important Remarks

1. During the initial isolation from bronchus, columnar cells with rapidly beating cilia should be observed indicating good cell viability. Beating cilia cells will disappear in the culture condition over time. The day after plating, isolated cells from bronchus, many red blood cells, and fibroblasts should be present in the culture and disappear after washing the flask with physiological saline solution. Small spots of epithelial cells should be observed.
2. HBEC from CF patients are frequently infected with antibiotic-resistant organisms. Particular care has been given to eradicate bacterial and fungal contamination with appropriate treatments.
3. During cell expansion HBEC and HNEC can be split up to four times to expand the number of cells available for functional studies. Further expansion may frequently lead to worsening of epithelia performance.
4. During expansion, special care must be taken when splitting cells into new flasks that the cell density reaches no more than 70% confluence. A higher density could induce a squamous differentiation (Sacco et al., 1992) that debars desired morphological and functional properties (**Figure 2**).
5. Measure transepithelial electrical resistance and potential difference with a voltohmmeter to check differentiation of polarized epithelia. We have observed that after 6 days of culture, the value of potential difference in CF epithelia is larger than non-CF.

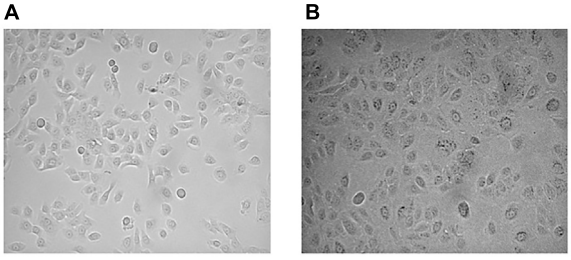

FIGURE 2 | Epithelial cell culture density in the proliferative phase. Epithelial cells with a good morphology and a high proliferation rate **(A)**. Epithelial cells with an increasing number of large squamous cells and decreased cell growth **(B)**.

6. The following remarks contribute to the development of polarized epithelia:
   - Detach cells at the proper growth density.
   - Seed the proper amount of cells on a permeable support.
   - Replace differentiation medium every day.

Recording Transepithelial Ion Transport

The airway epithelium balances Na^+ absorption and Cl^- secretion to carefully regulate the properties of the ASL in order to ensure efficient mucociliary clearance.

Here we present electrophysiological approaches to perform transepithelial ion transport measurements. Moreover, we highlight the applications, the strengths, and limitations of the techniques.

Short-Circuit Current Recordings Using the Ussing Chamber Technique

The Ussing chamber provides a system to measure the transport of ions across epithelial tissues.

Although it can be complicated to perform, it is considered the gold standard for electrophysiological experiments with the aim of studying epithelial transport.

In the most used configuration, the short-circuit current (Isc) technique, the transepithelial potential difference is clamped at zero with a voltage clamp amplifier and the resulting current is recorded.

The Ussing system consists of two hemi-chambers separated by the monolayer of epithelial cells grown on Snapwell supports, a perfusion system, an amplifier, and a data acquisition system.

Both hemichambers are fill with a Ringer's solution, continuously bubbled with a gas mixture containing 5% CO_2 −95% air that maintains the physiological pH. The temperature of the system is kept at 37°C. The voltage-clamp is connected to the apical and basolateral chambers with Ag/AgCl electrodes and agar bridges (two for voltage and two for current). The short-circuit current is recorded with a PowerLab 4/25 analog-to-digital converter connected to a computer.

A typical experiment is conducted as follows:

1. Fresh culture medium is replaced the day before the experiment. The medium may contain drugs or compounds. For example, epithelia from F508del patients may be treated with 1 μM lumacaftor to rescue mutant CFTR protein. As a control, cells are treated with an equal amount of control vehicle (usually DMSO at 0.1%; higher amounts of DMSO may significantly alter epithelial properties).
2. Snapwell support, carrying differentiated bronchial or nasal epithelia, is mounted in a vertical diffusion Ussing chamber.
3. Add 5 ml/hemichamber of warm Ringer's solution and connect each side with the latex tubes that supply the gas mixture for solution bubbling.
4. Insert the current and voltage electrodes into each hemichamber and connect to the voltage-clamp amplifier (follow the instructions of the amplifier manufacturer for correct procedures).
5. Under open-circuit conditions, measure the spontaneous transepithelial electrical potential difference. Then switch the amplifier to voltage-clamp configuration and measure the resulting Isc. Calculate the transepithelial electrical resistance from the values of the transepithelial electrical potential difference and the short-circuit current with Ohm's law. Low resistance values (below 500 Ω) may reflect a "leaky" epithelium that might increase paracellular ion flux.
6. Start recording and allow the transepithelial current to stabilize (10 min) before adding stimuli.
7. Add 10 μM amiloride to apical side to block the ENaC channel. The amplitude of the current drop caused by amiloride is dependent on the expression/function of ENaC. However, it should be kept in mind that experiments are carried out on an intact epithelium. Therefore, transepithelial ion transport is dependent on the coordinated activity of different ion channels and transporters. Changes in amiloride-sensitive current may also occur following modified expression/function of proteins that affect intracellular ion concentrations and membrane potential.
8. Stimulate CFTR channel by adding 5 μM forskolin or 10 μM CPT-cAMP (apical and basolateral sides). Both agents activate CFTR as cAMP agonists therefore they stimulate transepithelial Cl^- and bicarbonate transport. Anion secretion may be also promoted by activation of cAMP-dependent basolateral proteins (e.g., K^+ channels) that generate the required electrochemical driving force.
9. In the case of epithelia from CF patients with CFTR channel gating mutations, add 20 μM forskolin or 100 μM CPT-cAMP in order to maximize CFTR activation, and subsequently add a potentiator compound on apical side (e.g., 50 μM genistein or 1 μM ivacaftor) to further stimulate channel opening.
10. Once the CFTR-dependent current is stabilized, add the CFTR blocker $CFTR_{inh}$-172 (10 μM). The amplitude of the current drop elicited by the inhibitor reflects the extent of CFTR function. However, as discussed for ENaC, other proteins also contribute to CFTR-depedent anion secretion.
11. In the presence of amiloride and $CFTR_{inh}$-172, the activity of other channels can be recorded. For example, stimulation with Ca^{2+} agonists (e.g., apical UTP or ATP) elicits anion secretion through Ca^{2+}- dependent chloride channel (ANO1, a.k.a.TMEM16A).
12. Stop the recording and save the trace.

Ussing Chamber Technique Application

Using *ex vivo* airway epithelia, this technique gives a representation of the functional characteristics and processes involved in the physiological environment.

1. Measurement of physiological ion transport in non-CF epithelia.
2. Study of alterations to ion transport occurring in CF or other pathologies.

3. Evaluation of therapeutic interventions.

Important Remarks

1. Ussing chamber is a valuable technique but its disadvantages include technical expertise, the cost, and space needed for the equipment.
2. It is limited to polarized tissue and it does not allow analysis of a large number of samples, thus this technique has a relatively low throughput.
3. Ussing chamber is not suitable for measuring electroneutral transporters such as the Cl^-/HCO_3^- exchanger (e.g., pendrin protein).
4. Ussing chambers are made of plastic polymers, therefore some compounds used in the experiments (particularly those that work at very low concentrations such as ivacaftor) can penetrate plastic surfaces and be released in subsequent experiments thus altering the results. To overcome this contamination problem, we recommend that the chambers be washed at the end of each experiment in a sodium phosphate tribasic solution (15 g diluted in 1 l of deionized water) for 4 h and then in hydrochloric acid 0.1% solution for 15 min. After that, rinse in deionized water.

   Agar salt bridges represent another source of contamination. We suggest that the bridges be replaced at the end of each experiment.

Electrical Epithelial Properties Measured With the Trans-Epithelial Electrical Resistance (TEER) – Potential Difference (PD) Technique

TEER/PD is a rapid and simple method to quantify ion transport by measuring the electrical resistance and potential difference across the epithelium. TEER/PD is dependent on the expression/activity of channels situated on both apical and basolateral membranes as well as on the properties of tight junctions that control paracellular ion transport.

TEER/PD measurements are performed using the EVOM2 system and placing Ag/AgCl "chopstick" electrodes on both sides of an epithelial monolayer cultured on HTS Transwell permeable supports.

The method is simply based on the separate measurement of TEER and PD values for each epithelium, under resting conditions and following the application of agonists and inhibitors. The equivalent short-circuit current is then calculated from TEER and PD.

Considering the simplicity of the measurement and the data reproducibility compared with the Ussing chamber method, TEER/PD measurements are a good benchmark tool for high-throughput drug discovery screening in CF epithelia.

1. Culture bronchial or nasal epithelial monolayers in HTS Transwell inserts as previously described.
2. Replace the medium the day before the experiment. If needed, treatments can be included (e.g., lumacaftor or other correctors for CF epithelia).
3. Wash the chopstick electrodes with deionized water and equilibrate the electrodes in a Coon's modified solution.
4. Incubate both sides of the epithelia with Coon's modified solution for 1 h at 37°C.
5. Measure TEER and PD under basal conditions.
6. Add 10 μM amiloride solution on the apical side. Blocking ENaC should result in a higher TEER and lower PD due to inhibition of the sodium-dependent current.
7. Wait 10 min at 37°C before recording measurements.
8. Replace the apical and basolateral solution with new solution containing the CFTR activating cocktail (5–20 μM forskolin and, if needed, 50 μM genistein). The apical side should also contain amiloride. Wait 10 min before taking the measurement. It should be noted that genistein is preferred as a low potency CFTR potentiator instead of the high potency ivacaftor. Ivacaftor may stick to electrodes and affect subsequent experiments.
9. The final measurement is performed by adding 30 μM PPQ-102, a CFTR inhibitor, on the apical side. This is done by replacing the apical solution with a new one containing PPQ-102 together with amiloride and a CFTR-activating cocktail at the indicated concentrations. The change in equivalent current elicited by the inhibitor reflects the extent of CFTR function.
10. Wash electrodes with deionized water and 70% ethanol solution.

Important Remarks

1. In the TEER method, CFTR-inh172 does not significantly reduce conductance. We have tested different inhibitors and we have observed that PPQ-102 is the best one for completely blocking CFTR activity (Tradtrantip et al., 2009).

Fluid Absorption Rate Measurements

In CF airways defective ion transport causes a simultaneous reduction of fluid, chloride, and bicarbonate secretion. The altered properties of mucus may be studied in order to understand the pathological mechanisms and to evaluate treatments for recovering hydration of the airways.

Epithelia with serum-free medium reproduces ASL periciliary characteristics with mucus and fluid secretion.

In this section, we present a protocol that allows the measurement of fluid absorption rate.

1. Epithelia begins to secrete mucus and fluids on the apical side after nearly 15 days of culture on Snapwell supports. A fully differentiated epithelia is obtained after 4 weeks of culture.
2. After 15 days of culture, wash the apical surface once a week with D-PBS to remove secreted mucus.
3. Use some of the epithelia of the same cell preparation for short-circuit current recordings to check appropriate ion transport.
4. Apply 130 μl D-PBS on the epithelium surface. We suggest that the apical fluid to recovered between 3 and 48 h after D-PBS incubation.
5. Weigh an empty 1.5 ml collection tube (tare).

6. Carefully collect the fluid remaining after incubation with a pipette and place it in the collection tube (**Figure 3**).
7. Weigh the tube again and estimate the final apical volume collected:
   Vf = (Wf-Wi) × 000/N. Where Wf = tare and collected fluid weight; Wi = tare weight; N = number of pool samples.
8. Calculate fluid transport rate: Jw = (Vi−Vf)/A × t. Where Vi = initial apical volume (130 µl); Vf = final apical volume; A = area of the epithelium (for Snapwell insert: 1.13 cm^2); t = time interval.

Important Remarks

1. During the first days of ALI culture, it is normal to observe some liquid (from the basolateral medium) on the epithelium surface due to the fact that the tight-junctions are not completely formed. This condition will persist in a leaky epithelium.
2. CF secreted mucus is more viscous and difficult to pipette compared to non-CF mucus.

ANTICIPATED RESULTS

To overcome the scarcity of biological material available for primary culture, our group has developed a protocol to extract primary airway epithelial cells from lung resections or nasal brushing and subsequently culture and amplify these cells, maintaining most of the characteristics of the airway epithelium *in vivo*.

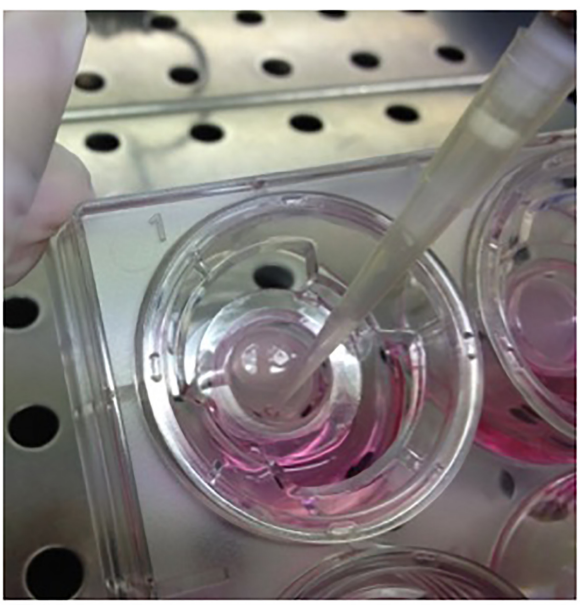

FIGURE 3 | Collection of fluid and mucus secreted from differentiated epithelia. Periciliar fluid collection from surface epithelia after D-PBS incubation.

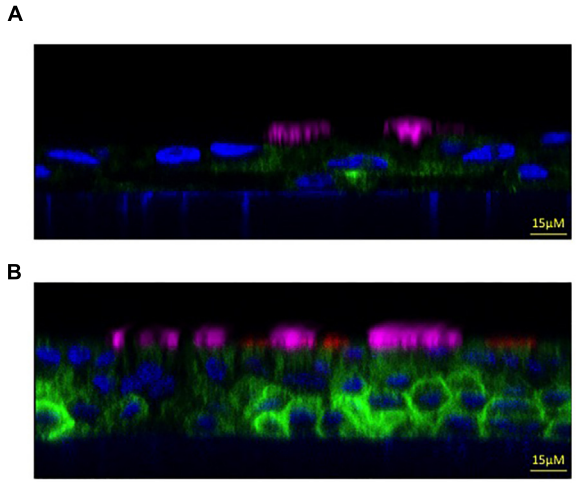

FIGURE 4 | Morphologic properties showing a mature airway epithelium. Representative *xz* immunofluorescence images taken with a confocal microscope (Leica SP8 Laser Scanning Confocal Microscope) to show non-CF bronchial epithelia differentiated with serum-supplemented medium **(A)** or with serum-free medium **(B)**. Cilia are labeled in magenta (anti-tubulin antibody), the goblet cells in red (anti-MUC5AC antibody), the apical membrane in green (anti-TMEM16A antibody) and the nucleus in blue (Hoechst dye). Images kindly provided by Dr. Paolo Scudieri (TIGEM, Pozzuoli, Italy).

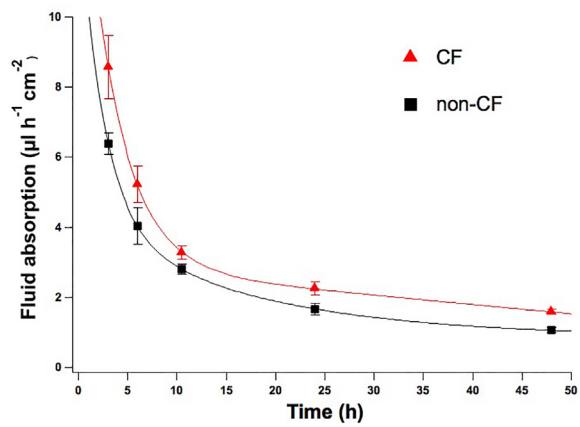

FIGURE 5 | Time course rate of fluid absorption of non-CF and CF epithelia. Net fluid absorption rate measured on non-CF (black squares) and CF (red triangles) epithelia up until 48 h of incubation with D-PBS. Mean values ± SEM are shown, at the timepoints of 24 and 48 h the differences between CF and non-CF epithelia are significant ($p < 0.05$).

The procedure starts by using a proliferative medium that promotes cell growth at a low density and a high proliferation rate in order to obtain a large number of polarized bronchial and nasal epithelia useful for the study of CF physiopathology.

This is followed by a differentiation phase. We have presented two protocols that both require seeding cells on permeable supports and the "ALI" condition, but they differ depending on the final aim of the designed experiments.

The serum-free medium protocol provides a fully differentiated epithelial monolayer that is useful for evaluating morphological and periciliary fluid properties in primary airway epithelia. However, a critical point of this protocol is the prolonged culture time and the cost of medium supplements.

Instead, we suggest that the serum-complemented medium be applied to the protocol for functional electrophysiologic assays, because it still provides a differentiated monolayer with transepithelial ion transport properties similar to *in vivo* conditions, but in a shorter time and with lower costs compared to the serum-free medium protocol. Therefore, this medium is optimal for functional electrophysiologic assays.

To compare the two protocols for evaluating epithelial morphological properties, we have used immunofluorescence combined with confocal microscopy. This technique provides high-resolution imaging of protein localization, expression patterns, and different cell types that characterize the airway of mature epithelium.

Cultured bronchial epithelia are analyzed by immunofluorescence for acetylated tubulin and MUC5AC to identify ciliated and goblet cells, respectively. To distinguish the apical from the basolateral membrane we have used an antibody against the ZO-1 tight junction marker.

The derived staining shows that epithelium differentiated with serum-supplemented medium is pseudo-stratified with a relatively small number of ciliated and goblet cells (**Figure 4A**).

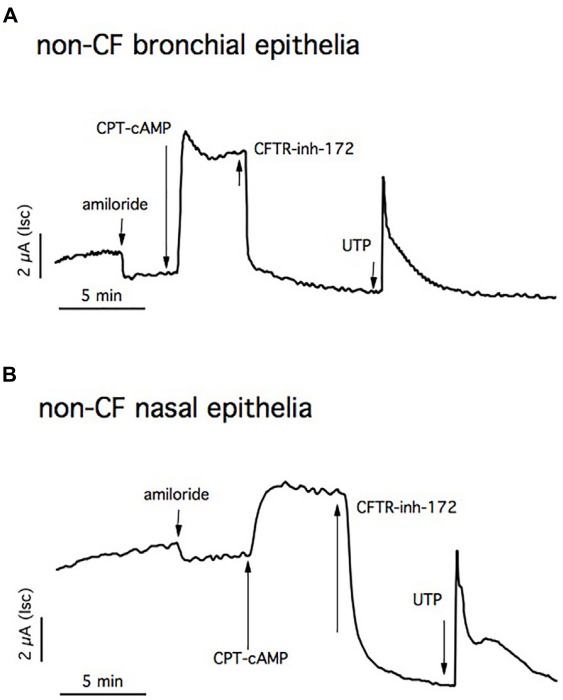

FIGURE 6 | Short circuit currents (Isc) measurements in non-CF epithelia. Representative traces from Ussing chamber recordings of non-CF bronchial **(A)** and nasal epithelia **(B)**. The ENaC channel is blocked with apical amiloride and CFTR activation and inhibition is obtained by adding CPT-cAMP and $CFTR_{inh}$-172, respectively. The Ca^{2+}- dependent chloride channel is stimulated with UTP.

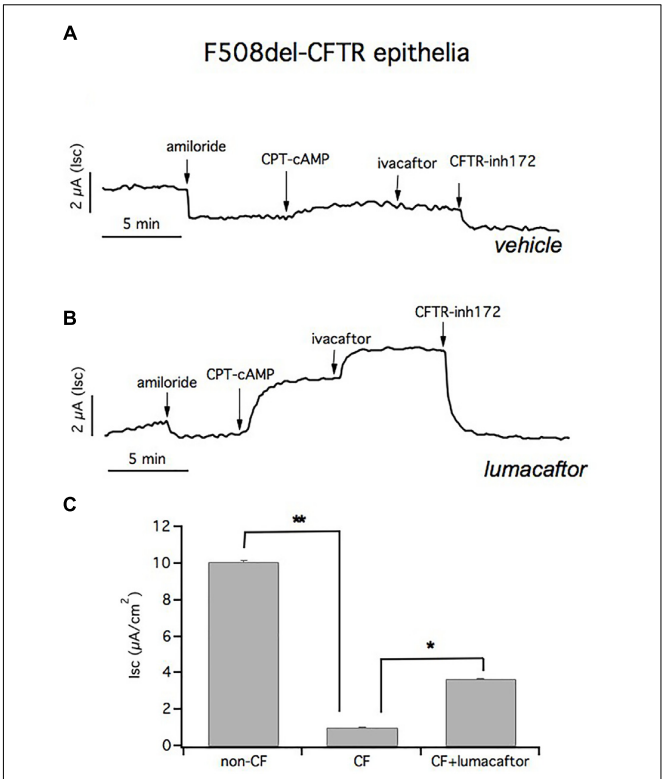

FIGURE 7 | Short circuit currents (Isc) in CF and in lumacaftor-treated CF epithelia. Representative traces from short circuit current (Isc) recordings of homozygous F508del-CFTR epithelia treated with vehicle **(A)** and lumacaftor **(B)**. The ENaC channel is blocked with apical amiloride and CFTR activation and inhibition is obtained by adding CPT-cAMP plus ivacaftor and $CFTR_{inh}$-172, respectively. **(C)** Graph bars summarizing CFTR-mediated currents from Ussing chamber recordings of HBEC derived from 4 non-CF donors (n = 22) and from 4 homozygous F508del-CFTR patients (n = 41). Mean values ± SEM are shown, * and ** indicate $p < 0.05$ or $p < 0.001$, respectively.

On the contrary, when using the serum-free medium protocol we obtained a higher pseudo-stratified epithelium characterized by a higher number of ciliated and goblet cells (**Figure 4B**). This result demonstrates that the latter culture condition provides most of the morphological characteristics of the airway epithelium *in vivo*, thus we suggest that it be used to analyse the expression and localization of selected proteins and airway cells.

In the CF lung, the decrease in CFTR-mediated chloride and fluid secretion are believed to cause the dehydration of the airway surface by altering the properties of periciliary mucus. To gather significant information, we have applied the serum-free medium protocol, that reproduces ASL periciliary characteristics with mucus and fluid secretion, to analyse the rate of fluid absorption of non-CF and CF airway mucus recovered from cultured HBEC.

Time course experiments confirmed that the different activities of CFTR in non-CF and F508del-CFTR epithelia resulted in significant differences in the net absorption rate after 24 and 48 h; reflecting the different ASL characteristics.

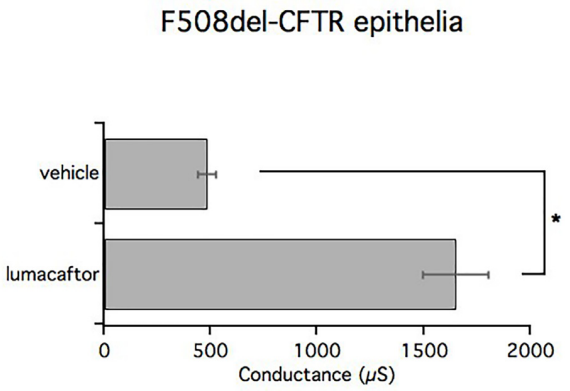

FIGURE 8 | TEER measurements in CF and lumacaftor-treated CF. Graph bars indicate TEER measurements performed on homozygous F508del-CFTR epithelia derived from 4 homozygous F508del-CFTR patients (n = 12) treated with vehicle or lumacaftor. The bar graphs show the electrical resistance values measured before and after CFTR inhibition, converted into their reciprocal conductance. Mean values ± SEM are shown, * indicate $p < 0.05$.

While non-CF epithelia absorbed after 24 and 48 h 1.67 ± 0.15 μl/h/cm^2 (n = 5) and 1.06 ± 0.06 μl/h/cm^2 (n = 7), respectively, in F508del-CFTR epithelia net absorpion rate was 2.25 ± 0.19 μl/h/cm^2 (n = 5) and 1.58 ± 0.07 μl/h/cm^2 (n = 7) (**Figure 5**).

Moreover, during recovery we observed that the CF fluid is more viscous and difficult to pipette compared to non-CF.

The development and validation of CF-HBEC aims to support drug discovery efforts to correct basic CF defects. For this purpose, electrophysiological approaches allow transepithelial ion transport to be measured in order to assess the efficacy of pre-clinical CFTR modulators in primary human airway cultures.

We have used the Ussing chamber technique to measure short-circuit current as an indication of net ion transport across bronchial and nasal epithelia monolayers maintained in serum-supplemented medium.

Figure 6A shows a representative trace experiment of short-circuit currents of differentiated non-CF bronchial epithelia. The basal current was inhibited by amiloride, indicating the presence of epithelial Na^+ channel (3.1 ± 0.6 μA/cm^2; n = 25). To assess the amount of CFTR-dependent Isc, cells are blocked with CFTRinh-172 after maximum CFTR activation using CPT-cAMP. The resulting chloride current is strongly sensitive to CFTRinh-172 (10.08 ± 0.04 μA/cm^2; n = 31). Application of UTP generates a very fast calcium-activated chloride current (3.8 ± 1 μA/cm^2; n = 29).

Primary human nasal monolayers recapitulate the transepithelial ion transport properties of bronchial cells, confirming the fact that this cell model can predict clinical treatment efficacy in patients (**Figure 6B**).

To further support the conclusion that our culture protocol allows F508del-CFTR-HBEC to reflect the ion transport defects in airway epithelia *in vivo*, we used cells from patients that were homozygous for this mutation; which causes a severe defect in CFTR function. As expected the response to CPT-cAMP and ivacaftor is markedly reduced in negative control cells (1.04 ± 0.023 μA/cm^2; n = 37) (**Figure 7A**). However, we have noted that lumacaftor treatment for 24 h induced a significant increase in chloride transport, as indicated by the current blocked by CFTRinh-172 (3.67 ± 0.07 μA/cm^2; n = 41) (**Figure 7B**).

Figure 7C summarizes the CFTR-mediated currents from Ussing chamber recordings of HBEC derived from 4 non-CF donors and 4 homozygous F508del-CFTR patients. Transepithelial current measurements showed that there was a significant difference in the CFTR-mediated Isc in cultured F508del-HBE cells compared to non-CF HBE cells, and incubation of CF epithelium with lumacaftor led to a significant improvement in CFTR-dependent chloride secretion.

TEER is a quantitative technique that is useful for predicting the ideal concentration of a drug to be tested, without damaging the epithelium, or to quantify ion transport by measuring the electrical resistance and potential difference across the barrier tissue.

We have performed TEER measurements to evaluate the ability of compounds to correct F508del-CFTR. Bronchial epithelia from patients that were homozygous for the F508del mutation were treated for 24 h with lumacaftor, or vehicle (DMSO), and then assayed. Transepithelial electrical resistance was measured before and after acute stimulation with forskolin and genistein to fully activate CFTR. Subsequently, transepithelial resistance was measured after the addition of the selective CFTR inhibitor, PPQ-102, used to completely block CFTR activity. The delta between the values of electrical resistance before and after CFTR inhibition were then converted into the reciprocal conductance.

In accordance with short-circuit current measurements, treatment with lumacaftor significantly increased transepithelial conductance (1650 ± 153.3 μS/cm^2) compared with the negative control (482 ± 42.2 μS/cm^2). The bar graph shows mean values obtained from four different omozygous F508del-CFTR patients (**Figure 8**).

In conclusion, we have provided evidence that our procedures provide highly differentiated airway epithelial monolayers with morphologic and functional characteristics similar to airway epithelia *in vivo*. These cell models are very useful for improving our knowledge about physiopathology mechanisms involved in CF and to support therapeutics strategies.

ETHICS STATEMENT

The procedure of the bronchus dissection and the informed consent from patients was approved by the Ethical Committee of the Gaslini Institute under the supervision of the Italian Ministry of Health.

AUTHOR CONTRIBUTIONS

AG and LD have performed the procedures and contributed equally to the work. EC has written the manuscript with the help of AG and LD.

ACKNOWLEDGMENTS

This work was supported by grants from the Italian Cystic Fibrosis Foundation (FFC no. CP/2012 with the contribution of "Delegazione FFC di Tradate Gallarate" and "Verbania-V.C.O.") and from European Union's Horizon 2020 research and innovation program under grant agreement no. 667079. We are grateful to Dr. Luis Galietta (TIGEM, Pozzuoli, Italy) for his ongoing support and Amber Philp (Universidad Austral de Chile, Valdivia, Chile) for english editing revision.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01176/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 19 December 2018
doi: 10.3389/fphar.2018.01490

Variable Responses to CFTR Correctors *in vitro*: Estimating the Design Effect in Precision Medicine

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: 03 October 2018
Accepted: 05 December 2018
Published: 19 December 2018

Citation:
Matthes E, Goepp J, Martini C, Shan J, Liao J, Thomas DY and Hanrahan JW (2018) Variable Responses to CFTR Correctors in vitro: Estimating the Design Effect in Precision Medicine. Front. Pharmacol. 9:1490. doi: 10.3389/fphar.2018.01490

Interest in precision medicine has grown in recent years due to the variable clinical benefit provided by some medications, their cost, and by new opportunities to tailor therapies to individual patients. In cystic fibrosis it may soon be possible to test several corrector drugs that improve the folding and functional expression of mutant cystic fibrosis transmembrane conductance regulator (CFTR) prospectively using cells from a patient to find the one that is best for that individual. Patient-to-patient variation in cell culture responses to correctors and the reproducibility of those responses has not been studied quantitatively. We measured the functional correction provided by lumacaftor (VX-809) using bronchial epithelial cells from 20 patients homozygous for the F508del-CFTR mutation. Significant differences were observed between individuals, supporting the utility of prospective testing. However, when correction of F508del-CFTR was measured repeatedly using cell aliquots from the same individuals, a design effect was observed that would impact statistical tests of significance. The results suggest that the sample size obtained from power calculations should be increased to compensate for group sampling when CFTR corrector drugs are compared *in vitro* for precision medicine.

Keywords: precision medicine, cystic fibrosis, correctors, lumacaftor, Orkambi, group sampling, design effect, power calculations

INTRODUCTION

Cystic fibrosis (CF) is a relatively common orphan disease caused by loss-of-function mutations in the gene encoding CFTR (cystic fibrosis transmembrane conductance regulator), a tightly regulated anion channel (Riordan, 2008). CFTR mediates secretion across many epithelia in the body and is required for efficient mucociliary clearance of inhaled pathogens from the lungs (Ratjen et al., 2015). CF modulators such as lumacaftor (VX-809) that partially correct the misfolding and/or potentiate the activity of mutant CFTR channels are available and more are in the pipeline, however, they are expensive and their clinical benefit varies between individuals (Boyle et al., 2014;

Hanrahan et al., 2017). With precision medicine one could potentially test multiple drugs on cells from a patient to identify the one that is most efficacious for that individual.

Although CF seems ideally suited for applying the precision medicine approach (Amaral, 2015; Martiniano et al., 2016; Burgener and Moss, 2018; Cholon and Gentzsch, 2018), some practical issues remain to be addressed. Foremost among these is whether differences in functional correction measured using primary cultured cells from different patients are statistically significant. This is obviously essential if prospective testing *in vitro* is to be useful for making prescribing decisions. The variable clinical benefit provided by Orkambi® is well known (Boyle et al., 2014), however, it remains unclear if similar variability exists at the level of epithelial cells and persists in cell culture. If cells from different patients vary in their response to drugs and the responses correlate with clinical benefit, a practical question arises as to how many assays would be needed to conclude that one corrector is more effective than another for that individual. The causes of variability are not yet understood therefore it is not possible to develop a statistical model capable of predicting responses to a drug. Nevertheless, methods for collecting and analyzing data are needed if efficacy in cell-based assays is to be useful for precision medicine.

Assays of CFTR function that utilize different cell types have been developed and could potentially be used to test the drug responsiveness of individual patients. Rescue of the mutant CFTR can be assayed directly by measuring Cl^- transport across tissue samples or primary cell cultures using electrophysiology (Van Goor et al., 2011; Brewington et al., 2018). CFTR function can also be assayed indirectly by measuring net fluid transport across intestinal organoids prepared from rectal biopsies (Dekkers et al., 2013, 2016) or airway epithelial spheroids prepared from cells that are obtained by brushing or curettage of the nasal or bronchial mucosa (Guimbellot et al., 2017; McCarthy et al., 2018). Induced pluripotent stem cells (iPSCs) may be useful if they can be made to differentiate fully and recapitulate variable drug responses between patients having the same CFTR genotype, which may depend on both genetic and epigenetic factors. Correlations have recently been reported when assaying CFTR function in the nasal epithelium *in vivo* and in cultured cells from patients with different CFTR mutations (Pranke et al., 2017). Similar responses have also been observed in nasal and bronchial epithelial cultures (Pranke et al., 2017; Brewington et al., 2018). Importantly, for seven patients homozygous for F508del-CFTR there was a correlation between mean functional rescue in nasal cell cultures and the clinical response to Orkambi measured as % FEV1 (% predicted forced expiratory volume in 1 s) (Pranke et al., 2017).

In this paper we start by quantifying variability in functional correction amongst patients having the same genotype (F508del-CFTR/F508del-CFTR) using well differentiated bronchial epithelial cell cultures. Then we explore the reproducibility of correction by repeatedly testing samples from large pools of cells from two individuals, analogous to sampling the airway *in vivo*. We observe a design effect caused by group sampling that needs to be considered when testing the statistical significance of differences in correction.

MATERIALS AND METHODS

Cells

Primary human bronchial epithelial (HBE) cells were obtained from the Primary Airway Cell Biobank at the McGill CF Translational Research centre (CFTRc) and cultured at the air liquid interface as described previously (Fulcher et al., 2005; Robert et al., 2007). Briefly, lung tissue from patients undergoing lung transplantation was obtained from the Biobank of respiratory tissues at the Centre Hospitalier de l'Université de Montréal and Institut de recherche cliniques de Montréal. Informed, written consent was obtained and all procedures were approved by the Institutional Review Board of McGill University (#A08-M70-14B) and followed Canadian Institutes of Health Research guidelines. Cells were isolated from bronchial tissue by enzyme digestion and cultured in bronchial epithelial growth medium (BEGM) on type I collagen-coated plastic flasks (Vitrogen 100, PureCol; Advanced BioMatrix), then trypsinized, counted, and used immediately for experiments (**Figures 1D,F**) or cryopreserved in aliquots of 2 million cells and used within 18 months (**Figures 1F** and **2A,B**). Bronchial epithelial cell growth medium (BEGM) was used during cell isolation and initial culture of the cells (i.e., passage 0, P0). BEGM consists of Laboratory of Human Carcinogenesis (LHC) Basal Medium (Invitrogen) and ∼20 supplements including bovine serum albumin (BSA) but not serum [see (Fulcher et al., 2005) for details]. Once cells had been thawed and seeded on porous supports they were cultured using air-liquid interface (ALI) medium to induce differentiation. The ALI medium was a 50:50 mixture of LHC Basal Medium and Dulbecco's modification of Eagle medium (D-MEM) with less added human epidermal growth factor (hEGF) but other supplements including BSA at similar levels to BEGM. Drug treatments were performed for 24 h in ALI medium without BSA or antibiotics.

Freshly isolated or thawed cells were seeded on collagen coated 6.5 mm Costar® 0.4 μm pore, polyester membrane inserts (Corning) and grown under submerged conditions for 4 days. The apical medium was then removed and cells were allowed to differentiate at the ALI for 4 weeks before use in correction assays. Isolation and growth media were supplemented with antibiotics that were selected based on recent patient microbiology reports. Only penicillin and streptomycin were added to ALI cultures. Cells were used at first passage (P1), and comparisons between patients used recently isolated cells that had not been frozen. Repeated sampling of two patients was performed using cells from large isolates that had been cryopreserved in aliquots of 2 million cells (Fulcher et al., 2005; Robert et al., 2010). Mature cultures were pseudostratified and appeared highly differentiated with ciliated, goblet and basal cells (**Figure 1A**).

Corrector Treatment and CFTR Functional Assays

When comparing different patients, three well differentiated monolayers were pretreated for 24 h at 37°C with vehicle (0.1% DMSO dimethyl sulfoxide) and three were pretreated

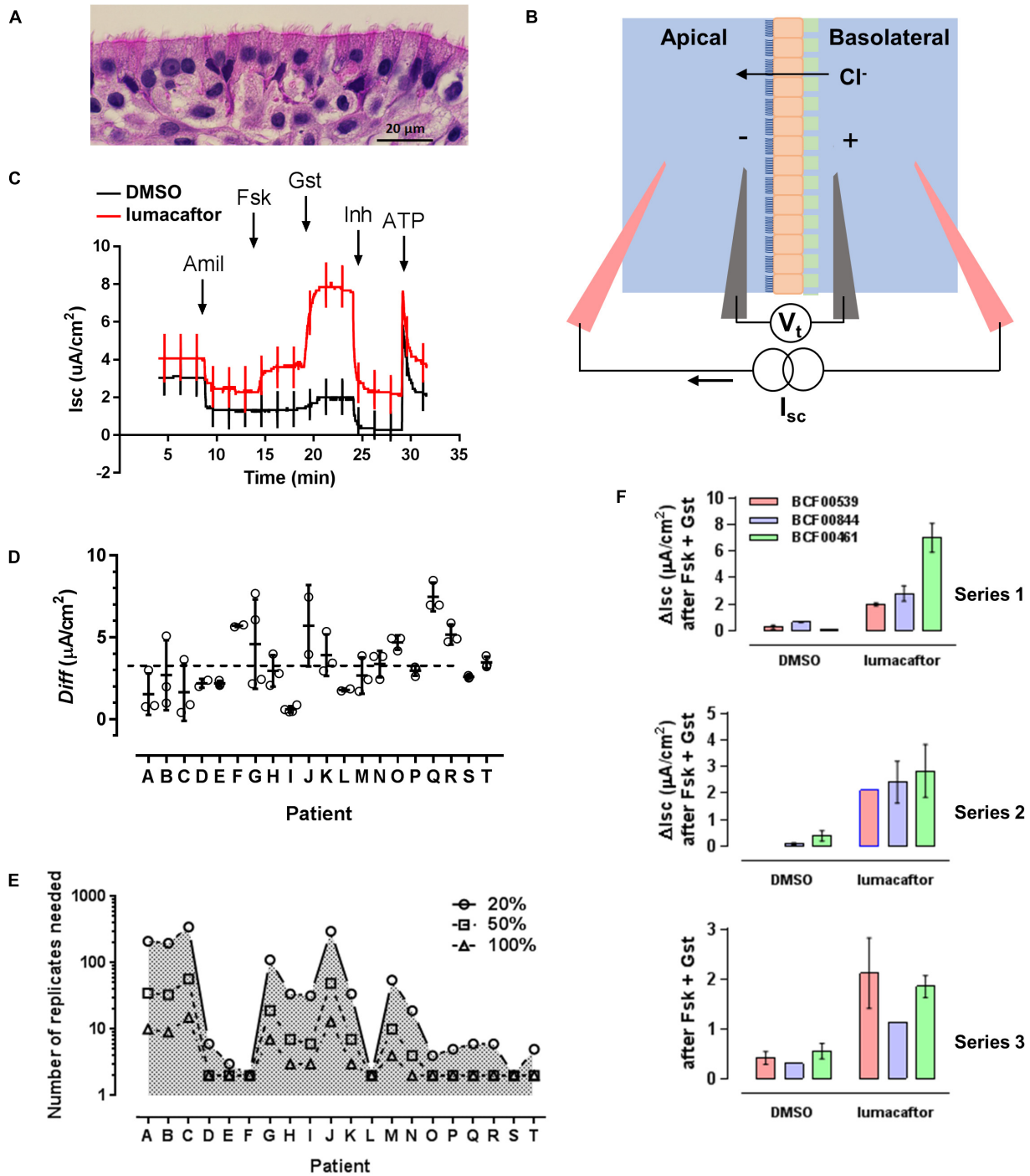

FIGURE 1 | An assay for testing F508del-CFTR corrector drugs. **(A)** Well differentiated primary human bronchial epithelial (HBE) cells cultured at the air-liquid interface. **(B)** Electrophysiological measurement of CFTR function as short-circuit current (I_{sc}), the current needed to clamp the transepithelial voltage (V_t) at 0 mV. Epithelial cells (orange) cultured on a porous support (green) are mounted in modified Ussing chambers. A basolateral-to-apical Cl^- gradient is imposed to generate a secretory flux through rescued mutant CFTR channels. **(C)** Representative recordings of cells pretreated for 24 h with DMSO (vehicle) or lumacaftor (corrector), then exposed sequentially to Na^+ channel blocker amiloride (10 µM Amil, apical), forskolin (10 µM Fsk, bilateral, activator), genistein (50 µM Gst, apical, potentiator), $CFTR_{inh}$-172 (10 µM Inh, apical, CFTR inhibitor), and ATP (10 µM, apical, purinergic agonist to stimulate Ca^{2+}-activated Cl^- channels as a positive control for viability). Current deflections show responses to brief voltage steps to +/–1 mV to monitor transepithelial resistance. **(D)** Response of cells from 20 patients to lumacaftor shown as the difference (*Diff*) in ΔI_{sc} stimulated by forskolin + genistein when cells were pretreated with lumacaftor or DMSO. (means +/– s.d., $n = 3$ for each condition). **(E)** Predicted number of replicates needed to detect a 20, 50, and 100% change in correction, calculated for each patient. **(F)** Three series of assays performed independently on the same three patients under identical conditions. In each series, cells from the same patients were exposed in triplicate to vehicle (DMSO) or corrector (lumacaftor).

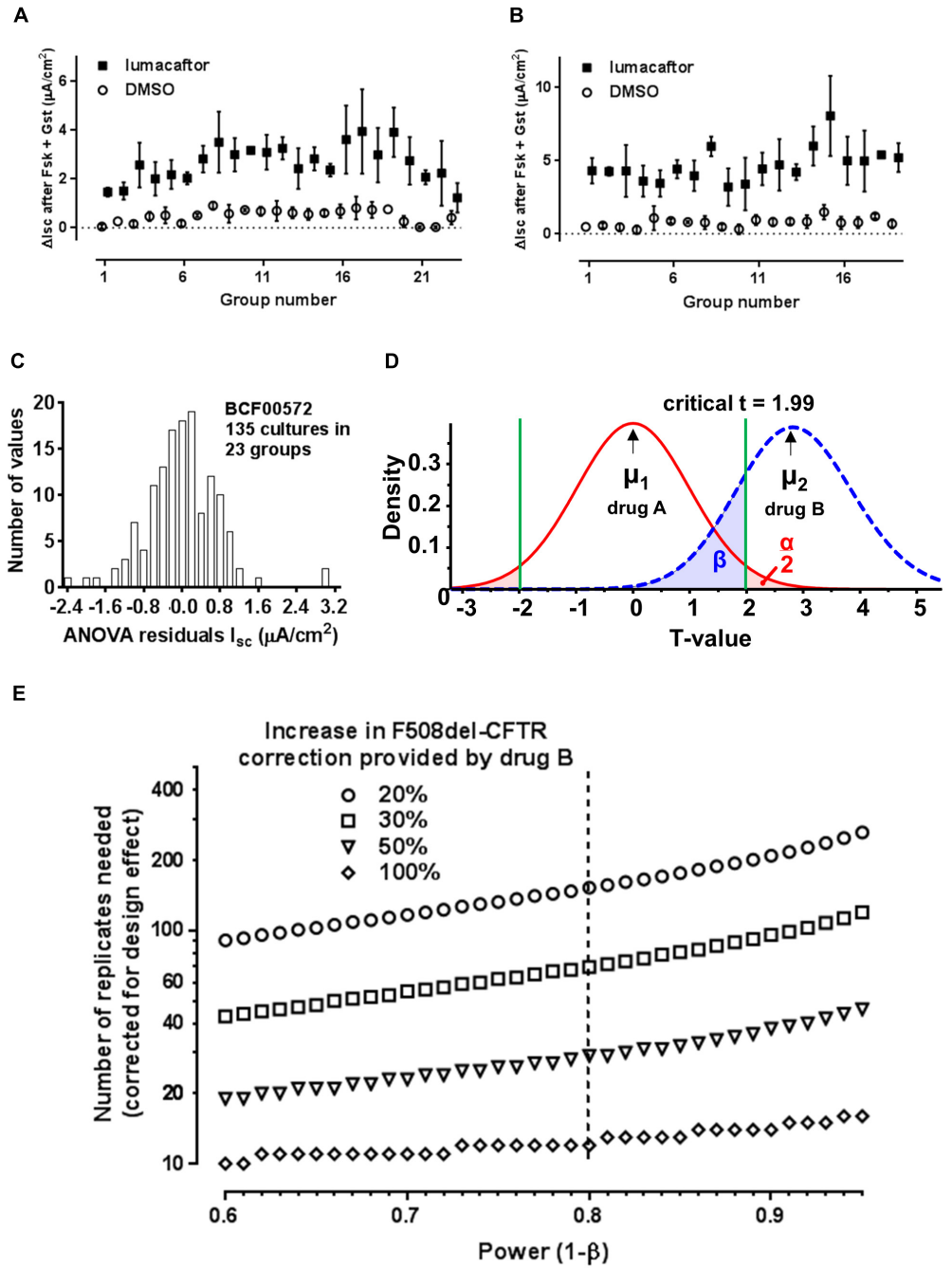

FIGURE 2 | Impact of group sampling when comparing two correctors. **(A)** Simulation of group sampling by measuring F508del-CFTR functional correction in aliquots of the same cell population (patient BCF00572). Response to forskolin + genistein after treatment with vehicle (open circles) or corrector (closed squares) in 23 groups of 3–9 cultures (technical replicates). **(B)** Same as panel **A** but with cells from patient BCF00710. **(C)** Distribution of the residuals from one-way random effects ANOVA of lumacaftor treated cell cultures from patient BCF00572. **(D)** Predicted *t*-distributions for two correctors, where hypothetical drug B gives a mean F508del-CFTR correction μ_2 that is 20% higher than corrector A (μ_1). The calculations assume $\alpha = 0.05$, $\beta = 0.8$, mean response to forskolin + genistein after pretreatment with corrector A = 3.3 μA/cm^2, and s.d. = 1.0 μA/cm^2 for both drugs. **(E)** Number of replicates needed to detect a significant change in correction by drug B compared to drug A, assuming there is a real improvement of 20, 30, 50, or 100%, after compensation for the design effect due to group sampling. The number of replicates is shown for power 0.60–0.95 (i.e., probability of a false negative of 0.4–0.05).

with lumacaftor (1 μM; Selleck Chemicals, Houston, TX, United States) and the same final concentration of DMSO. The six monolayers were then mounted in modified Ussing chambers and short-circuit current (I_{sc} in μA cm^{-2}) was measured to assay CFTR function in all six cultures simultaneously (**Figure 1B**). Transepithelial voltage was clamped at 0 mV

except for 2 s bipolar pulses to ±1 mV at 100 s intervals to monitor transepithelial resistance. A basolateral-to-apical NaCl chloride gradient was imposed to increase the signal-to-noise of the current response; apical (in mM): 1.2 NaCl, 115 Na-gluconate, 25 $NaHCO_3$, 1.2 $MgCl_2$, 4 $CaCl_2$, 2.4 KH_2PO_4, 1.24 K_2HPO_4, 10 Glucose; basolateral (in mM): 115 NaCl, 25 $NaHCO_3$, 1.2 MgCl, 1.2 $CaCl_2$, 2.4 KH_2PO_4, 1.24 K_2HPO_4, 10 Glucose. After the short-circuit current had stabilized (typically 2–3 min), Fsk (10 μM) was added to both sides to raise intracellular cAMP. This was followed by sequential additions of the potentiator genistein (Gst, 50 μM), the CFTR inhibitor $CFTR_{inh}$-172 (10 μM) and the purinergic agonist ATP (100 μM) to the apical side to stimulate Ca^{2+}-activated Cl^- channels as a positive control and to confirm cell viability (Matthes et al., 2016). Potentiation by 50 μM genistein was similar to that produced by 0.1 μM ivorcaftor (VX-770 or Kalydeco®) and was used because it was more easily washed from the chambers. The stimulation of I_{sc} (taken as the increase from the steady-state baseline level before adding forskolin to the maximum current after genistein addition; ΔI_{sc}) was used to measure of F508del-CFTR functional expression (**Figure 1C**). Assays using CF mouse intestine were performed as described previously (Robert et al., 2010). Rotterdam delF508/delF508−CFTR mice (*Cftr*$^{tm1\ Eur}$), FVB inbred, 14–17 weeks old, 24–30 g) were used (van Doorninck et al., 1995; Scholte et al., 2004). All procedures followed Canadian Institutes of Health Research guidelines and were approved by the faculty Animal Care Committee at McGill University (#2012-7119). The ileum was stripped of muscle and mounted in mini-Ussing chambers (Physiological Instruments, San Diego, CA, United States). Tissues were bathed with William's E−Glutamax (x1; Gibco) supplemented with insulin (10 μg/ml) and dexamethasone (20 μg/ml). Short-circuit current and resistance were measured before and after sequential additions of forskolin (10 μM) and genistein (50 μM) to the apical side. After steady-state stimulation these were washed from the chambers and lumacaftor or vehicle (0.1% DMSO) was added for 4 h. Forskolin and genistein were then re-assayed and the difference between the I_{sc} responses before and after exposure to lumacaftor or vehicle (which served as a time control) were used as a measure of correction. Reagents were from Sigma unless otherwise indicated.

Statistics

Results are expressed as the mean ± S.E.M. of *n* observations. F508del-CFTR correction was displayed as *Diff* (the difference between maximum I_{sc} response to forskolin + genistein when cells were pretreated with lumacaftor vs. DMSO

$$Diff = \Delta Isc_{\ treated} - \overline{\Delta Isc}_{\ ctrl} \quad (1)$$

Although the same number of cultures was exposed to drug and to vehicle, the measurements were unpaired and all three DMSO controls were equally applicable to each drug-treated culture. *Diff* was calculated and plotted for clarity to show correction but was not used in statistical tests, which were based on the raw data collected under each condition.

The impact of group sampling was determined as the intraclass correlation coefficient (ICC, also called intracluster correlation coefficient or "rho" ρ) the ratio of the variance between groups to the sum of variances between and within groups (Donner and Klar, 2000; Killip et al., 2004). ρ can range from 0 when there is no effect of group sampling to 1 when the replicates within groups are perfectly consistent and variation is entirely between groups. The results of ANOVA were then used to determine $\rho(\Delta Isc_{\ treated})$ as:

$$\rho = \frac{MS_B - MS_W}{MS_B + (n_o - 1)MS_W} \quad (2)$$

where MS_B and MS_W are the mean squares between and within groups, respectively, and η_o is the average number of replicates per group calculated as:

$$\frac{\begin{array}{l}(\text{total \# of replicates} \\ \quad - \text{ sum of the squared \# of replicates in each group} \\ \quad /\text{total \# of replicates})\end{array}}{\text{total \# of groups} - 1} \quad (3)$$

Residuals for 22 of the 23 group samples passed the Kolmogorov-Smirnov normality test with α = 0.05 using GraphPad Prism v. 6.04 (GraphPad Software, La Jolla, CA, United States)[1]. When the more stringent D'Agostino-Pearson K^2 omnibus test (D'agostino et al., 1990) was applied to groups having $n \geq 9$ (the minimum needed) they passed the normality test. When residuals for samples from all groups were pooled there was deviation from normality due to positive kurtosis, however, the distribution was symmetric and well fitted by a Gaussian curve ($r^2 = 0.9944$) after excluding two bins as outliers (**Figure 2C**). The robustness of ANOVA when there are moderate departures from normality (Motulsky, 2014) suggests the ANOVA results are valid. The standard deviation of the I_{sc} response to drug ($\Delta Isc_{\ treated}$) was used in the program G*Power v3.9.1.2 when estimating the number of replicates needed to determine if lumacaftor causes significant correction for each of the 20 patients and for different effect sizes (Faul et al., 2007).

RESULTS

Cystic fibrosis bronchial epithelial cells from all 20 patients were more strongly stimulated by forskolin + genistein after 24 h pretreatment lumacaftor (**Figure 1D**). The average stimulation after treatment with lumacaftor was 11.4% that measured in non-CF cells expressing wild-type CFTR (pooled data from 11 non-CF donors). This is similar to a previous report in which cells from seven patients that had been cultured in medium supplemented with Ultroser G and assayed acutely using a different potentiator (Van Goor et al., 2011).

The I_{sc} responses were ∼15-fold more variable after drug pretreatment than after pretreatment with vehicle. The variation was quantified using a mixed, two-way analysis of variance (ANOVA) of all data in both groups (i.e., baseline I_{sc} and

[1]www.graphpad.com

maximum I_{sc} after stimulation by forskolin + genistein). Most (51.0%) of the variability was associated with the effect of drug pretreatment while 19.3% could be attributed to patients and 11.9% was explained by interaction between these factors; i.e., patient-to-patient differences in the response to lumacaftor (parameter of interest for precision medicine). Despite the small sample size, the null hypothesis that cells from different patients respond similarly to lumacaftor could be rejected with $P = 0.0001$. This is consistent with a previous study using a linear random effects model in which most variability in the response to lumacaftor was due to inter-patient rather than intra-patient variability (Pranke et al., 2017). Our power calculations to estimate the number of replicates needed for lumacaftor responses to reach statistical significance for a given effect size (i.e., increase of 20, 50, or 100%) yielded variable results when based on triplicate assays (**Figure 1E**) suggesting a larger sample size is needed to reliably estimate the standard deviation. Nevertheless, the results provide support for precision medicine in CF in that they show significant variability in drug responses between airway epithelial cells cultured from different individuals.

To assess the reproducibility of F508del-CFTR correction we compared the stimulation by forskolin + genistein on 3 successive weeks using different ALI cultures. The cells were prepared from different patients, cryopreserved and then thawed at 1 week intervals and cultured under identical conditions for 1 month for assays. Cells from one patient responded similarly to forskolin + genistein after pretreatment with lumacaftor in all three sets of experiments. However, ΔI_{sc} was more variable when cells from the other two patients (**Figure 1F**).

To explore this variability further we simulated *in vivo* sampling by dividing a pool of 170 million HBE cells from one patient into aliquots of 2 million. Each aliquot was considered a group sample from the airway surface epithelium to mimic repeated collection of small samples *in vivo* by bronchial brushing, but under well controlled conditions. Multiple ALI cultures were prepared using 23 randomly chosen aliquots (2–18 cultures per aliquot) and the ΔI_{sc} response to forskolin + genistein was measured using equal numbers of vehicle- and lumacaftor-treated replicates from each aliquot. Cells that had been treated with lumacaftor were always more responsive to forskolin + genistein than DMSO controls as expected (**Figure 2A**). One-way random effects ANOVA of ΔI_{sc} revealed significant variation between the groups ($P < 0.0001$), despite coming from the same original pool of cells.

Since prospective testing for the response to a CF drug will likely involve analyzing small cell samples *in vitro,* we examined the statistical consequences of such group sampling on correction. We calculated the intraclass correlation coefficient (ICC, also called ρ rho), which is the ratio of the variance between groups to the sum of the variances between and within groups. This was done when correction by lumacaftor was assayed repeatedly using 135 cultures from one patient (BCF00572). On average, each group consisted of $\eta_o = 5.86$ replicates and had $\rho = 0.377$, yielding a design effect $[1 + \rho(\eta_o - 1)] = 2.83$. ICC was somewhat higher for vehicle-treated cells ($\rho = 0.4466$), in agreement with the lower variability of DMSO controls in **Figure 2A**.

To determine if the design effect is observed generally when assaying F508del correction we analyzed cells from a second patient (BCF00710). We selected 19 aliquots of cells (2 million cells each) at random from a pool of 280 million cells, cultured them at the ALI for 1 month, and assayed their stimulation by forskolin + genistein with and without lumacaftor pretreatment as before (**Figure 2B**). In this experiment there were $\eta_o = 4.34$ replicates per lumacaftor treatment group and equation (2) yielded $\rho = 0.2816$ and a design effect of 1.94. Plotting the residuals from a one-way random effects ANOVA of all data from lumacaftor-treated cultures from patient BCF00572 gave a bell-shaped frequency distribution that showed positive kurtosis but only moderate deviation from normality, supporting the validity of ANOVA (**Figure 2C**). In summary, the results from both patients indicate that variability of drug responses measured using group samples underestimates the true variation in the original population. Correcting for the design effect is necessary when comparing drug responses *in vitro* for precision medicine; two- to three-fold more replicates than estimated by regular power calculations are needed to draw conclusions based on two-tailed *t*-tests.

With this in mind we asked "How many replicates are needed to conclude that corrector B is more effective than corrector A using cells from one individual that have been collected and cultured *in vitro*?" To answer this we assumed a typical stimulation by forskolin + genistein after pretreatment with corrector A (lumacaftor; $\Delta I_{sc} = 3.3\ \mu A/cm^2$, s.d. $= 0.962\ \mu A/cm^2$; the mean from 20 patients studied under our conditions). This response was compared to that generated by hypothetical "corrector B," which was assumed to have the same variance as lumacaftor. We set $\alpha = 0.05$ (probability of Type I error or false positive when testing the null hypothesis H_o that correctors A and B have the same efficacy) and $\beta = 0.2$ (probability of a Type II error or false negative, corresponding to a statistical power of $1-\beta = 0.8$; see **Figure 2D**). The number of replicates needed was then calculated as a function of power when corrector B would provide a real improvement in F508del-CFTR correction that is 20, 30, 50, or 100% higher than corrector A. Replicates were assumed to be independent samples, then the predicted number of replicates was then multiplied by 2.83 to correct for the effect of group sampling (**Figure 2E**).

The results indicate that if hypothetical corrector B has twice the efficacy of corrector A (i.e., increases F508del-CFTR rescue by 100%), 12 replicates from a group sample would be sufficient to conclude there is a significant difference between correctors. However, more replicates are needed if the real change in efficacy is less dramatic, e.g., 153 when corrector B increases F508del-CFTR function by only 20%.

DISCUSSION

The present results demonstrate statistically significant variation in responses to lumacaftor when assayed using cultured cells

from different patients. Variable corrector responses have been observed previously (Van Goor et al., 2011; Eckford et al., 2014) and were analyzed for homozygous F508del CFTR patients in one study (Pranke et al., 2017), however, the implications for comparing efficacies of different drugs have not been explored. We also found considerable variability between cell samples from the same patient and explored its consequences when distinguishing between correctors. Prospective drug testing *in vitro* will likely begin with the collection of a small sample of epithelial cells from the patient. We used bronchial epithelial cells, however, similar results would be expected when small numbers of epithelial cells are harvested from the nasal or bronchial mucosa or by rectal biopsy. Since group samples have reduced standard deviation compared to the original epithelial cell population, they are expected to cause underestimation of the sample size needed for statistical testing, although this estimate can be corrected by determining the design effect.

Air-liquid interface cultures are considered to be the "gold standard" for testing CFTR modulators and have been accepted by the FDA when evaluating new drug applications (Durmowicz et al., 2018). Using this model to assay samples from a single large population of airway cells we estimated the design effect to range between 2 and 3. The sources of variability between group samples and replicates within groups are unknown and may reflect heterogeneity in the cell isolate and/or subtle differences during differentiation in prolonged culture. Cells were handled identically according to detailed standard operating procedures, nevertheless we cannot exclude slight variations in cell viability after thawing that could affect seeding density, or volume when the cells were fed with fresh medium. Cells were treated with corrector for 24 h in our study, however, lumacaftor is hydrophobic and longer exposures (48–72 h) are expected to increase its uptake and might reduce variability. The efficacy of Orkambi appears to be limited mainly by the modest efficacy of lumacaftor, therefore we have focussed on it in this study and pretreated cells only with lumacaftor. Simultaneous exposure to another drug such as ivacaftor could increase variability since variances add, although it will be important to test prolonged exposure to ivacaftor at clinically-relevant, low nanomolar free concentrations to avoid adverse effects on CFTR functional rescue by lumacaftor (Matthes et al., 2016). When multiple corrector drugs become available for CF they may be compared *in vitro* for precision medicine on the assumption that more functional expression *in vitro* will correlate with better symptoms *in vivo*. Such a correlation is observed between mean responses of multiple homozygous F508del-CFTR patients to Orkambi *in vivo* and nasal epithelial cultures from the same patients treated with ivorcaftor + lumacaftor (Pranke et al., 2017). Those results are also consistent with the ability of ivorcaftor alone to increase Cl^- conductance in cells expressing G551D-CFTR but not F508del-CFTR (Van Goor et al., 2009) and to improve lung function in patients with G551D (Accurso et al., 2010) but not in F508del homozygotes (Flume et al., 2012). Why correction varies between patients with the same CFTR genotype remains uncertain. Variation in genetic background (i.e., genes other than CFTR) may contribute since genome wide association studies have identified several genes that can modify CF disease severity (Cutting et al., 1990; Wright et al., 2011; Sun et al., 2012; Blackman et al., 2013; Corvol et al., 2015). Epigenetic variation caused by exposure to environmental factors could also contribute to variable corrector responses *in vitro*. Measuring correction *in vitro* probably excludes some off-target drug effects, however, the underlying genetic and epigenetic factors that affect F508del-CFTR expression, folding and trafficking may persist.

We were interested to compare the variability of correction in cells from patients and in F508del-CFTR homozygous mice (van Doorninck et al., 1995; Wilke et al., 2011). The genomes and epigenomes of CF mice are expected to be more similar than humans because they are inbred and housed under identical conditions. We examined correction in the ileum because the CF phenotype is stronger in the intestine than in the lung in mice. In preliminary experiments we isolated small pieces of ileum from CF mice ($Cftr^{tm1\ Eur}$) (van Doorninck et al., 1995) in quadruplicate and measured the forskolin-stimulated ΔI_{sc} after exposing tissues to DMSO or lumacaftor for 4 h *ex vivo* as described previously (Robert et al., 2010). Mixed, two-way ANOVA showed that the variability of F508del-CFTR correction was less in inbred mice than in patients. The interaction term (i.e., variability in drug response between individuals) accounted for ∼6.5% of the variance in mouse intestinal assays vs. 11.9% in HBE cultures from patients but was still significant ($P < 0.0001$). We believe this variation reflects the health of the mice or the condition of tissues after dissection, but cannot exclude that genetic and/or epigenetic variations persist in the mice despite inbreeding and the same environment.

The application of personalized medicine in CF has a bright future, however, the predictive value of *in vitro* assays remains to be established when there are alternative drugs and multiple patients carrying the same mutation. The present results indicate that patient-to-patient differences in F508del-CFTR correction can be assayed using airway cell cultures, however, a design effect due to group sampling needs to be compensated by increasing the number of replicates. Hopefully, CF correctors and corrector combinations will continue improving until they become so effective for all patients that precision medicine is no longer necessary (Hanrahan et al., 2017).

AUTHOR CONTRIBUTIONS

JH, EM, and DT: conceptualization. EM, JG, CM, JS, JL, and JH: investigation, and validation. JH: writing—original draft. JH, EM, JG, CM, and DT: writing—review and editing. JH and DT: funding acquisition. JG, CM, JL, and EM: resources.

FUNDING

This work was supported by grants from the Canadian Institutes of Health Research to JH (MOP-287879) and DT (MOP-119341)

and from Cystic Fibrosis Canada to the Primary Airway Cell Biobank (PACB) at McGill (2997).

ACKNOWLEDGMENTS

We thank Dr. Andrea Benedetti (McGill University) for helpful discussions and Dr. Vanessa Dumeaux (Concordia University) for comments on the manuscript. We also thank the Biobank of respiratory tissues at the Centre Hospitalier de l'Université de Montréal and Institut de Recherche Cliniques de Montréal (Respiratory Health Network of Fonds de Recherche du Québec-Santé) for providing lung samples. We are grateful to Dr. Bob Scholte, Dr. Hugo de Jonge, and Dr. Martina Wilke (Erasmus MC, Rotterdam, Netherlands) for providing F508del-CFTR mice (*Cftr*$^{tm1\ Eur}$) and a protocol for assaying correction in mouse intestine.

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Conflict of Interest Statement: CF Translational Research Centre seminar series is supported by Vertex Pharmaceuticals Inc. JH is a mentor for the Vertex CF Research Innovation Award program. Vertex had no input into this study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 26 October 2018
doi: 10.3389/fphar.2018.01221

Identification and Characterization of Novel CFTR Potentiators

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *26 July 2018*
Accepted: *08 October 2018*
Published: *26 October 2018*

Citation:
Gees M, Musch S, Van der Plas S, Wesse A-S, Vandevelde A, Verdonck K, Mammoliti O, Hwang T-C, Sonck K, Stouten P, Swensen AM, Jans M, Van der Schueren J, Nelles L, Andrews M and Conrath K (2018) Identification and Characterization of Novel CFTR Potentiators. Front. Pharmacol. 9:1221. doi: 10.3389/fphar.2018.01221

There is still a high unmet need for the treatment of most patients with cystic fibrosis (CF). The identification and development of new Cystic Fibrosis Transmembrane conductance Regulator (CFTR) modulators is necessary to achieve higher clinical benefit in patients. In this report we describe the characterization of novel potentiators. From a small screening campaign on F508del CFTR, hits were developed leading to the identification of pre-clinical candidates GLPG1837 and GLPG2451, each derived from a distinct chemical series. Both drug candidates enhance WT CFTR activity as well as low temperature or corrector rescued F508del CFTR, and are able to improve channel activity on a series of Class III, IV CFTR mutants. The observed activities in YFP halide assays translated well to primary cells derived from CF lungs when measured using Trans-epithelial clamp circuit (TECC). Both potentiators improve F508del CFTR channel opening in a similar manner, increasing the open time and reducing the closed time of the channel. When evaluating the potentiators in a chronic setting on corrected F508del CFTR, no reduction of channel activity in presence of potentiator was observed. The current work identifies and characterizes novel CFTR potentiators GLPG1837 and GLPG2451, which may offer new therapeutic options for CF patients.

Keywords: CF, CFTR, potentiator, bronchial epithelial cells, electrophysiology

INTRODUCTION

Cystic fibrosis (CF) is the most common genetic (autosomal recessive) disease in Caucasians, with an estimated 80,000 CF diagnosed cases worldwide (Lubamba et al., 2012; Hanrahan et al., 2013; Merk and Schubert-Zsilavecz, 2013; De Boeck et al., 2014). Current strategies for treatment of CF patients can be broadly divided into two main categories: agents that target downstream effects of Cystic Fibrosis Transmembrane conductance Regulator (CFTR) dysfunction (i.e., symptomatic treatment) and agents that target the root cause of the disease, i.e., CFTR modulators which address the absent or dysfunctional CFTR in epithelial membranes. The treatments in the latter category are further classified as either correctors, which increase the levels of CFTR present on the cell surface, or potentiators which enhance the function of CFTR channels, There are three approved CFTR modulator treatments available for CF patients, namely the potentiator Ivacaftor (Kalydeco®) or VX770, the corrector Lumacaftor or VX809 and the corrector Tezacaftor or VX661, The Ivacaftor/Lumacaftor combination therapy (Orkambi®) or the Ivacaftor/Tezacaftor combination therapy (Symedeko®) are available for the treatment of patients homozygous for the F508del CFTR

mutation. However, these treatments result in only minor benefit to these patients (Wainwright et al., 2015; Taylor-Cousar et al., 2017) and thus there is a demand for improved combinations to further improve clinical benefit for CF patients with the F508del mutation.

Potentiators can only function if CFTR is already present at the cell membrane, and work by increasing the opening probability (Po) of CFTR (Moran, 2010). VX770 is a potentiator which improves the channel opening of CFTR mutants with gating or conductance defects (class III and IV mutations, respectively) such as G551D (Van Goor et al., 2009; Yu et al., 2012; Jih and Hwang, 2013) and R117H (Yu et al., 2016). Overall, these two classes of mutations that could benefit from VX770 represent approximately 8–10% of the total patient population. Clinical trials indeed showed significant benefit in these patient populations with VX770 including an improvement of lung function by 10.6% (Ramsey et al., 2011; Hadida et al., 2014). The availability of additional potentiator molecules with improved potency, efficacy, and safety could provide further benefit to that provided by VX770.

Here we describe the characterization of two chemically distinct clinical candidate potentiators. This characterization was performed by evaluating their activity using higher throughput methodologies such as yellow fluorescent protein (YFP)-halide assays, low throughput patch clamp assays and more clinically relevant assays using primary cells derived from CF patients.

MATERIALS AND METHODS

Cell Cultures

CFBe41o- cells were cultured in Eagle's minimal essential medium (MEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine and 500 μg/ml hygromycin B. The cells were grown on culture flasks coated with 0.01% bovine serum albumin (BSA) (Sigma), 30 μg/ml Purecol (Nutacon), and 0.001% human fibronectin (Sigma). A CFBe41o- cell line stably expressing F508del CFTR harboring an HRP-tag in the 4th extracellular loop was obtained from Professor Gergely Lukacs (Department of Physiology, McGill University, Montreal, Canada) (Veit et al., 2012). Cells were grown in MEM supplemented with 10% FBS, 1% L-glutamine (Life Technologies), 10 mM HEPES (Life Technologies), 200 μg/ml geneticin (Life Technologies) and 3 μg/ml puromycin (Sigma) on culture flasks coated as for CFBe41o-. HEK293 cells were cultured in uncoated flasks using Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) supplemented with 10% FBS and 1% penicillin/streptomycin. CHO cells were cultured in DMEM containing 10% FBS.

Human Bronchial Epithelial (HBE) Cell Culture

Bronchial epithelial cells isolated from transplanted lungs from wild-type (WT), CF patients homozygous for the F508del mutation or heterozygous G551D/F508del or R334W/F508del patients, were obtained from McGill University (Montreal, Canada) and University of North Carolina (Chapel Hill, NC, United States). Cells were isolated from lungs obtained from donors undergoing a planned transplantation. These primary cells were cultured directly on type IV collagen-coated polycarbonate Transwell supports with a diameter of 6.5 mm and pore size of 0.4 μm (Costar, #3397) for 18–25 days in air liquid (ali) interface essentially as previously described (Fulcher et al., 2005).

Yellow Fluorescent Protein (YFP) Halide Assay (CFBE, Low Temperature Corrected)

CFBe41o- cells were plated in black 384-well microplates at a density of 1,500 cells per well. After 24 h, cells were transduced with adenoviruses containing F508del CFTR and YFP (H148Q/I152L/) F47L using a multiplicity of infection of 30 infectious units per cell for each adenovirus. The next day, for the low temperature corrected assay, cells were incubated at 27°C for 24 h, for the chemically corrected assay cells were treated with corrector for 24 h at 37°C. On the day of analysis, cells were washed five times with Dulbecco's Phosphate-Buffered Saline (DPBS) containing Ca^{2+} and Mg^{2+} (Life Technologies) using a Microplate Washer (BioTek). Then, cells were treated with 10 μM forskolin and the desired concentration of potentiator in a volume of 30 μl and the plates were incubated at room temperature for 10 min, a timepoint optimized in previous experiments showing good window (positive control/negative control > 2) and signal to background ratio. The YFP fluorescence was recorded for 2 min, starting immediately before addition of 30 μl NaI buffer (375 mM NaI, 7.5 mM KI, 1.76 mM KH_2PO_4, 10.1 mM Na_2HPO_4, 13.75 mM glucose) to the wells, using a FDSS/μCell (Hamamatsu) fluorescence reader with a 480 nm excitation filter and a 540 nm emission filter. The capacity of potentiators to increase CFTR channel function was expressed as 1-[fluorescence 36 s after NaI addition (F)/fluorescence before NaI addition (F0)], a timepoint optimized previously, resulting in an optimal signal window. Data was normalized using the formula: normalized response = 100* (absolute response – negative control response)/(VX770 response – negative response) as such, VX770 response corresponds to 100. For Dose-response experiments, data was fitted using a 4 parameter hill function of the form Response = Bottom + (Top-Bottom)/(1 + 10^((LogEC50-concentration)*HillSlope)) to determine EC_{50} values.

YFP Halide Assay (HEK293)

HEK293 cells were transfected with 10–80 ng of plasmid encoding G551D, G178R, G1349D, S549N, R117H, R334W, or wild type CFTR and 20 ng of plasmid encoding YFP (H148Q/I152L/F47L) using jetPEI (Polyplus transfection). Directly after transfection, cells were seeded in black 96-well plates coated with Poly-D-lysine at a density of 70,000 cells per well. The next day, cells were incubated at 27°C for 24 h, for low temperature correction. When evaluating in a chronic setting, the potentiator concentration range was also added to the cells for 24 h. After this incubation period, cells were washed twice with

DPBS with Ca^{2+} and Mg^{2+}. Subsequently, cells were treated with 10 μM forskolin and the desired concentration of potentiator in a volume of 40 μl and incubated at room temperature for 10 min, a timepoint optimized in previous experiments resulting in a good window (positive control/negative control > 2) and signal to background ratio. YFP fluorescence was measured using an EnVision plate reader (PerkinElmer). The signal was recorded for 7 s, starting just before injection of 110 μl NaI buffer (137 mM NaI, 2.7 mM KI, 1.7 mM KH_2PO_4, 10.1 mM Na_2HPO_4, 5 mM D-glucose) into the well with a speed of 150 μl/s, resulting in a final volume of 150 μl. The excitation wavelength was 485 nm and the emission wavelength 530 nm. The capacity of potentiators to increase CFTR channel function was expressed as 1-[fluorescence 7 s after NaI addition (F)/fluorescence before NaI addition (F0)]. Data was normalized using the formula: normalized response = 100* (absolute response – negative control response)/(VX770 response – negative response) as such, VX770 response corresponds to 100. For Dose-response experiments, data was fitted using a 4 parameter hill function of the form Response = Bottom + (Top-Bottom)/(1 + 10^((LogEC50-concentration)*HillSlope)) to determine EC_{50} values.

Cell Surface Expression Assay

CFBE41o$^-$ TetON cells expressing HRP tagged F508del-CFTR (Veit et al., 2012) were seeded at a density of 4,000 cells per well in white 384-well plates (Greiner). Medium containing 500 ng/ml doxycycline was used to induce expression of the F508del-CFTR–HRP construct. After 3 days, cells were treated with corrector and/or potentiator compounds and transferred to an incubator at 33°C. On day 4, cells were washed 5 times with PBS containing Ca^{2+} and Mg^{2+} using a Bio-Tek plate washer and incubated with a chemiluminescent HRP substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific) for 15 min. Chemiluminescence was measured using an Envision plate reader (Perkin Elmer).

TECC Experiments

Trans-epithelial clamp circuit (TECC) recordings were performed using the TECC instrument developed and sold by EP Design (Bertem, Belgium). During the recording, the epithelial cells were bathed in a NaCl-Ringer solution (120 mM NaCl, 20 mM HEPES, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.8 mM KH_2PO_4, 0.8 mM K_2HPO_4, 5 mM glucose, pH 7.4) on both the basolateral (640 μl) and the apical side (160 μl) and kept at 37°C. Apical amiloride was used to inhibit the endogenous ENaC currents (100 μM) while forskolin (10 μM) was applied on both the apical and basolateral sides to stimulate CFTR. All triggers and compounds used during the experiment were first dissolved in DMSO to a 1000 X concentrated solution, just prior to treatment a 10 X stock was prepared in the NaCl-Ringer solution which was used for addition of the correct concentration of trigger and or compound during the experiment. When using the F508del CFTR mutant, VX809 (3 μM) or GLPG2222 (1 μM) corrector was added on the basolateral side for 24 h to partially rescue F508del CFTR before adding the potentiator. For chronic experiments, the potentiator range was also added to the cells for 24 h and reapplied in the NaCl-ringer buffer prior to the electrophysiological recording. Measurements were done during a 20 min timeframe with recordings every 2 min. The transepithelial potential (PD) and transepithelial resistance (R_t) were measured in an open circuit and transformed to I_{eq} using Ohm's law. The increase in I_{eq} (ΔI_{eq}) was used as a measure for the increased CFTR activity. EC_{50} values were generated by measuring impact of different concentrations of compound on I_{eq} in primary cells. For this purpose each transwell was treated with a different compound concentration. CFTRInh-172 was used at 10 μM to assess the specificity of the response. Each compound concentration was tested in duplicate or triplicate and standard error of mean (SEM) was calculated except for R334W/F508del which was single replicate but had a large concentration range showing a clear concentration-responsive effect. Data was fitted using a 3 parameter hill function of the form Response = Bottom + (Top-Bottom)/(1 + 10^((LogEC50-concentration))) to determine EC_{50} values.

Patch-Clamp Electrophysiological Recording

A more complete description of the patch-clamp methodology can be found in a recent publication (Lin et al., 2016). Briefly, CHO (Chinese Hamster Ovary) cells were transiently transfected with pcDNA plasmids containing various CFTR constructs and pEGFP-C3. After transfection, cells were trypsinized and plated onto 35 mm tissue culture dishes dispersed with sterilized glass chips. Electrophysiological experiments were performed 3–7 days following transfection.

Patch-clamp experiments were performed at room temperature with an EPC-9 patch clamp amplifier (HEKA Instruments, Holliston, MA, United States). Recording microelectrodes were made from borosilicate capillary glass with a two-stage pipette puller (Narishige, Tokyo, Japan). The pipette tip was polished with a home-made microforge before use. The pipette solution contained (in mM) 140 NMDG (*N*-methyl-d-glucamine)-Cl (Fisher Biotec), 2 $MgCl_2$ (Fisher Biotec, Perth, Australia), 5 $CaCl_2$ (Fisher Biotec), and 10 HEPES (Fisher Biotec), pH 7.4, with NMDG (see Lin et al., 2016 for details). The pipette resistance when filled with the regular pipette solution was 3–5 MΩ. Once the tip of the pipette and the cell membrane established GΩ resistance, the electrode was quickly pulled away from the cell to form an inside-out patch, where the cytosolic side of the membrane was exposed to the perfusion solution. Cells were perfused with a bath solution having (in mM) 145 NaCl (Fisher Biotec), 5 KCl (Fisher Biotec), 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose (Fisher Biotec), 5 HEPES, and 20 sucrose (Fisher Biotec), pH 7.4, with NaOH (Fisher Biotec). After establishing an inside-out configuration, the patch was perfused with a standard perfusion solution (i.e., intracellular solution) containing (in mM) 150 NMDG-Cl, 2 $MgCl_2$, 10 EGTA (Fisher Biotec), and 8 Tris (Fisher Biotec), pH 7.4, with NMDG (see Lin et al., 2016 for details) containing the catalytic subunit of protein kinase A (PKA) and 2 mM ATP. Once the phosphorylation-dependent

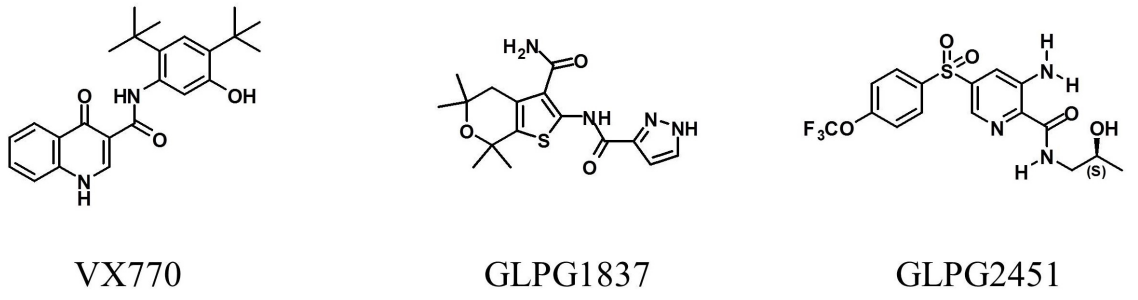

FIGURE 1 | Structure of VX770, GLPG1837, and GLPG2451.

activation of CFTR reached a plateau, 3 μM GLPG1837 or 10 μM GLPG2451 was applied in the continuous presence of 2 mM ATP until a steady state was attained.

Microscopic kinetic analysis was performed with a program provided by Dr. László Csanády (Semmelweis University, Budapest, Hungary) (Csanády, 2000). Experiments were performed 4-5 times and averages and SEM were calculated. The resulting Po values and single-channel kinetic parameters were compared with paired *t*-test (Excel, Microsoft); $P < 0.05$ is considered statistically significant.

RESULTS

Small compound libraries, selected on the basis of molecular shape and electrostatic similarity to known potentiators and correctors, were screened for their ability to improve channel activity of low temperature rescued F508del CFTR using a YFP halide based assay. Several hits were identified including several series of structurally related compounds. Two of these series were selected for further medicinal chemistry efforts and optimization, resulting in the molecules GLPG1837 (described by Van der Plas et al., 2018) and GLPG2451. The chemical structures of both molecules and VX770 as comparator are represented in **Figure 1**.

Both molecules effectively potentiate low temperature rescued F508del CFTR with an EC_{50} of 11.1 ± 3.6 nM (n = 10) and 3.5 ± 0.2 nM (n = 31) for GLPG2451 and GLPG1837 (a representation of the YFP quenching with time in the assay is represented in **Supplementary Figure 1**), respectively. Evaluation in a cell surface expression assay (Veit et al., 2014) shows that both molecules are not able to increase F508del CFTR levels at the plasma membrane and as such show no corrector activity (**Figure 2**).

Characterization of Novel Potentiators on Class III Mutations Using YFP Halide Assay

Both compounds were characterized for their ability to increase channel open probability of various gating defective CFTR mutants. **Figure 3**, represents a comparison of the potency and activity of VX770, GLPG1837, and GLPG2451 on various Class III CFTR mutations. A concentration-dependent increase in activity of G178R, S549N, G551D, and R117H CFTR was observed in the YFP halide assay. In this setting, GLPG1837 shows both a higher potency and a higher efficacy on all mutants tested when compared to VX770. The extent of the increased efficacy varies between the different mutations, 154% for G178R, 137% for S549N, 260% for G551D and 120% for R117H. The behavior of GLPG2451 is slightly different, being more potent on F508del CFTR when compared to VX770, but having similar potency on the other CFTR mutants evaluated. At saturation, the maximal ion channel activity is not as high as that seen for GLPG1837, but is similar to or higher than for VX770 (106% for G178R, 109% for S549N, 171% for G551D and 105% for R117H).

Characterization Using Patient Derived Bronchial Epithelial Cells

Compound activity was then tested in a more physiologically relevant system, i.e., primary bronchial epithelial cells derived from CF patients. The effect on the function of VX809 (3 μM) corrected F508del/F508del CFTR was determined using

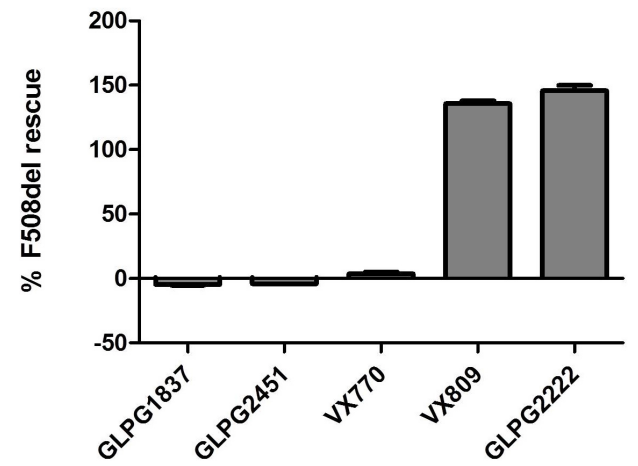

FIGURE 2 | Cell Surface Expression assay: Rescue of HRP-tagged F508del CFTR in CFBe41O- cells was evaluated in presence of (from left to right), 10 μM GLPG1837, GLPG2451, VX770, VX809, or GLPG2222. Percent of cell surface rescue of F508del CFTR was calculated using an internal reference compound 15 (derived from same chemical series as GLPG2222). (*n* between 1 and 155 for different samples).

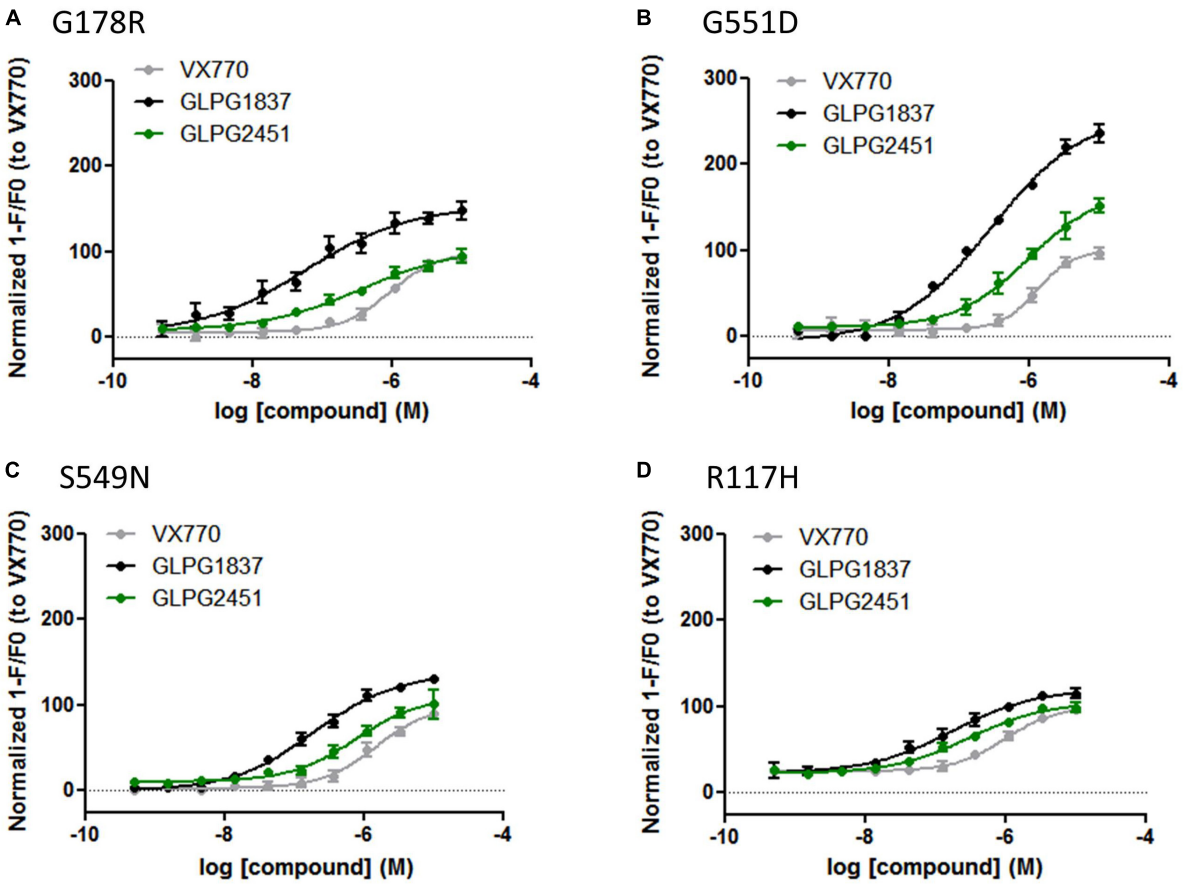

FIGURE 3 | YFP-Halide assay on HEK cells expressing different CFTR mutants: Effect of a concentration range of GLPG1837, GLPG2451, or VX770 on **(A)** G178R CFTR, **(B)** G551D CFTR, **(C)** S549N CFTR, **(D)** R117H CFTR. 10 μM Forskolin was used for channel activation. The measured YFP fluorescence quenching was normalized to VX770 response (*n* between 2 and 8 for each concentration tested).

an electrophysiological readout (TECC) and the data were compared to the data generated in the YFP halide assay. This showed the potency on VX809 corrected F508del/F508del HBEs to be lower (i.e., higher EC_{50}) than on low temperature rescued F508del CFTR in a YFP halide assay [46.6 ± 14.2 nM (n = 6) vs. 3.5 ± 0.2 nM (n = 31) for GLPG1837 and 68.9 ± 25.7 nM (n = 4) compared to 11.1 ± 3.6 nM (n = 10) for GLPG2451 (representative curves in **Figure 4A**)]. This could be the result of differences in the cell background or assay sensitivity but could also result from subtle differences in the structures of low temperature corrected and chemically corrected F508del CFTR resulting in different compound potencies. Consistent with the latter interpretation, VX770 showed a similar potency in both assay formats implying it is not simply an assay difference and that these novel potentiators may act in a mechanistically different manner to VX770 (80.7 ± 21.7 nM (n = 7) (TECC) compared to 126.2 ± 10.9 nM (n = 56) (YFP halide assay), data not shown). To further understand this difference, a YFP halide assay was developed using a GLPG2222-like compound (1 μM) to chemically correct F508del CFTR which allows to determine the potency of the novel potentiators in a setting more similar to the HBE setting. In this case, the potency of both GLPG1837 and GLPG2451 corresponded closely to the potency obtained on chemically corrected F508del HBE cells (**Supplementary Figure 2**), suggesting that the potentiators can differentiate between chemical and temperature corrected F508del CFTR while this is not the case for VX770. Evaluation of low temperature corrected F508del HBE cells was not feasible.

The data in HBE cells were generated by simultaneous addition of potentiators and forskolin, to activate CFTR channels, a protocol referred to as "acute setting." We also tested the compounds ability to potentiate F508del CFTR when incubated with the cells 24 h prior to the addition of forskolin, a setting called "chronic." Under "chronic" conditions the potency of GLPG1837 and GLPG2451 improved ∼6 fold as compared to the "acute" setting (representative curves in **Figure 4B**).

As performed in the YFP halide assay, the effect of GLPG1837 and GLPG2451 on different gating defects was then investigated by TECC using G551D/F508del and R334W/F508del bronchial epithelial cells derived from CF patients.

On G551D/F508del cells, GLPG1837 had an EC_{50} value of 159 nM and an efficacy level of 173% of that of VX770. GLPG2451 had an EC_{50} value of 675 nM and an efficacy level

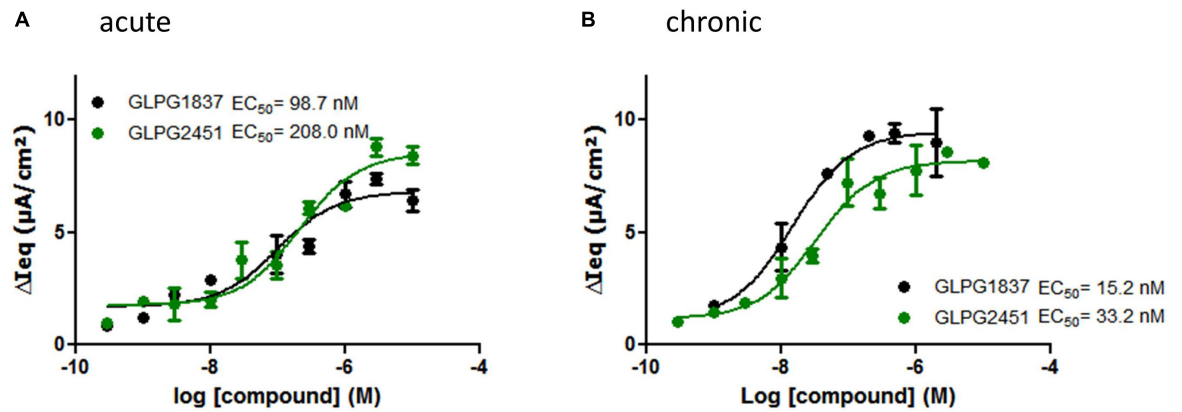

FIGURE 4 | TECC assay on F508del/F508del HBEs: effect of a concentration range of GLPG1837, GLPG2451 on GLPG2222 corrected F508del CFTR. **(A)** Potentiators were added to the cells in "acute" setting, correctors were added 24 h prior to electrophysiological recording. **(B)** Corrector GLPG2222 and a concentration range of the potentiators were added to the cells 24 h prior to electrophysiological recording ("chronic" setting). Data represented is net current obtained from the combined FSK and potentiator treatment (tested in duplicate for each concentration, single F508del/F508del HBE donor shown).

of 147% of that of VX770 (**Figures 5A,B**). The R334W/F508del mutant has residual activity, but improved channel activity can be obtained by addition of a potentiator. In this setting a potency similar to that for corrected F508del CFTR was found (EC_{50} value of 40.7 and 40.3 nM for GLPG1837 and GLPG2451, respectively) again with a higher channel opening

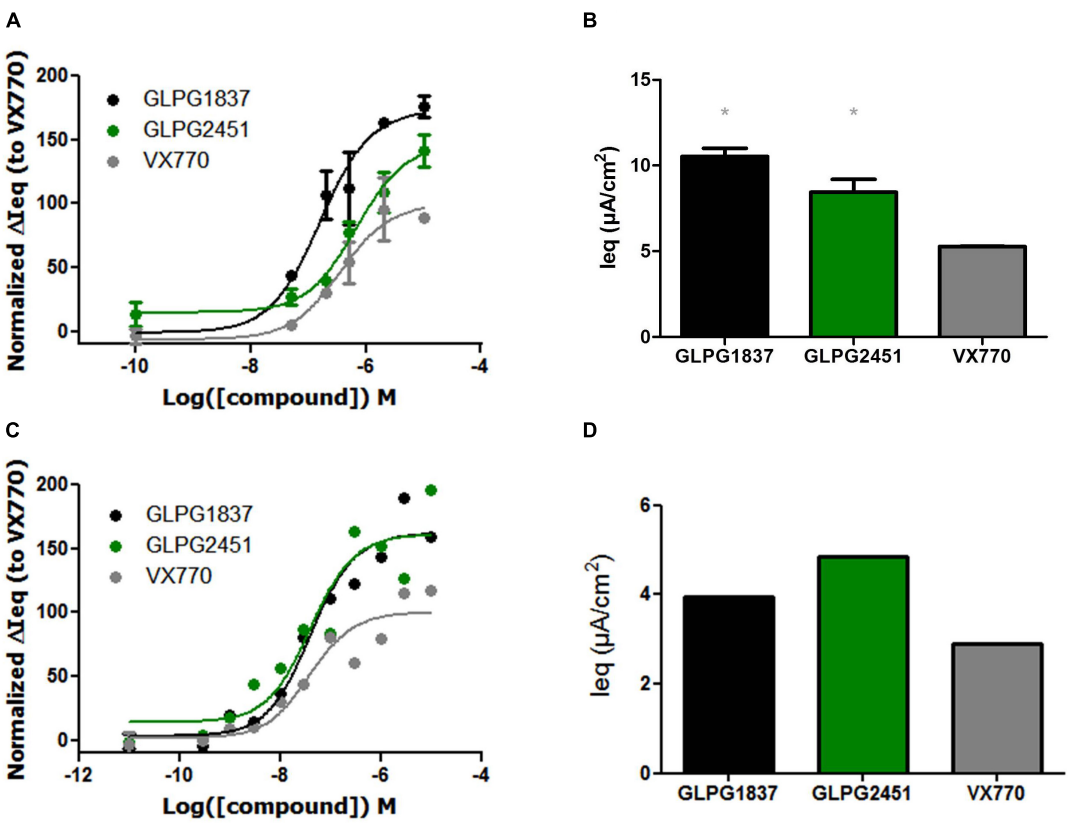

FIGURE 5 | Effect of a concentration range or maximal efficacy at 3 μM of GLPG1837, GLPG2451, and VX770 on G551D/F508del **(A,B)** and R334W/F508del **(C,D)** primary human bronchial epithelial cells. (n = 2 for each tested concentration in G551D/F508del, n = 1 for each tested concentration in R334W/F508del), data in **(A,C)** was normalized to VX770. * denotes a $P < 0.05$ compared to VX770 treatment. **(A)** Contains data for VX770 and GLPG1837 adapted with permission from Van der Plas et al. (2018). Copyright (2018) American Chemical Society.

when compared to VX770 (162% for GLPG1837 and 161% for GLPG2451) (**Figures 5C,D**).

GLPG1837 and GLPG2451 Activation of Wild Type (WT) CFTR

Potentiators influence the open probability of CFTR and are expected to interact directly with CFTR. It has been demonstrated by Yu et al. (2012) that VX770 is able to improve Cl-current carried by WT CFTR. We investigated whether GLPG1837 and GLPG2451 could also improve WT CFTR channel activity. For this, a YFP halide assay was set up using WT CFTR and the assay was optimized to have maximal sensitivity by transfecting with low amounts of WT CFTR plasmid to avoid saturation of the YFP quenching signal. Upon activation of WT CFTR with 10 μM forskolin, GLPG1837 and GLPG2451 were able to increase the open probability with a potency of 103.5 and 79.7 nM, respectively, which is slightly weaker than their potency on F508del CFTR (**Figure 6A**). In primary cells derived from a WT CFTR donor, EC_{50} values of 88.0 and 102.5 nM after activation with 0.1 μM forskolin were found for GLPG1837 and GLPG2451, respectively, similar to the observations in the YFP halide assay (**Figure 6B**).

GLPG1837 and GLPG2451 Improve CFTR Channel Function

The impact of GLPG1837 and GLPG2451 on the CFTR channel activity was further investigated with the patch-clamp technique using WT and/or F508del CFTR. Inside-out patches were excised from cells transiently expressing WT or F508del CFTR, and application of the compounds was found to result in reversible potentiation of the activity of the channels when pre-activated with PKA and ATP. This activation can be seen immediately (within seconds) after addition of the compounds (an example using GLPG2451 is shown in **Supplementary Figure 3**). For WT CFTR, the Po before potentiator stimulation was 0.39 ± 0.04 (with τ = 0.97 ± 0.34 s and τ_c = 1.35 ± 0.27 s, n = 5), in the presence of 3 μM GLPG1837 the Po increased to 0.78 ± 0.04 (n = 5) with open time (τ_o) and closed time (τ_c) constants of 1.48 ± 0.41 s and 0.29 ± 0.03 s, respectively, a result comparable to those seen with VX770 (Jih and Hwang, 2013). For F508del, the Po was also dramatically increased to 0.55 ± 0.05 (n = 5) with τ_o = 3.29 ± 0.82 s and τ_c = 2.18 ± 0.52 s with GLPG1837 (Pre-potentiator Po was 0.04 ± 0.01 with τ_o = 1.16 ± 0.15 s and τ_c = 17.86 ± 1.90 s). Addition of GLPG2451 to F508del CFTR resulted in an increase in Po to 0.57 ± 0.05 (n = 4) with τ_o = 5.05 ± 1.82 s and τ_c = 4.44 ± 2.23 s (Pre-potentiator Po was 0.05 ± 0.01 with τ_o = 2.14 ± 0.86 s and τ_c = 47.88 ± 20.41 s) (**Figure 7**). When determining the channel opening probability impact on F508del CFTR, GLPG1837, and GLPG2451 both behave in a manner comparable to that of VX770, i.e., reducing the closed time and increasing the open time of the channel resulting in a net increase in Po to the comparable level. As there is always an uncertainty regarding the number of functional channels in the patch, the estimated Po should be considered as a maximal value.

Are GLPG1837 and GLPG2451 Additive to Other Potentiators?

A next question we wanted to address is whether the novel potentiators would be able to increase further the CFTR activity when combined with each other or VX770. Whilst using the YFP halide assay to look into additivity/synergy seemed straightforward, none of the combinations resulted in higher CFTR (WT of F508del) activity when compared to the single components (data not shown). The absence of

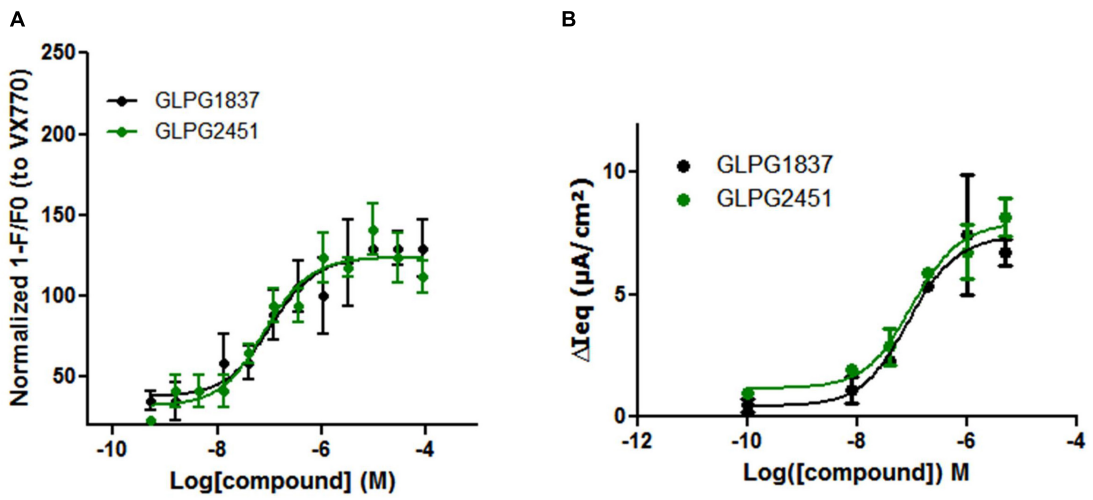

FIGURE 6 | Effect of a concentration range of GLPG1837, GLPG2451 on WT CFTR activity. **(A)** Effect of GLPG1837, GLPG2451 on WT CFTR using a YFP halide assay in HEK293 cells. Data is normalized to the response observed for VX770 set at 100% and each condition was tested in triplicate. **(B)** Effect of GLPG1837 and GLPG2451 on WT CFTR expressed by primary human bronchial epithelial cells (tested in duplicate for each concentration, potentiator response is shown after FSK treatment).

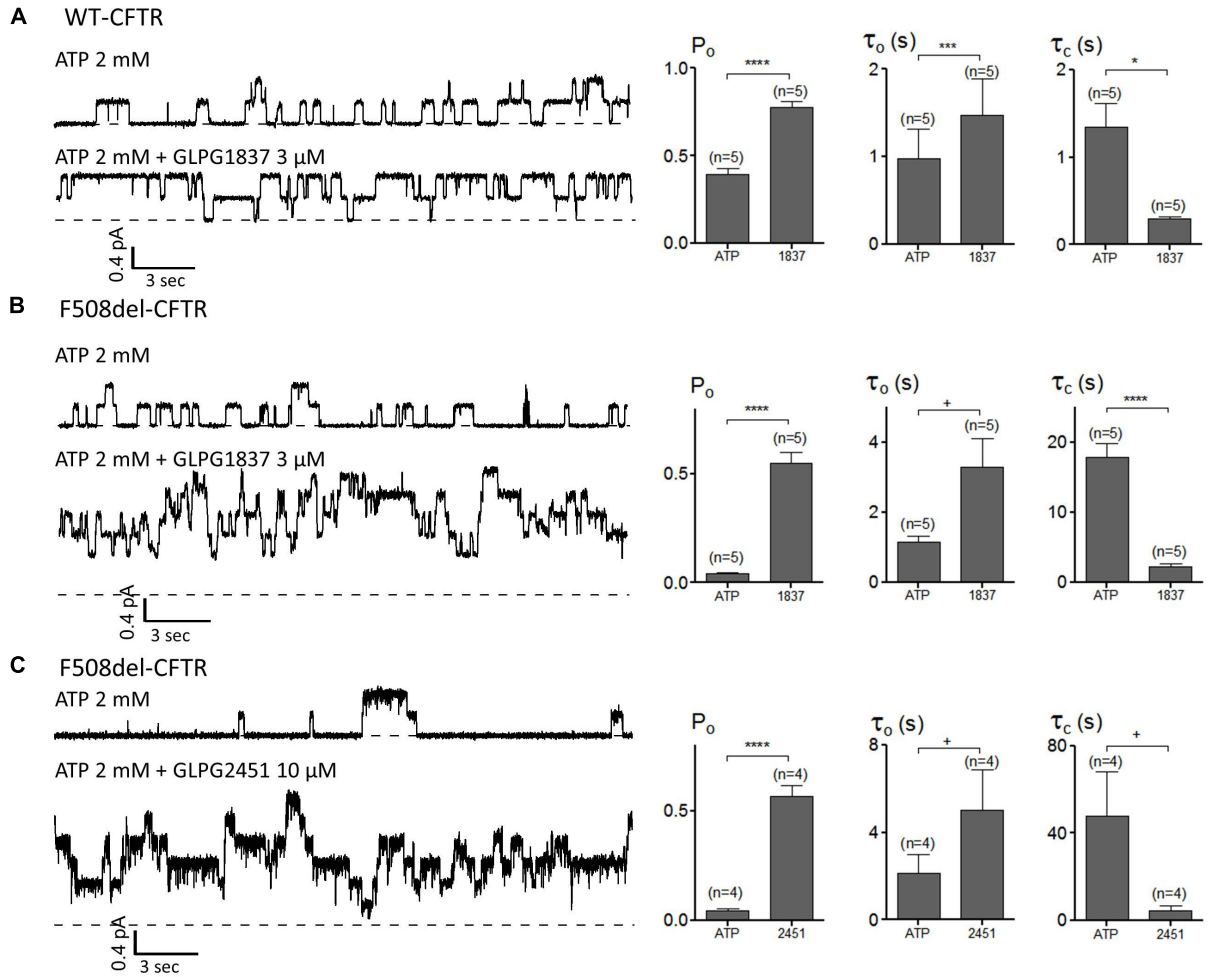

FIGURE 7 | Patch clamp studies on excised patches using CHO cells showing the effect of GLPG1837 on **(A)** WT (Po, τ_o, and τ_c), **(B)** F508del (Po, τ_o, and τ_c) and of GLPG2451 on **(C)** F508del (Po, τ_o, and τ_c) CFTR. The statistical analysis of single-channel kinetic parameters was described in Materials and Methods, every condition was tested at least 4 times. $^*P < 0.05$; $^{***}P < 0.005$; $^{****}P < 0.001$; $^{+}P > 0.05$.

any additional effect on CFTR activity could be due to both potentiators having the same binding site, as molecules are then competing to bind CFTR or due to the dynamic window of the YFP halide assay which might not allow us to see further increase in CFTR activity. Since the maximal effect on WT or F508del CFTR with different potentiators was similar, but different in the case of G551D, it was decided to evaluate potentiator effects on G551D CFTR using the YFP halide assay (**Figure 8**). The experiments showed that GLPG2451 and GLPG1837 failed to show any additive effect, suggesting that both compounds either bind in the same area or are hampering each other's binding (**Figure 8C**). The two potentiators also did not show any additive effect to VX770 (**Figures 8A,B**). A possible limitation to this assay is that a Po of 1 could be reached and therefore no additivity would be observed. However, single channel patch clamp suggests that a Po of only 0.78 ± 0.04 is reached for WT CFTR and 0.55 ± 0.05 for F508del (**Figure 7**). While single channel studies on G551D CFTR were unsuccessful due to the low Po, it is highly unlikely that it would be reaching 1 when treated with either GLPG1837 or GLPG2451. Secondly, it could be that the signal in the assay itself reaches saturation, resulting in an apparent lack of additivity; we therefore performed similar experiments at a lower FSK concentration of 0.3 μM (data not shown). While these experiments resulted in a reduced assay window with higher variability, the data support a similar conclusion as presented with the higher forskolin concentration. In the case of GLPG1837, these data support the observations from Yeh et al. (2017) which suggested GLPG1837 and VX770 may share the same binding site.

GLPG1837 and GLPG2451 Have a Greatly Reduced Detrimental Effect on Surface Expression of Corrected F508del CFTR After Chronic Exposure

Recently, several groups published data on the potential inhibitory effect of chronically exposed VX770 on VX809

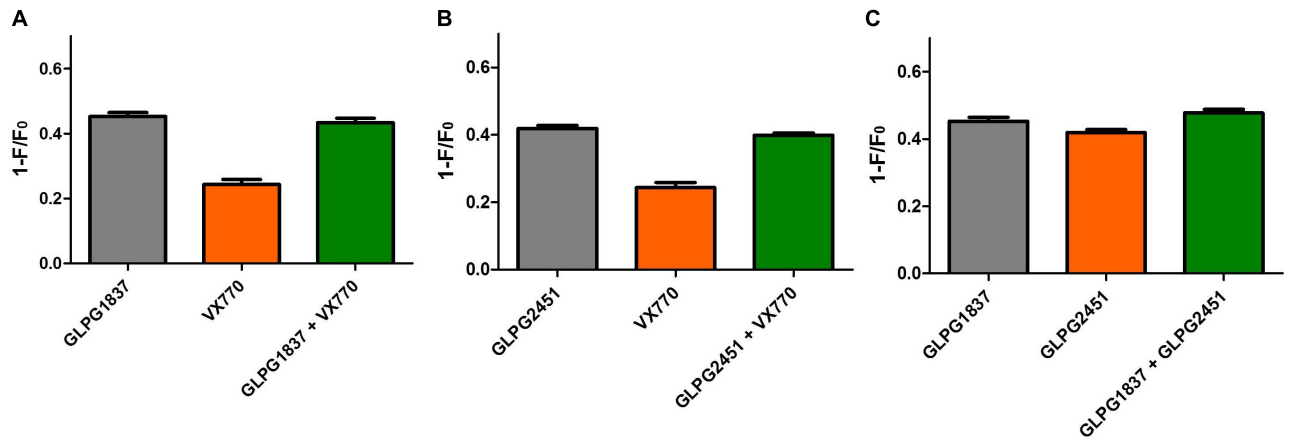

FIGURE 8 | Evaluation of additivity of potentiators on G551D CFTR in a YFP-Halide assay using HEK cells. **(A)** Effect of 90 μM GLPG1837, 10 μM VX770 and combination of both using 1 μM forskolin for channel activiation. **(B)** Effect of 90 μM GLPG2451, 10 μM VX770 and combination of both using 1 μM forskolin for channel activation. **(C)** Effect of 90 μM GLPG1837, 90 μM GLPG2451 and combination of both using 1 μM forskolin for channel activiation. $n = 9$ for each condition.

corrected F508del CFTR leading to reduced amounts of corrected VX809 F508del CFTR on the plasma membrane (Cholon et al., 2014; Veit et al., 2014; Avramescu et al., 2017). The translation to clinical efficacy of these observations at the concentrations evaluated is improbable, however not all types of potentiators show this effect under similar conditions (Veit et al., 2014). We thus included this analysis here. Addition of 10 μM of either GLPG1837, GLPG2451, or VX770 to VX809 corrected F508del/F508del HBE cells in a "chronic" and "acute" setting shows a decrease in F508del CFTR channel activity using VX770 in "chronic" setting, but this negative drug–drug interaction was not observed for GLPG2451 and GLPG1837 in similar experimental conditions (**Figure 9A**). A concentration range of the three potentiators was also evaluated in the cell surface expression assay using F508del CFTR tagged with HRP. F508del CFTR was first partially rescued with VX809 and co-incubated with various concentrations of potentiators. After 24 h co-incubation, the amount of rescued F508del CFTR present at the plasma membrane was determined by measuring the horseradish peroxidase activity. **Figure 9B** shows concentration-dependence of these potentiators on surface expression of F508del CFTR. While VX770 reduces the level of cell surface expressed F508del CFTR at all concentrations tested, a higher concentration of GLPG2451 or GLPG1837 is required to see this detrimental effect. In addition, between GLPG2451 and GLPG1837, GLPG1837 appears to be slightly more potent in decreasing the effect of corrector VX809.

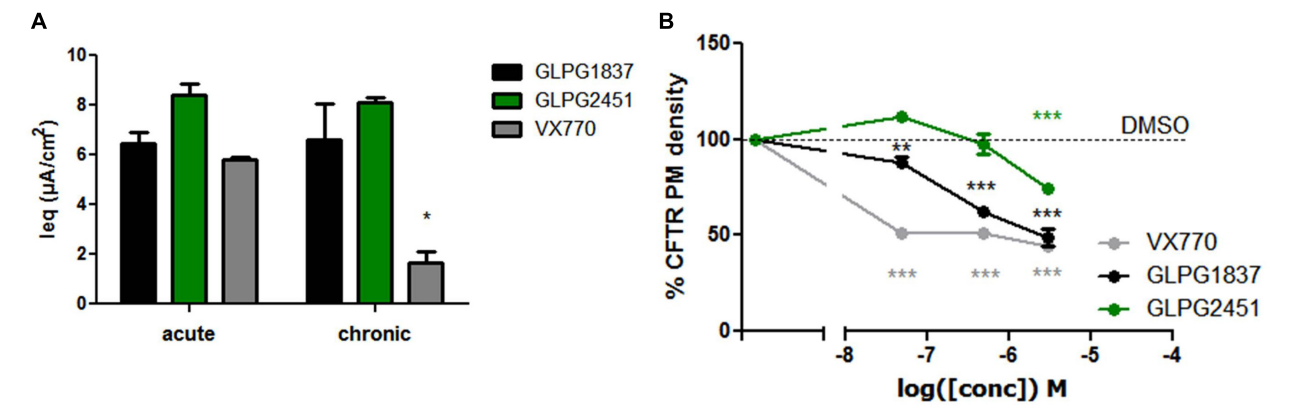

FIGURE 9 | Treatment of F508del CFTR expressing cells corrected with VX809 or GLPG2222 with potentiator in acute or chronic setting. **(A)** Activity of F508del CFTR in primary HBE cells treated for 24 h with 3 μM corrector VX809 (for evaluation with VX770) or 0.15 μM GLPG2222 (in combination with GLPG1837 or GLPG2451). Potentiators were added at 10 μM together with the corrector ("chronic") or just prior to the electrophysiological recording ("acute"). $N = 3$ for each condition, * denotes a $P < 0.05$ compared to similar compound treatment in the acute condition. **(B)** Rescue of HRP-tagged F508del CFTR in CFBe41O- cells treated for 24 h with 10 μM VX809 in combination with a concentration range of VX770, GLPG1837, or GLPG2451. Data was normalized to cells treated with VX809 only. In black, results for GLPG1837 are shown, in green, results for GLPG2451 and in gray results for VX770 ($n > 2$ for each point). $^{**}P < 0.01$; $^{***}P < 0.005$ compared to the control condition without compound treatment.

DISCUSSION

There are currently three approved CFTR modulator treatments available for cystic fibrosis patients, namely Ivacaftor, Orkambi and Symdeko. Of these, Ivacaftor (VX770) is a potentiator which improves the channel function for CFTR mutants with a gating or a conductance defect (Van Goor et al., 2009; Yu et al., 2012). In a clinical trial for patients carrying the G551D mutation, Ivacaftor improved the lung function by 10.6% (Ramsey et al., 2011). Orkambi and Symdeko on the other hand are combination therapies containing VX770 and VX809 or VX661, respectively, and are used for the treatment of patients homozygous for the F508del CFTR mutation. These later treatments show only minor benefit to the patients (Wainwright et al., 2015). Thus, there is a demand for the identification and development of novel correctors and potentiators, or more potent combinations of CFTR modulators for more effective therapy for CF patients with the F508del mutation.

This paper described the characterization of two chemically distinct potentiators GLPG1837 and GLPG2451 which have recently been identified (Van der Plas et al., 2018). Whilst other potentiators with interesting biological activities (absence of detrimental effect on the surface expression of corrected F508del CFTR and good efficacy on G551D CFTR) have recently been reported (Phuan et al., 2015; Park et al., 2016), these are not suitable for clinical development.

Both GLPG1837 and GLPG2451 are able to potentiate F508del, and some of the Class III and IV mutant CFTR. The pharmacological effects measured with YFP halide assays were found to correlate well to the results from more physiologically relevant assays using patient derived primary cells. In the case of F508del CFTR, the potency observed in the YFP halide assay on low temperature rescued CFTR was higher compared to chemical rescued F508del CFTR in both YFP halide and primary HBE cells. This difference was not observed for VX770 used in the same assay settings, suggesting that the novel potentiators may act on CFTR by a different binding mechanism. However, patch clamp data on F508del CFTR showed both GLPG1837 and GLPG2451 enhance the open probability by prolonging the open time and reducing the closed time, similar to that measured in a similar setting for VX770 (Jih and Hwang, 2013). The extent of activation of, e.g., G551D observed with GLPG1837 and GLPG2451 was higher compared to that seen with VX770 supporting a potentially different mechanism of action for the potentiators. Yeh et al. (2017) reported that GLPG1837 induced a 27.5-fold increase of macroscopic G551D CFTR current in comparison to a 10-fold increase by VX770 described in Jih and Hwang (2013). This increase in macroscopic current on G551D is in line with the observed higher channel activity observed in G551D/F508del bronchial epithelial cells in TECC.

We investigated whether both potentiators could act additively to each other or to other potentiators. On F508del CFTR, no further increase in CFTR was observed after addition of any second potentiator to GLPG1837 or GLPG2451. These data suggest that the potentiators cannot bind at the same time to F508del CFTR, or if they do, no further activation of the channel is possible. Yeh et al. (2017) suggested GLPG1837 and VX770 both bind to a similar epitope on CFTR using the patch clamp technique. Since for all potentiators a similar maximal signal is observed when using WT or F508del CFTR, use of these constructs does not allow for the assessment of whether they compete for a similar binding site.

On G551D CFTR, however, several of the potentiators yielded a different maximal quenching rate and therefore we investigated possible interactions between potentiators. GLPG1837 and GLPG2451 didn't show any additive effect to each other nor to VX770, suggesting that the potentiators compete for the same binding site or binding of one hampers binding of the other.

All these data demonstrate that the interactions between potentiators and the channel are complex and additional experiments including structural as well as mechanistic studies would be needed to better understand the binding site and mode of action of these potentiators.

Recently, two groups published data on the potential inhibitory effect of chronically dosed VX770 on VX809 corrected F508del CFTR leading to reduced levels of VX809 corrected F508del CFTR at the plasma membrane (Cholon et al., 2014; Veit et al., 2014; Avramescu et al., 2017). The clinical impact of this observation remains, however, unknown. In this study; the impact of chronic incubation of GLPG1837, GLPG2451, or VX770 together with VX809 was assessed in two different types of assays. When adding a high concentration of potentiator (10 μM) together with VX809 in primary bronchial epithelial cells derived from a F508del/F508del homozygous patient, the detrimental effect of VX770 on VX809 rescued F508del CFTR was observed, comparable to the data presented previously by Cholon et al. (2014). In the same setting, both GLPG1837 and GLPG2451 did not show this effect. Similar effects of VX770 were noted when looking into the effect of the potentiators using a cell surface expression assay as described by Veit et al. (2014). In the same assay, GLPG1837 also showed some reduction of partial rescued F508del CFTR but with a concentration response curve shifted to the right when compared to VX770. GLPG2451 on the other hand showed a minor effect on F508del CFTR rescued protein only at the highest concentration evaluated. These data show that there is a difference in sensitivity toward the negative effect of potentiators on partial rescued F508del CFTR, depending on the assay format used. Overall, GLPG1837 and GLPG2451 reduce membrane density of VX809 rescued F508del CFTR to a lesser extent than VX770 but to a slightly higher extent compared to the potentiator P5 described by Veit et al. (2014).

Based on the more desirable properties of GLPG1837 and GLPG2451 as potentiators, further testing was performed to determine suitability for use in the clinic. GLPG1837 was evaluated in humans and was well tolerated in healthy volunteers, with single doses up to 2,000 mg and 14 days dosing up to 800 mg (Vanhoutte et al., 2015). Similarly, GLPG2451 was evaluated in humans and was well tolerated in healthy volunteers (Brearley et al., 2017). These data supported progression of GLPG1837 and GLPG2451 into Phase 2 studies. Two studies have been designed based on the *in vitro* characteristics of GLPG1837 on Class III CFTR mutants. GLPG1837 has been dosed in patients harboring a G551D or S1251N CFTR mutation. A phase 1b study evaluating the triple combination of GLPG2451, GLPG2222, and GLPG2737 in F508del CF patients is currently ongoing.

In summary, having additional therapeutic options available is of high interest to the CF community. Here, we have characterized two CFTR potentiators with distinct chemical structures GLPG1837 and GLPG2451, the hallmark of which is their higher efficacy for promoting Class III mutant channel gating in comparison to VX770. Having these molecules available to the CF field will enable a better understanding of the molecular mechanism of defects associated with different CFTR variants. Moreover, further testing and optimization of these may pave the way for the development of additional treatment regimens for patients with CF.

AUTHOR CONTRIBUTIONS

MG, SM, KC and JVdS conceptualized the data. MG, SM, SVdP, A-SW, KV, AV, KS, OM, T-CH, PS, AS, MJ, LN, and MA investigated the data. KC wrote the original draft. MG, SM, and MA wrote, reviewed, and edited the manuscript. MG, SM, and KC visualized the data.

ACKNOWLEDGMENTS

We would like to acknowledge Niels Foolen, Moussa El-Abdellati for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01221/full#supplementary-material

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Conflict of Interest Statement: MG, SM, SVdP, A-SW, AV, KV, KS, PS, MJ, JVdS, LN, MA, and KC are employees of Galapagos and may own stock options in that company. AS was an employee of AbbVie.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 01 October 2018
doi: 10.3389/fphar.2018.01112

Role of the SLC26A9 Chloride Channel as Disease Modifier and Potential Therapeutic Target in Cystic Fibrosis

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The solute carrier family 26, member 9 (SLC26A9) is an epithelial chloride channel that is expressed in several organs affected in patients with cystic fibrosis (CF) including the lungs, the pancreas, and the intestine. Emerging evidence suggests SLC26A9 as a modulator of wild-type and mutant CFTR function, and as a potential alternative target to circumvent the basic ion transport defect caused by deficient CFTR-mediated chloride transport in CF. In this review, we summarize *in vitro* studies that revealed multifaceted molecular and functional interactions between SLC26A9 and CFTR that may be implicated in normal transepithelial chloride secretion in health, as well as impaired chloride/fluid transport in CF. Further, we focus on recent genetic association studies and investigations utilizing genetically modified mouse models that identified SLC26A9 as a disease modifier and supported an important role of this alternative chloride channel in the pathophysiology of several organ manifestations in CF, as well as other chronic lung diseases such as asthma and non-CF bronchiectasis. Collectively, these findings and the overlapping endogenous expression with CFTR suggest SLC26A9 an attractive novel therapeutic target that may be exploited to restore epithelial chloride secretion in patients with CF irrespective of their *CFTR* genotype. In addition, pharmacological activation of SLC26A9 may help to augment the effect of CFTR modulator therapies in patients with CF carrying responsive mutations such as the most common disease-causing mutation F508del-CFTR. However, future research and development including the identification of compounds that activate SLC26A9-mediated chloride transport are needed to explore this alternative chloride channel as a therapeutic target in CF and potentially other muco-obstructive lung diseases.

Keywords: cystic fibrosis, epithelial ion transport, chloride channels, SLC26A9, pharmacology

OPEN ACCESS

Edited by:
Nicoletta Pedemonte, Istituto Giannina Gaslini (IRCCS), Italy

Reviewed by:
John W. Hanrahan, McGill University, Canada
Yukiko Sato, McGill University, Canada, in collaboration with reviewer JH.
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *01 August 2018*
Accepted: *10 September 2018*
Published: *01 October 2018*

Citation:
Balázs A and Mall MA (2018) Role of the SLC26A9 Chloride Channel as Disease Modifier and Potential Therapeutic Target in Cystic Fibrosis. Front. Pharmacol. 9:1112. doi: 10.3389/fphar.2018.01112

INTRODUCTION

Cystic fibrosis (CF) is a severe life-shortening multiorgan disease caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) chloride channel, which plays a fundamental role in salt and fluid transport across the surfaces and ducts of many epithelial organs including the lungs, pancreas, and gastro-intestinal tract. In patients with CF, CFTR dysfunction results in impaired epithelial ion and water transport that leads to a characteristic multi-organ

pathology (Mall and Hartl, 2014). More, than 2000 *CFTR* variants have been identified in CF patients, with F508del being the most common accounting for 70% of all CF mutations (Sosnay et al., 2013). Disease-causing mutations may lead to various molecular defects in CFTR protein production, processing, channel function, and stability at the plasma membrane (Welsh and Smith, 1993; Mall and Hartl, 2014). Drug development efforts aiming to restore CFTR function have yielded mutation specific pharmacotherapies that are now available for a subgroup of patients with CF (Gentzsch and Mall, 2018). These include the CFTR potentiator compound ivacaftor (VX-770) that can improve channel activity in CFTR gating mutations present in ∼5% of CF patients and the combination of ivacaftor with the CFTR corrector compound lumacaftor (VX-809) that has been approved for F508del homozygous patients (Ratjen et al., 2017). The large number of disease-causing variants and the variability of biological effects of CFTR mutations and their responsiveness to CFTR modulator therapies argues that targeting an alternative chloride channel may be an attractive therapeutic strategy in CF (Li et al., 2017). Emerging evidence suggests that the alternative chloride channel SLC26A9 is a modulator of CFTR function and a potential candidate to circumvent the primary ion transport defect in several organs affected by CF including the lungs, the pancreas, and the gastrointestinal tract independent of the *CFTR* genotype (Li et al., 2017; Gentzsch and Mall, 2018). In this review, we summarize the basic physiological and pharmacological properties of SLC26A9, outline its regulation and molecular interaction with CFTR, detail the role of SLC26A9 as a disease modifier in CF and potentially in other diseases, and finally discuss the impact of these discoveries on future research that is needed to explore this alternative chloride channel as a potential therapeutic target in the clinical arena.

PHYSIOLOGY, PHARMACOLOGY, AND REGULATION OF SLC26A9

SLC26A9 is a member of the solute-linked carrier 26 (SLC26) anion transporter family that functions uniquely as a chloride channel with minimal conductance to bicarbonate (Dorwart et al., 2007; Loriol et al., 2008). SLC26A9 is mainly expressed in epithelial cells of the respiratory tract, stomach, duodenum, ileum, and the pancreas, while transcripts were also detected in the salivary gland, kidney, brain, heart, prostate, testis, ovary, and skin (Lohi et al., 2002; Xu et al., 2005; Chang et al., 2009b; Lee et al., 2015; Liu et al., 2015; Consortium, 2017). SLC26 members share a conserved structural organization: N-terminally the SLC26/sulphate permease transmembrane domain and C-terminally the cytoplasmic sulphate transporter and anti-sigma factor antagonist (STAS) domain (Geertsma et al., 2015). The STAS domain plays a role in membrane targeting, interaction with scaffolding proteins and other ion channels (detailed later). Furthermore, structural variants in the STAS domain were reported to associate with diseases linked to SLC26 transporters (Sharma et al., 2011). SLC26A9 also has a type-I PDZ-binding domain at the C-terminus (T-A-L), which may promote vesicular trafficking or assembly of macromolecular complexes as seen in other SLC26 members (Lohi et al., 2003; Rossmann et al., 2005). The human SLC26A9 transcript is alternatively spliced, such that inclusion of an exon results in a 96 amino acid extension at the C-terminus (isoform 2). The shorter splice form of 791 residues was designated as canonical isoform, which was also mainly used in heterologous expression systems; however, the isoform-ratio in specific tissues has not yet been elucidated. Similarly to the glycosylation pattern of CFTR, SLC26A9 also displays both core- and complex N-glycosylated forms, which may be important for plasma membrane targeting or function (Li J. et al., 2014; Salomon et al., 2016; Thomson et al., 2016; Bertrand et al., 2017).

The function of SLC26A9 has been widely investigated in heterologous expression systems. Whole-cell patch clamp studies showed that SLC26A9 is a constitutively active chloride channel displaying linear current–voltage characteristics (Dorwart et al., 2007; Loriol et al., 2008; Bertrand et al., 2009; Avella et al., 2011b; Salomon et al., 2016), while the current amplitude was not affected by cAMP stimulation or increase in intracellular Ca^{2+} (Bertrand et al., 2009; Salomon et al., 2016). Interestingly, SLC26A9-expressing epithelial monolayers studied in Ussing chambers displayed constitutive and forskolin stimulated chloride currents, which may be attributed to the enhanced transepithelial electrochemical gradient generated by activation of basolateral potassium channels by cAMP/PKA signaling (Mall et al., 2000; Salomon et al., 2016). Moreover, studies in primary human bronchial epithelial (HBE) cells elegantly demonstrated the presence of a SLC26A9-mediated transepithelial chloride conductance, which contributed to basal and to cAMP/PKA stimulated chloride currents (Bertrand et al., 2009, 2017). These finding support that SLC26A9 could provide an alternative pathway for chloride secretion that may compensate for CFTR dysfunction in CF epithelia. In addition, several studies suggested that SLC26A9 also functions as a chloride-bicarbonate exchanger (Xu et al., 2005; Chang et al., 2009b; Demitrack et al., 2010) and sodium transporter (Chang et al., 2009b). However, the contribution of SLC26A9 to epithelial bicarbonate and sodium transport is not completely understood (see discussion at stomach) and it is possible that transport modes depend on the cell and tissue context.

In the absence of sensitive and specific antibodies for immunolocalization studies, investigations of SLC26A9 largely relied on pharmacological tools. Multiple compounds were found to inhibit SLC26A9 at various potency and selectivity. Highly effective inhibitors include several non-selective chloride channel blockers, such as flufenamic acid, niflumic acid, GlyH-101, and 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), while anion-exchanger inhibitor 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) only partially inhibited SLC26A9 (Dorwart et al., 2007; Loriol et al., 2008; Salomon et al., 2016). On the other hand, SLC26A9 is not sensitive to diphenylamine-2-carboxylic acid (DPC), glibenclamide or CFTRinh172, which makes differentiation from CFTR-mediated chloride currents possible (Loriol et al., 2008; Bertrand et al., 2009;

Salomon et al., 2016). Despite the lack of highly selective inhibitors, this characteristic "pharmacological fingerprint" enabled investigation of SLC26A9-mediated chloride secretion in primary epithelial cultures and native tissues (Anagnostopoulou et al., 2012; Bertrand et al., 2017).

The constitutive channel activity suggests that SLC26A9-mediated chloride secretion is controlled by the insertion and stability of SLC26A9 channels in the plasma membrane. Previous studies demonstrated that SLC26A9 cell surface expression is influenced by WNK (with no lysine [K]) kinases that have been implicated in the regulation of cell volume homeostasis and ion transport processes in epithelial cells (Dorwart et al., 2007; Salomon et al., 2016). WNK kinases uniquely function as intracellular chloride and/or osmolality sensors that activate downstream kinases serine/threonine-protein kinase 39 (SPAK) and oxidative stress-responsive 1 protein (OSR1), which can directly modulate ion transporters by phosphorylation (Alessi et al., 2014; Bazúa-Valenti et al., 2015). Furthermore, WNKs can also act as scaffolds that recruit other proteins that regulate the activity or plasma membrane abundance of ion channels (Rodan and Jenny, 2017). This kinase-independent function of WNKs has been demonstrated to affect plasma membrane trafficking of SLC26A9, as well as CFTR, by mechanisms that may involve lysosomal targeting or endosomal sorting (Dorwart et al., 2007; He et al., 2007; Yang et al., 2007; Zhou et al., 2010).

Notably, there is a large overlap in the expression pattern of SLC26A9 and CFTR in epithelial tissues and emerging evidence suggests a complex, multifaceted interaction between the two channels, both under physiological and pathophysiological conditions. First, there is a functional interaction between the two channels mediated by the STAS domain of SLC26A9 and the R domain of CFTR. Although an inhibitory relationship has also been suggested (Chang et al., 2009a), the nature of this molecular interaction is likely stimulatory, as SLC26A9 contributes to cAMP-stimulated chloride currents in HBE cells and co-expression of CFTR with SLC26A9 results in larger chloride currents than expression of CFTR alone (Loriol et al., 2008; Avella et al., 2011b). Interestingly, the cellular background and level of differentiation may be important determinants of this functional interaction between SLC26A9 and CFTR, as suggested by a study that showed a different behavior in human embryonic kidney (HEK293) cells compared to polarized airway epithelial cells (Ousingsawat et al., 2012). Of note, opposite functional interactions between CFTR and SLC26A9 were also reported in two heterologous expression studies that both used HEK cells (Bertrand et al., 2009; Ousingsawat et al., 2012). While these conflicting results are more difficult to reconcile, we speculate that the observed differences in CFTR/SLC26A9 interactions may be related to differences in the cellular background of the HEK cell clones used in the different laboratories.

Biochemical and functional studies of two other SLC26 family members, SLC26A6 and SLC26A3, showed that following cAMP-dependent stimulation, the phosphorylated R domain can bind to STAS domain, which leads to mutual activation of both CFTR and SLC26 transporters (Ko et al., 2004). Coding mutations located in the STAS domain of SLC26A9 may disrupt this stimulatory interaction, as demonstrated by functional analysis of variant L683P (Avella et al., 2011a). Interestingly, STAS domain variant V622L and domain adjacent variant V744M decreased chloride currents of SLC26A9, which could be partially attributed to decreased plasma membrane expression (Chen et al., 2012). Although no disease-association has been reported for the variants above, these studies demonstrate the role of SLC26A9 STAS domain in protein expression, channel function, and stimulation of CFTR currents.

Second, a recent study demonstrated a physical interaction between SLC26A9 and CFTR, which is mediated by PDZ proteins that facilitate trafficking and stabilization of both proteins at the cell surface (Bertrand et al., 2017). As shown by co-localization studies, this interaction is not restricted to the plasma membrane suggesting a common regulatory mechanism along the secretory pathway (Avella et al., 2011b; Bertrand et al., 2017). SLC26A9 shows affinity to a number of PDZ proteins, such as Na^+/H^+ exchanger-3 regulatory factor 1 (NHERF1) and CFTR-associated ligand (CAL), which display antagonistic effects in CFTR trafficking (Bertrand et al., 2017). CAL, also known as GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein) facilitates ubiquitination and lysosomal degradation of CFTR, as well as endoplasmic reticulum (ER)-associated degradation of F508del-CFTR, whereas NHERF1 tethers CFTR to the plasma membrane thus promoting its cell surface expression (Cheng and Guggino, 2013; Bergbower et al., 2018). Moreover, F508del-CFTR could be rescued by NHERF1 overexpression or CAL silencing (Wolde et al., 2007; Castellani et al., 2012). Constitutive SLC26A9-mediated chloride secretion is diminished in human bronchial epithelium from CF donors carrying F508del-CFTR and recent evidence suggests a PDZ-domain sensitive, CAL-dependent underlying mechanism (Bertrand et al., 2009, 2017). Due to proteasomal degradation of F508del-CFTR, association of CAL with SLC26A9 increases, which in turn hampers its forward trafficking. Furthermore, restoration of F508del-CFTR by the small molecule CFTR corrector compound VX-809 increased CFTR as well as SLC26A9 cell surface expression indicating that rescue of intracellular processing of CFTR has a positive influence on SLC26A9 membrane targeting. Consistent with this notion, SLC26A9-mediated chloride currents were not affected by co-expression with the CFTR gating mutation G551D, which is inserted into the plasma membrane, but has impaired CFTR chloride channel regulation (Bertrand et al., 2017).

Third, numerous genetic association studies and investigations utilizing genetically modified mouse models supported an important role of SLC26A9 in the pathophysiology of several organ manifestations in CF, as well as other chronic lung diseases such as asthma and non-CF bronchiectasis (Anagnostopoulou et al., 2012; Sun et al., 2012; Bakouh et al., 2013; Blackman et al., 2013; Miller et al., 2015; Strug et al., 2016). The results of these studies are consistent with an important role of SLC26A9 as a disease modifier in CF and potentially other

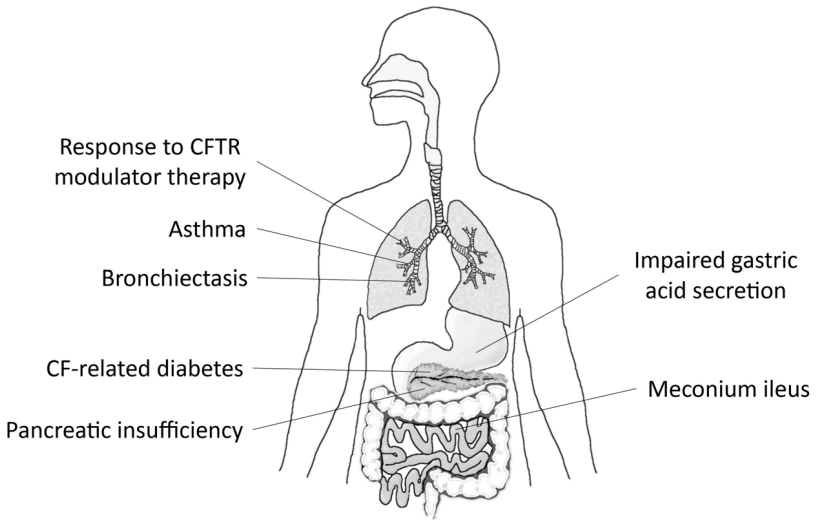

FIGURE 1 | SLC26A9 is a modifier of disease severity in multiple organs affected by CF. Graphical summary depicting associations of variants in *SLC26A9* with exocrine pancreatic insufficiency, CF-related diabetes mellitus (CFRD) and meconium ileus, and emerging data supporting a role of SLC26A9 as modifier of response to CFTR modulator therapy in patients with CF. Beyond CF, SLC26A9 has been implicated in the pathogenesis of asthma, diffuse bronchiectasis (DB), and impaired gastric acid secretion.

muco-obstructive lung diseases and are discussed in more detail below (**Figure 1**).

SLC26A9 CHLORIDE CHANNEL AS A DISEASE MODIFIER IN CF

Lung

Muco-obstructive lung disease remains the major cause of morbidity and mortality in patients with CF. The basic CF ion transport defect causes airway surface dehydration, impaired mucociliary clearance and mucus obstruction, which triggers chronic inflammation and infection, and ultimately leads to progressive lung damage (Button et al., 2016). There is a large heterogeneity in lung disease severity among CF patients, which is influenced by genetic and environmental factors. It is estimated that ∼50% of the genetically determined variability can be attributed to the CFTR genotype and the other ∼50% to CF modifier genes (Cutting, 2015). In an effort to identify genetic determinants of CF lung disease, genome-wide association studies (GWAS) were performed in large CF patient cohorts (Wright et al., 2011; Corvol et al., 2015). These studies revealed a number of loci and candidate genes that may influence lung function, but initially, these investigations did not find association with SLC26A9. However, more recently, SLC26A9 was reported as a modifier of lung function and response to CFTR modulator therapy in patients carrying the CFTR gating mutation G551D (Strug et al., 2016). In a cohort of over 1,700 F508del homozygous CF patients and 70 patients with at least one G551D allele the authors examined the effect of SLC26A9 SNP rs7512462, which was previously reported to associate with CF related diabetes, pancreatic insufficiency and meconium ileus. The minor C allele of rs7512462 positively influenced lung function in G551D CF patients, but had no effect in F508del homozygotes. Furthermore, improvement of lung function, as determined from FEV1 response to therapy with the small molecule CFTR potentiator ivacaftor (VX-770) was markedly higher in patients carrying at least one protective C allele. This study also assessed the effect of rs7512462 in primary HBE cultures from F508del homozygous CF patients and found that rescue of cAMP-stimulated currents in cultures pretreated with lumacaftor (VX-809) increased with each additional C allele (Strug et al., 2016). In the presence of an *in vitro* modulatory effect, the lack of SLC26A9 association with lung function in F508del homozygous patients is difficult to reconcile. One explanation may entail the F508del dependent trafficking defect of SLC26A9 and the necessity of CFTR plasma membrane targeting for SLC26A9 function discussed above (Bertrand et al., 2017). On the other hand, age-dependent expression may also be involved. Interestingly, a recent study in a Brazilian cohort of 188 CF patients revealed association of rs7512462 with several outcome measures of pulmonary function (Pereira et al., 2017). Taken together, these data support that SLC26A9 is a modulator of response to CFTR targeted therapies, which in the future may help predict patient outcomes and optimize tailored treatment regimens for individual patients with CF.

Idiopathic diffuse bronchiectasis (DB) is a chronic lung disease involving airway mucus obstruction, recurrent infections, airway inflammation, and remodeling that resembles CF lung disease in many aspects (Bergougnoux et al., 2015). Interestingly, a previous study reported rare loss-of-function SLC26A9 mutations in patients with DB suggesting that SLC26A9 may be implicated in disease pathogenesis. In a study comparing 147 patients with DB, 78 patients with CF and 50 healthy controls, exon sequencing of SLC26A9 revealed coding variants R575Y and V486I in two DB patients (Bakouh et al., 2013). Functional analysis showed

that both mutations abolished chloride currents and that R575Y located in the STAS domain also interfered with the stimulatory interaction with CFTR.

Further insights on the importance of SLC26A9 in airway epithelial chloride secretion, airway surface hydration, and mucus clearance came from studies in mice with allergic airway disease. In a murine model of allergic asthma, it was shown that type 2 airway inflammation induced SLC26A9-mediated Cl^- secretion, which prevented airway mucus plugging in presence of mucus hypersecretion (Anagnostopoulou et al., 2010, 2012). In the absence of allergic airway inflammation, *Slc26a9*-deficient (*Slc26a9*$^{-/-}$) mice displayed normal lung morphology and no differences in epithelial ion transport when compared to wild-type mice. However, in the presence of allergic inflammation *Slc26a9*$^{-/-}$ mice lacked upregulation of chloride secretion and mucus hypersecretion led to airway mucus plugging. Furthermore, three SNPs at 3′ UTR of SLC26A9 were identified that were associated with a risk for childhood asthma, with an odds ratio of 1.48. Functional investigations revealed that one of these non-coding variants altered a putative micro-RNA response element in the 3′ UTR of SLC26A9 and lead to micro-RNA mediated translational repression *in vitro*, indicating that decreased SLC26A9 protein expression may be the underlying mechanism for disease association (Anagnostopoulou et al., 2012).

Stomach

Investigations in the stomach revealed an essential role of SLC26A9 in the regulation of gastric acid secretion. In the gastric mucosa SLC26A9 is expressed in the surface epithelial cells and in the parietal cells of the deep gastric gland. *Slc26a9*$^{-/-}$ mice displayed structural and ultrastructural changes, such as distended gastric glands, hypoplasia of parietal cells, and loss of tubulovesicular system inside the parietal cells (Xu et al., 2008). Furthermore, H-K-ATPase expression was decreased. *Slc26a9*$^{-/-}$ mice had complete achlorhydria, which could be explained by the absence of transmucosal chloride secretion and/or the role of Slc26a9 in the maintenance and maturation of the secretory pathway that regulates the H-K-ATPase trafficking. Alternatively, Slc26a9 could also promote gastric bicarbonate secretion and regulation of luminal pH to protect against injury by gastric acid (Xu et al., 2005; Demitrack et al., 2010). It is currently debated how SLC26A9 could contribute to bicarbonate secretion. One possibility would be that SLC26A9-mediated chloride secretion is paralleled by chloride-bicarbonate exchange via apical anion exchangers, similarly to the proposed model of pancreatic fluid secretion, where CFTR works tandem with anion exchangers to secrete bicarbonate (Stewart et al., 2009). The second possibility is that SLC26A9 itself can operate as an anion exchanger, which was supported by two studies on gastric epithelium (Xu et al., 2005; Demitrack et al., 2010), however, most heterologous expression data suggest that SLC26A9 functions as an ion channel with minimal bicarbonate conductance. Third, the channel conductance to bicarbonate could also be modulated, similarly to the regulation of CFTR bicarbonate permeability by WNK1-OSR1/SPAK pathway (Park et al., 2010). Notably, SLC26A9 expression was upregulated in *Helicobacter pylori* infection, as well as upon chronic gastritis induced by interleukin-11 in mice, indicating that SLC26A9 is a key player in gastric mucosal defense under pathological conditions (Henriksnäs et al., 2006; Howlett et al., 2012).

Intestine

Obstruction of the ileum due to highly viscous and sticky bowel content, also known as meconium ileus, affects ∼15% of newborns with CF. Sibling and twin studies demonstrated a high heritability for this disease phenotype and a number of genetic studies reported association of SLC26A9 with meconium ileus (Blackman et al., 2006; Sun et al., 2012; Li W. et al., 2014; Miller et al., 2015). SLC26A9 expression decreases along the GI tract with relatively high levels in the stomach, medium levels in the proximal duodenum, low levels in the ileum and no detectable expression in the colon, whereas CFTR shows an inverse pattern with low expression in the stomach and high levels in the duodenum, small intestine and colon (Liu et al., 2015). *Cftr*-deficient (*Cftr*$^{-/-}$) mice show a severe meconium ileus-like intestinal phenotype characterized by severe intestinal plugging. When *Cftr*$^{-/-}$ mice were crossbred with *Slc26a9*$^{-/-}$ mice, mortality highly increased, indicating that SLC26A9-mediated anion secretion ameliorates meconium ileus (Liu et al., 2015). *Slc26a9*$^{-/-}$ mice also failed to elicit duodenal bicarbonate secretion in response to acid load, which may signify the physiological role of SLC26A9 to neutralize gastric acid in the duodenum (Singh et al., 2013).

Pancreas

Exocrine pancreatic insufficiency (PI) in CF is present from early life in nearly all patients with severe CFTR genotypes. CFTR is expressed in the pancreatic ductal epithelium and regulates the secretion of an alkaline, bicarbonate rich pancreatic fluid that washes out digestive enzymes produced by acinar cells. CFTR dysfunction leads to an acidic, low volume, protein rich secretion that can slow down or even block the outflow of zymogens from the ductal tree (Kopelman et al., 1988). Early histopathological changes include dilatation of ducts filled with inspissated zymogen material and destruction of acinar cells (Tucker et al., 2003). In advanced stages, fibrotic and adipose tissue replaces acinar mass and destroys Langerhans-islands (Gibson-Corley et al., 2016). PI shows a strong, but incomplete correlation with CF genotype and CFTR chloride channel function, while SLC26A9 can largely explain the remainder of genetic variability (Cystic Fibrosis Genotype-Phenotype Consortium, 1993; Hirtz et al., 2004; Li W. et al., 2014; Miller et al., 2015). These studies found correlation between circulating levels of immunoreactive trypsinogen (IRT), an early biomarker for exocrine damage, and SLC26A9 SNPs that also associated with meconium ileus and CF-related diabetes mellitus (CFRD). The physiological function of SLC26A9 in the pancreas is largely unexplored. Emerging data suggest that SLC26A9 may enhance ductal anion transport and fluid secretion (Li et al., 2016). Impaired SLC26A9-mediated secretion may thus aggravate the defective ductal wash-out mechanism and ductal plugging in CF. Reduced fluid secretion and ductal

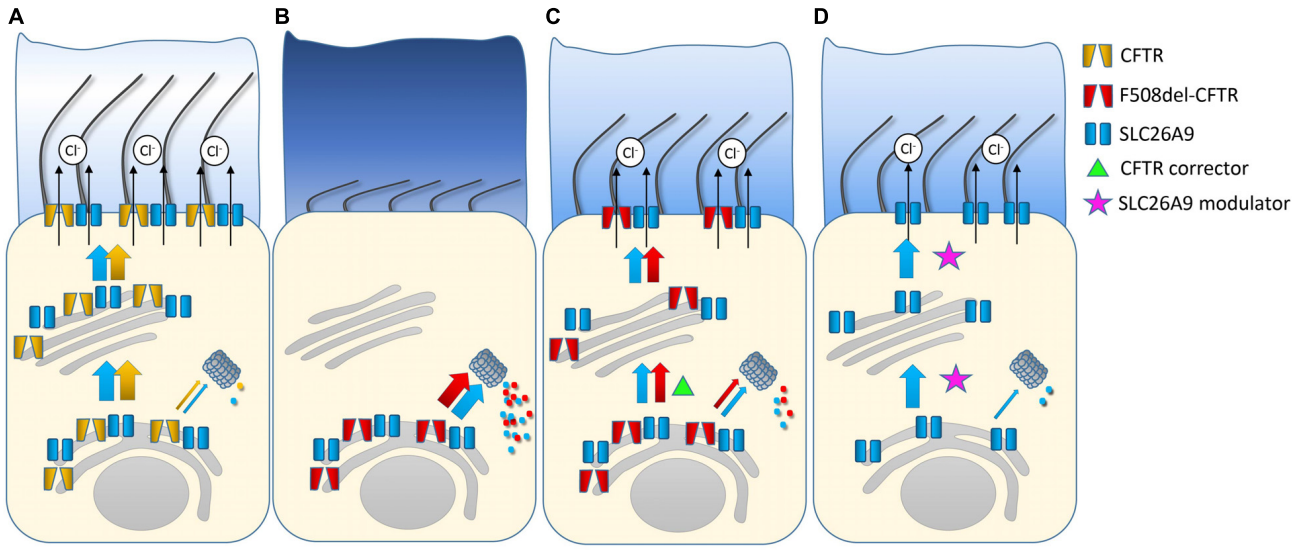

FIGURE 2 | Role SLC26A9 in healthy airways and as a therapeutic target in cystic fibrosis (CF). Healthy airway epithelia express SLC26A9 and CFTR that are co-trafficked from the ER to the plasma membrane, where SLC26A9 functions as a constitutive and CFTR as a cAMP/PKA-regulated chloride channel. Further, SLC26A9 and CFTR interact reciprocally to augment transepithelial chloride/fluid secretion essential to maintain proper airway surface hydration and effective mucociliary clearance **(A)**. In airway epithelial cells from patients with CF carrying the most common disease-causing mutation F508del-CFTR, impaired folding of F508del leads to its retention in the ER and degradation by the proteasome. The F508del trafficking defect also hampers co-trafficking of SLC26A9 and its insertion into the plasma membrane. Lack of SLC26A9 chloride channels may aggravate deficient chloride secretion in CF **(B)**. Pharmacological rescue of F508del-CFTR trafficking with the CFTR corrector lumacaftor (VX-809) restores co-trafficking and insertion of SLC26A9 chloride channels into the apical plasma membrane **(C)**. Compounds that facilitate trafficking of SLC26A9 in the absence of CFTR may improve chloride secretion and airway surface hydration in all patients with CF regardless of the type of CFTR mutation. Alternatively, chloride secretion may also be facilitated by compounds that potentiate SLC26A9 channels that are present and/or newly trafficked to the plasma membrane **(D)**.

mucus obstruction are also present in chronic pancreatitis, a disease that shares many key pathogenic features with CF of the pancreas (Maléth et al., 2015; Balázs et al., 2018). It is conceivable that SLC26A9 may also act as a modifier in these disorders.

Pancreatic insufficiency confers a major risk for CFRD. CFRD is an age-dependent complication that affects 2% of children and 50% of adults with high heritability (Blackman et al., 2009; Moran et al., 2009). A GWAS identified major association of SLC26A9 variants with CFRD, with a hazards ratio of 1.47. Interestingly, meta-analysis of two GWAS studies in type II diabetes showed evidence for association with the same SLC26A9 SNPs, although the risk allele was different (Blackman et al., 2013). There are two prevailing hypotheses on CFRD development: one is that CFRD is driven by the exocrine damage that stresses endocrine cells and causes islet remodeling (Rotti et al., 2018). This is supported by a Mendelian randomization study utilizing a disease-associated SLC26A9 SNP, where a causal relationship between PI and CFRD risk was determined (Soave et al., 2014). Alternatively, loss of CFTR function might have a direct role in insulin secretion (Guo et al., 2014) and/or result in an intrinsic defect that impairs endocrine function before acinar damage occurs (Gibson-Corley et al., 2016). Given its function as chloride channel, it is possible that SLC26A9 may regulate membrane potential and insulin secretion in β-cells, however, there are no data available that support expression of SLC26A9 in the endocrine pancreas.

CONCLUSION AND FUTURE DIRECTIONS

In summary, SLC26A9 constitutes an alternative chloride channel that is implicated in coordinated ion and fluid secretion in various epithelial tissues affected in CF including the airways, the pancreas and the gastro-intestinal tract (Lohi et al., 2002; Dorwart et al., 2007; Bertrand et al., 2009). Demonstration of its role as a disease modifier, as well as overlapping expression of SLC26A9 and CFTR in several affected organs, suggest SLC26A9 as an attractive alternative therapeutic target to bypass the primary ion transport defect in CF (Sun et al., 2012; Blackman et al., 2013; Miller et al., 2015; Strug et al., 2016). In the airways, activation of SLC26A9-mediated chloride/fluid secretion is predicted to counteract airway surface dehydration that sets the stage for mucus plugging and mucociliary dysfunction and constitutes an important disease mechanism in CF lung disease (Mall and Hartl, 2014). Similarly, in the gastro-intestinal tract, activation of SLC26A9 chloride channels is expected to improve the hydration of intestinal surfaces and facilitate pancreatic fluid secretion thus counteracting important extra-pulmonary organ dysfunctions in CF. Therefore, SLC26A9 is an attractive target not only for the treatment of CF lung disease, but also for systemic therapy that may be beneficial for the prevention and/or treatment of several extrapulmonary disease manifestations including meconium ileus and distal intestinal obstruction syndrome (DIOS), exocrine pancreatic insufficiency

and CFRD. Further, pharmacological activation of SLC26A9-mediated chloride/fluid secretion may also be beneficial in other muco-obstructive lung diseases and in chronic pancreatitis (Mall, 2016; Balázs et al., 2018). Importantly activation of an alternative chloride channel is expected to be beneficial for all patients with CF, irrespective of their *CFTR* genotype, including patients with non-sense or splicing mutations, where no CFTR protein is made, or other rare CFTR mutations that do not respond to emerging CFTR modulator therapies (Li et al., 2017). Given the reciprocal interaction with CFTR (**Figures 2A–C**), pharmacological activation of SLC26A9 may also augment functional rescue of mutant CFTR achieved by CFTR modulator therapies (Strug et al., 2016; Bertrand et al., 2017). However, to date, no compounds that activate SLC26A9 chloride channel function have been reported and the development of such SLC26A9 modulators remains a major challenge for further testing of these concepts in preclinical models and in the clinical arena. In this context, the negative impact of the common CFTR mutation F508del present on at least one allele in ∼90% of CF patients on SLC26A9 trafficking indicates that an ideal SLC26A9 modulator compound should overcome this interaction and facilitate trafficking of SLC26A9 from the ER to the plasma membrane independent of CFTR co-trafficking (Strug et al., 2016; Bertrand et al., 2017; **Figure 2D**). In addition to compounds that facilitate SLC26A9 trafficking to increase the number of channels, SLC26A9-mediated chloride secretion may be augmented by compounds that enhance the open probability of the channel or its stability in the plasma membrane. Single channel recordings demonstrated SLC26A9 channels with open and closed states and a conductance of ∼3.2 pS (Chang et al., 2009b) suggesting that it may be possible to increase the open probability by SLC26A9 potentiator compounds. Taking the development of CFTR modulators as a model for drug development, such SCL26A9 modulators may be identified by high-throughput screening of compound libraries in SLC26A9-expression epithelial cells (Gentzsch and Mall, 2018). In addition, a better understanding of SLC26A9 regulation in epithelial tissues, including its interaction network and signaling pathways, may help to explore SLC26A9 as a novel therapeutic target in CF and potentially other muco-obstructive lung diseases.

AUTHOR CONTRIBUTIONS

AB and MM conceived and designed the review, contributed to analysis and interpretation of published literature, and drafted the article and revised it critically for important intellectual content.

FUNDING

MM was supported in part by the German Federal Ministry of Education and Research (82DZL004A1) and a grant from the Einstein Foundation Berlin (EP-2017-393).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 26 July 2018
doi: 10.3389/fphar.2018.00828

SLC26A9 Gene Is Associated With Lung Function Response to Ivacaftor in Patients With Cystic Fibrosis

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Ivacaftor is a drug used to treat cystic fibrosis (CF) patients carrying specific gating *CFTR* mutations. Interpatient variability in the lung response has been shown to be partly explained by rs7512462 in the Solute Carrier Family 26 Member 9 (*SLC26A9*) gene. In an independent and larger cohort, we aimed to evaluate whether *SLC26A9* variants contribute to the variability of the lung phenotype and if they influence the lung response to ivacaftor. We genotyped the French CF Gene Modifier Study cohort (n = 4,840) to investigate whether *SLC26A9* variants were involved in the lung phenotype heterogeneity. Their influence in the response to ivacaftor was tested in the 30 treated patients who met the inclusion criteria: older than 6 years of age, percent-predicted forced expiratory volume measured in 1 s (FEV_{1pp}) in the 3 months before treatment initiation ranging between 40 and 90%. Response to treatment was determined by the change in FEV_{1pp} from baseline, averaged in 15–75 days, and the 1st-year post-treatment. We observed that *SLC26A9* variants were not associated with lung function variability in untreated patients and that gain of lung function in patients treated with ivacaftor was similar to clinical trials. We confirmed that rs7512462 was associated with variability in ivacaftor-lung response, with a significant reduction in lung function improvement for patients with the C allele. Other *SLC26A9* SNPs also contributed to the ivacaftor-response. Interindividual variability in lung response to ivacaftor is associated with *SLC26A9* variants in French CF patients. Pharmacogenomics and personalized medicine will soon be part of CF patient care.

Keywords: cystic fibrosis, lung, gene modifier, SLC26A9, ivacaftor, pharmacogenetic, individualized medicine

OPEN ACCESS

Edited by:
Miquéias Lopes-Pacheco, Universidade de Lisboa, Portugal

Reviewed by:
Fernando Augusto Lima Marson, Universidade Estadual de Campinas, Brazil
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *04 May 2018*
Accepted: *09 July 2018*
Published: *26 July 2018*

Citation:
Corvol H, Mésinèle J, Douksieh I-H, Strug LJ, Boëlle P-Y and Guillot L (2018) SLC26A9 Gene Is Associated With Lung Function Response to Ivacaftor in Patients With Cystic Fibrosis. Front. Pharmacol. 9:828. doi: 10.3389/fphar.2018.00828

INTRODUCTION

Cystic fibrosis (CF) is the most common, severe, autosomal recessive genetic disease in Caucasians. It is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR), a chloride channel expressed in epithelial cells throughout the body (Riordan et al., 1989). The disease affects several organs such as the lungs, pancreas, intestine, and liver. Over 2,000 variations in the *CFTR* gene have been described, including 312 CF-causing variant [The Clinical and Functional TRanslation of CFTR (CFTR2)[1]], which are usually classified into six classes, according to their resulting effect on the protein (Corvol et al., 2016). The most common

[1]http://cftr2.org

mutation (70% of alleles) is p.Phe508del (F508del), which prevents normal CFTR expression at the apical surface of epithelia. *CFTR* genotype strongly influences pancreatic function, which is either deficient (PI for pancreatic insufficiency), or normal (PS for pancreatic sufficiency). It is recognized that in the major part of cases, patients carrying two PI-associated severe *CFTR* mutations have a classical form of CF, whereas others have a milder form of disease associated with PS (Corvol et al., 2016).

Until recently, treatment of CF was only symptomatic. However, in recent years, considerable efforts have led to the development of therapies that target the CFTR protein. Since 2012, patients carrying the *CFTR* gating mutation p.Gly551Asp (G551D) and who are older than 6 years can be treated with ivacaftor, a molecule called a potentiator, which targets CFTR directly to increase the probability of the channel being open (Van Goor et al., 2009). Significant clinical benefits of ivacaftor, such as gain of lung function and reduced number of pulmonary exacerbations, were initially observed in patients older than 12 years and carrying at least one G551D *CFTR* mutation (Ramsey et al., 2011). Subsequently, ivacaftor was approved for other *CFTR*-gating mutations: p.Gly1244Glu (G1244E), p.Gly1349Asp (G1349D), p.Gly178Arg (G178R), p.Gly551Ser (G551S), p.Ser1251Asn (S1251N), p.Ser1255Pro (S1255P), p.Ser549Asn (S549N), and p.Ser549Arg (S549R) (De Boeck et al., 2014) and younger patients (Davies et al., 2013). Now, ivacaftor is approved for patients with CF older than 2 years carrying at least one of these gating mutations (Davies et al., 2016).

The Solute Carrier Family 26 Member 9 gene, *SLC26A9,* was recently shown to modulate the airway response to CFTR-directed therapeutics. In particular, in CF patients carrying at least one CFTR-G551D mutation, the single nucleotide polymorphism (SNP), rs7512462, in the *SLC26A9* gene was shown to explain 28% of the response variability to ivacaftor (Strug et al., 2016). In that study, rs7512462 was also associated with the lung function variability of patients carrying a *CFTR*-gating mutation. Moreover, *SLC26A9* variants have been previously shown in genome wide association studies (GWAS) to contribute to the phenotype variability of meconium ileus (Sun et al., 2012) (rs7512462, rs4077468, rs4077469, rs7419153, rs12047830, rs12741299) and CF-related diabetes (CFRD, rs4077468, rs4077469, rs1874361) (Blackman et al., 2013).

In the current study, we examine the French cohort ($n = 4,840$) of the French CF Gene Modifier Study to investigate whether *SLC26A9* variants firstly contribute to the variability of the lung phenotype, and secondly influence the response to ivacaftor.

MATERIALS AND METHODS

Study Subjects and Lung Phenotype

Patients with CF treated in 38 out of the 47 French CF centres between January 2004 and January 2017 were enrolled in the French CF Modifier Gene Study. As of January 1, 2017, 4,840 patients with CF had been included (corresponding to ∼75% of all French patients with CF) (Vaincre la Mucoviscidose and Ined, 2017). The study was approved by the French ethical committee (CPP n°2004/15), and the information collection was approved by the Commission Nationale de L'informatique et des Libertés (n°04.404). Informed consent in writing was obtained from each patient and/or guardian.

Measurements of the forced expiratory volume measured in 1 s (FEV_1) were either expressed as percent-predicted values (FEV_{1pp}) using the Global Lung Function Initiative (GLI) equations (Quanjer et al., 2012) or transformed to the Kulich Normalized Mortality Adjusted CF-specific lung phenotype (SaKnorm *Z*-value) (Kulich et al., 2005; Taylor et al., 2011). This quantitative phenotype allows indeed direct comparison of lung phenotypes between patients with CF and accounts for differential survival.

Details on the 4,840 CF patients are reported in **Table 1** and in the Flowchart (**Figure 1**). Only patients with severe *CFTR* mutations were considered (pancreatic sufficient patients excluded). Among those, 119 carried at least one gating mutation for which ivacaftor therapy has been approved in Europe (i.e., G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, and S549R), 81 were prescribed ivacaftor. Finally, 60 patients on ivacaftor had lung function measurements available before and after treatment initiation. To assess the association of *SLC26A9* with lung function response to ivacaftor, we included the 30 patients older than 6 years of age and with FEV_{1pp} in the 3 months before treatment initiation ranging between 40 and 90%; their *CFTR* genotypes are depicted in Supplementary Table S1. The response to treatment was determined by the change in FEV_{1pp} from baseline, averaged in the 15–75 days after treatment, as well as that averaged over the 1st-year post-treatment, as used in an earlier study (**Table 1**) (Strug et al., 2016). Besides,

TABLE 1 | Baseline characteristics of the patients.

Patient characteristics	*CFTR* variants		
	F508del/ F508del	Gating[$]/ other	G551D/ other
Pancreatic Insufficient ($n = 4,045$)	$n = 2,143$	$n = 119$	$n = 72$
Sex (male/female)	1,125/1018	64/55	42/30
European origin, *n* (%)	2,061 (96)	105 (88)	67 (93)
Treated by ivacaftor ($n = 81$)			
FEV_{1pp} (baseline) [£]		$n = 60$	$n = 40$
>90%		17	16
40–90%		30	16
<40%		13	8
FEV_{1pp} change analysis ($n = 30$)		$n = 30$	$n = 16$
Age, mean (*SD*)		20.0 (14.0)	17.6 (12.4)
Sex (male/female)		18/12	10/6

[$]*Ivacaftor-approved CFTR gating mutations: G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, and S549R;* ***FEV_{1pp}** *(forced expiratory volume in 1 s percent-predicted) value in the 3 months before treatment. Data are means (SD) or numbers (%) unless otherwise indicated. CFTR: Cystic Fibrosis Transmembrane Conductance Regulator.*

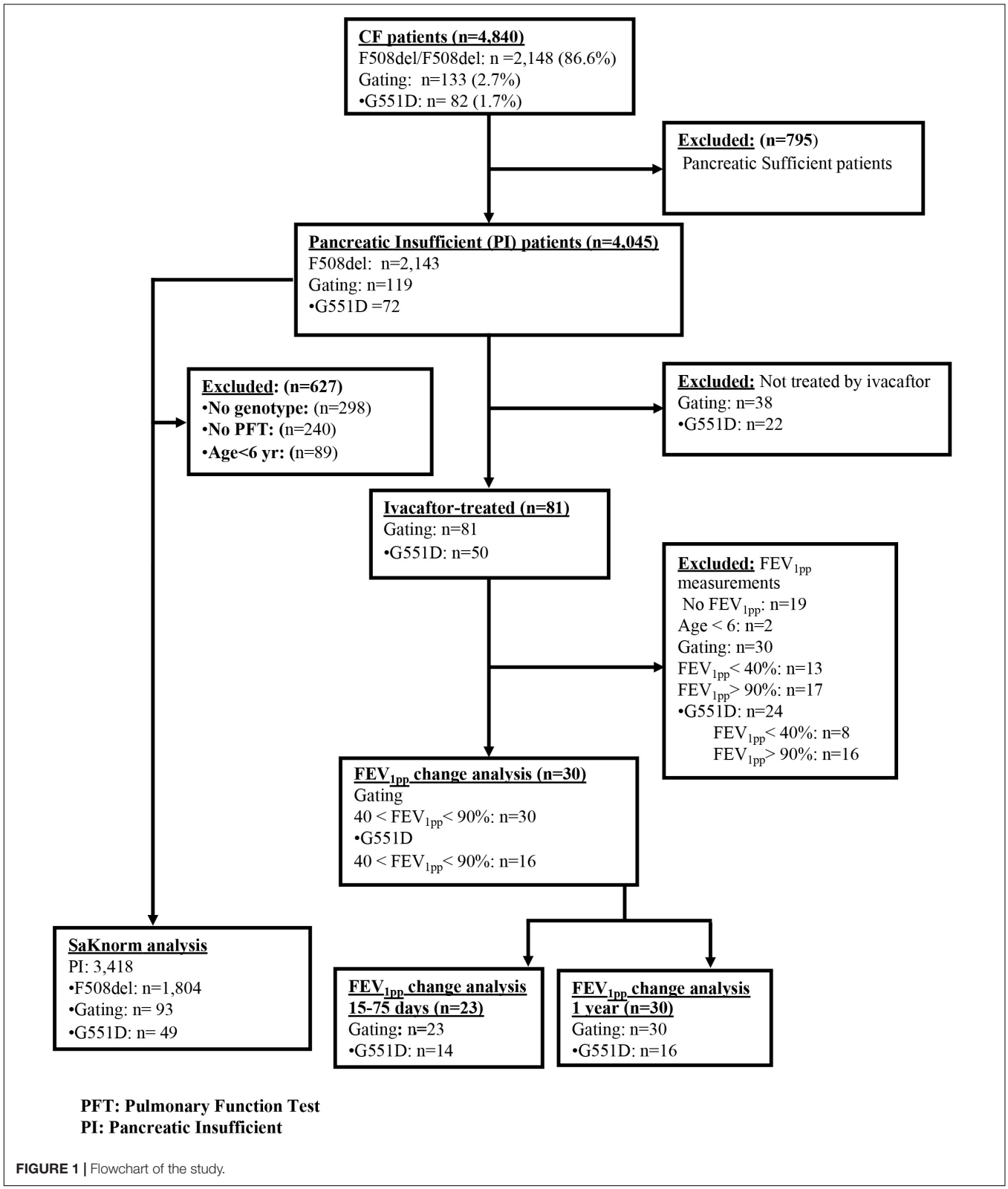

FIGURE 1 | Flowchart of the study.

these two timelines were chosen to evaluate: (1) an "early" response (15–75 days), as it takes several days for ivacaftor to reach a maximal response; and (2) a "long-term" response (1 year), computed by averaging the FEV_{1pp} over the 1st year of ivacaftor treatment, illustrating the overall response of the patients.

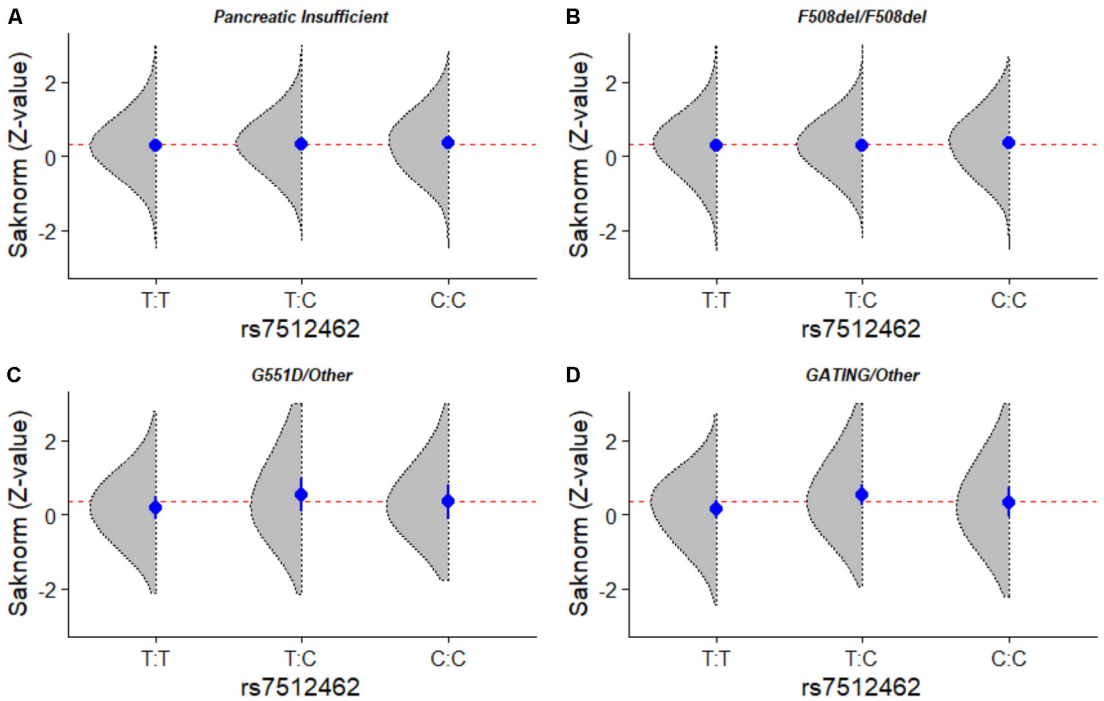

FIGURE 2 | Lung phenotype distribution according to *SLC26A9* rs7512462 variant in patients with cystic fibrosis. Smoothed histograms of SaKnorm *Z*-values by *SLC26A9* rs7512462 genotypes (gray areas), genotype specific mean ± SE (dot and bars) and overall mean (dashed). **(A)** All pancreatic insufficient CF patients (n = 3,418), **(B)** *CFTR* F508del homozygous CF patients (n = 1,804), **(C)** CF patients with at least one G551D allele (n = 49) and **(D)** CF patients with at least one ivacaftor-approved gating mutation (n = 93).

Genotyping

The genotyping of *SLC26A9* SNPs (rs7512462, rs4077468, rs7419153, rs12047830, rs4077469, rs12741299, and rs1874361) was carried out using Kompetitive Allele Specific PCR (KASP) genotyping chemistry (LGC, Teddington, United Kingdom).

Statistical Analysis

Lung function was analyzed as FEV_{1pp} (GLI) (Quanjer et al., 2012) or CF-specific quantile-Z value (SaKnorm *Z*-value) (Kulich et al., 2005; Taylor et al., 2011). For each patient, FEV_{1pp} measurements in the 15-75 days following treatment initiation were averaged to determine the early response. We used the trapezoidal rule to compute average FEV_{1pp} over the 1st year to account for irregular measurements. The change in FEV_{1pp} from baseline was then analyzed by linear regression, adjusting for baseline measurement. We used additive coding to estimate the effect of SNPs in *SLC26A9*. Reference alleles were taken from annotations of the human genome[2]. Among SNPs in *SLC26A9*, rs7512462 had previously shown association with treatment response and, therefore, was analyzed independently from the other SNPs. We also analyzed five other SNPs in *SLC26A9*, adjusting the *P*-values for multiplicity in this situation.

Conformance of the allele frequencies with the Hardy–Weinberg equilibrium (HWE) was tested using a Fisher's exact test. As shown in Supplementary Table S2, the population did not deviate significantly from the HWE indicating no issue with the genotyping method or population stratification.

We reconstructed haplotypes using the EM algorithm with all patients (n = 4,045) keeping loci in their physical order on chromosome 1 (using haplo.stats package in the R software) (Supplementary Table S3) (Lake et al., 2003). We analyzed the association of haplotypes with FEV_{1pp} using additive haplotype coding (see Supplementary Material).

A *P*-value of less than 5% was interpreted as evidence of a statistically significant difference or association. Multiple comparisons utilized the Bonferroni correction. All association analyses were carried out using the software, R (version 3.4.0[3]).

RESULTS

SLC26A9 Gene Variants and Lung Function

In the absence of ivacaftor treatment, the effect of rs7512462 on lung function did not reach statistical significance for any *CFTR* genotype group. We considered all patients (n = 3,418), F508del homozygous patients (n = 1,804), carriers of at least one ivacaftor-approved gating mutation (n = 93), and carriers of one G551D allele (n = 49) (**Figure 2** and **Table 2**). The

[2]www.ensembl.org

[3]http://www.R-project.org/

TABLE 2 | Mean differences in lung function according to *CFTR* and *SLC26A9* variants.

	All pancreatic insufficient patients (*n* = 3,418)		F508del/F508del (*n* = 1,804)		G551D/other (*n* = 49)		Gating[$]/other (*n* = 93)	
***SLC26A9* variants**[£]	**Mean change in CF SaKnorm***	***P*-value**	**Mean change in CF SaKnorm***	***P*-value**	**Mean change in CF SaKnorm***	***P*-value**	**Mean change in CF SaKnorm ***	***P*-value**
rs7512462	0.03 ± 0.02	0.20	0.03 ± 0.03	0.29	0.14 ± 0.16	0.40	0.13 ± 0.12	0.27
rs1874361	−0.02 ± 0.02	0.30	−0.00 ± 0.03	0.97	−0.07 ± 0.16	0.66	−0.17 ± 0.11	0.13
rs12741299	0.01 ± 0.03	0.70	−0.02 ± 0.04	0.64	−0.06 ± 0.33	0.86	0.23 ± 0.23	0.32
rs4077468	0.05 ± 0.02	0.01	0.05 ± 0.03	0.07	0.22 ± 0.18	0.22	0.14 ± 0.13	0.29
rs4077469	0.05 ± 0.02	0.01	0.05 ± 0.03	0.08	0.22 ± 0.17	0.21	0.14 ± 0.13	0.26
rs12047830	0.04 ± 0.02	0.03	0.03 ± 0.02	0.19	0.37 ± 0.18	0.05	0.27 ± 0.13	0.05
rs7419153	−0.02 ± 0.02	0.359	−0.02 ± 0.03	0.47	−0.35 ± 0.18	0.06	−0.18 ± 0.14	0.20

£*See description Supplementary Table S2;* $*Ivacaftor-approved CFTR gating mutations: G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, and S549R; *change per minor allele (linear regression of SaKnorm Z-value with additive model). SaKnorm is normalized function of FEV_1 (forced expiratory volume in 1 s) adjusted for age, sex, height, and cohort-specific survival.*

results were similar with other SNPs in the *SLC26A9* gene (i.e., rs1874361, rs12741299, rs4077468, rs4077469, rs12047830, rs7419153) (**Table 3**).

Change in FEV_1 With Ivacaftor

In total, 30 pancreatic insufficient CF patients had at least one ivacaftor-approved gating mutation, baseline FEV_{1pp} between 40 and 90%, and post-treatment data (see Flowchart, **Figure 1**). FEV_{1pp} measurements within 15–75 days post-treatment and in the 1st year on ivacaftor were available for 23 and 30 patients, respectively, among whom 14 and 16, respectively, were carriers of at least one G551D allele (see Flowchart, **Figure 1**). Overall, patients showed an improvement in FEV_{1pp} after 15–75 days and in the 1st year on ivacaftor. For individuals with ivacaftor-approved gating mutations, the baseline-adjusted change in FEV_{1pp} was +11.72% (95% CI: 7.32–16.06) at 15–75 days and +9.83% (95% CI: 4.91–14.77) in the 1st year of treatment ($P < 0.0001$, **Table 3** and **Figure 3**). In patients with at least one G551D allele, the improvement in FEV_{1pp} was +14.39% (95% CI: 7.89–20.66) at 15–75 days and +15.06% (95% CI: 7.1–23.14) over the 1st year of treatment ($P < 0.0001$, **Table 3** and **Figure 3**).

SLC26A9 Variants and Ivacaftor Treatment Response

In patients carrying at least one G551D allele, the response to treatment changed with the *SLC26A9* rs7512462 genotype, with less change in FEV_{1pp} (−7.7% over 15–75 days and −7.8% over 1 year of treatment) for each C allele ($P = 0.0007$ and 0.006, respectively; **Table 4** and **Figure 4**). Other *SLC26A9* variants showed similar associations with the changes in FEV_{1pp}. In particular, the following SNPs demonstrated significant association after adjustment for multiplicity: with reduced FEV_{1pp} over 15–75 days: rs4077468, −7.9 FEV_{1pp} ($P_{adj} = 0.0007$); and rs4077469, −9.3 FEV_{1pp} ($P_{adj} = 0.0035$); or with increased FEV_{1pp} over 15–75 days: rs7419153, +9.8 FEV_{1pp} ($P_{adj} = 0.0049$) (**Table 4**).

The results for individuals with gating mutations were similar. Indeed, the response to treatment also changed with the *SLC26A9* rs7512462 genotype, with less change in FEV_{1pp} (−5.9% over 15–75 days and −5.2% over 1 year of treatment) for each C allele ($P = 0.0031$ and 0.0042, respectively; **Table 5** and **Figure 4**). Moreover, other *SLC26A9* variants also showed similar associations with, in particular, significant association after adjustment for multiplicity for the following SNPs with reduced FEV_{1pp} over 15–75 days: rs4077468, −7.0 FEV_{1pp} ($P_{adj} = 0.0231$); and rs4077469, −7.8 FEV_{1pp} ($P_{adj} = 0.0490$) (**Table 5**).

In these patients, the most frequent haplotypes (7 SNPs in physical order, Supplementary Table S2) were CCCGTAG (35%) and TACACGA (18%) (Supplementary Table S3). In agreement with the direction of association in the SNP analysis, FEV_{1pp} in carriers of at least one CCCGTAG haplotype increased on average by 17 ± 12% over 15–75 days and by 17 ± 17% over 1 year of treatment, while the FEV_{1pp} of the carriers of at least one TACACGA increased by 6 ± 5% over 15–75 days and 4 ± 5% over 1 year of treatment. Overall, this analysis did not provide significant evidence for a heterogeneity in FEV_{1pp} change with haplotypes ($P = 0.2$ over 15–75 days, $P = 0.08$ over 1 year), but was underpowered given the large diversity of haplotypes in the sample (Supplementary Table S3).

DISCUSSION

We have shown that the response to ivacaftor measured as lung function modulation varied between individuals and is associated with *SLC26A9* variants, as previously described (Strug et al., 2016). *SLC26A9* is a key candidate in CF as it has been shown to play a pleiotropic role across CF phenotypes, associated with meconium ileus (Sun et al., 2012; Li et al., 2014), immunoreactive trypsinogen at birth (Soave et al., 2014; Miller et al., 2015), pancreatic damage (Li et al., 2014), and CFRD (Blackman et al., 2013; Soave et al., 2014). With the development of new curative treatments, such as CFTR-targeted therapies,

TABLE 3 | Change in lung function evolution after 15–75 days and after 1 year on ivacaftor.

	n	Before**	After	Difference	95% CI	*P*-value*
Average FEV_{1pp} measures within 15–75 days on ivacaftor						
Gating[$]/other	23	66.13	80.85	11.72	7.32–16.06	<0.0001
G551D/other	14	70.66	85.05	14.39	7.89–20.66	0.00012
Average FEV_{1pp} measures in the 1st year on ivacaftor						
Gating[$]/other	30	69.07	78.90	9.83	4.91–14.77	<0.0001
G551D/other	16	69.14	84.2	15.06	7.10–23.14	<0.0001

[$]*Ivacaftor-approved CFTR gating mutations: G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, and S549R; **FEV_{1pp} (forced expiratory volume in 1 s percent-predicted) value in the 3 months before treatment. *Paired Mann–Whitney–Wilcoxon test.*

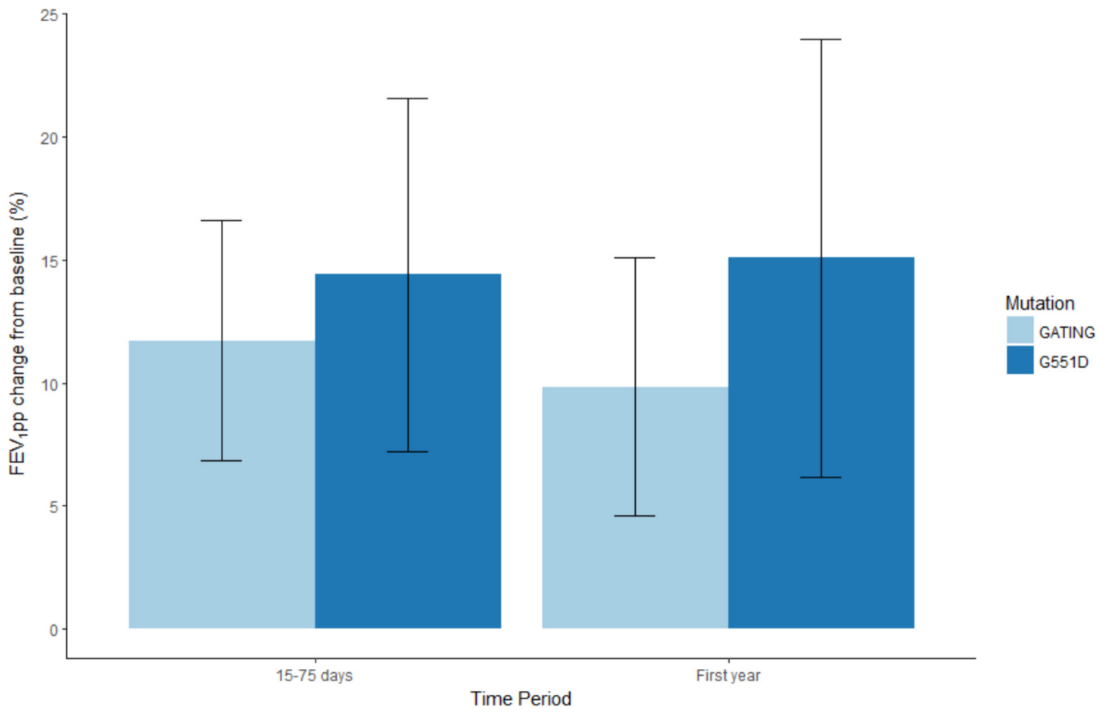

FIGURE 3 | FEV_{1pp} change from baseline in patients carrying at least one ivacaftor-approved gating mutation (light blue) or one G551D variant (dark blue) within the first 15–75 days and over the 1st year of ivacaftor.

TABLE 4 | Change in FEV_{1pp} within 15–75 days and over 1 year on ivacaftor treatment according to *SLC26A9* variants in patients carrying at least one G551D mutation.

Period of evaluation	**15–75 days after ivacaftor treatment start**			**Over the 1st year of ivacaftor treatment**		
***SLC26A9* variants[£]**	**G551D/other (*n* = 14)**			**G551D/other (n = 16)**		
SNP	**FEV_{1pp} change***	***P*-value**	**Adjusted *P*-value****	**FEV_{1pp} change***	***P*-value**	**Adjusted *P*-value****
rs7512462	−7.7 ± 1.7	0.0007	0.0049	−7.8 ± 2.4	0.0063	0.0441
rs1874361	4.4 ± 1.5	0.0107	0.0749	3.1 ± 3.0	0.3129	1.0000
rs12741299	2.9 ± 6.0	0.6336	1.0000	2.1 ± 5.9	0.7331	1.0000
rs4077468	−7.9 ± 1.3	0.0001	0.0007	−8.4 ± 1.7	0.0003	0.0027
rs4077469	−9.3 ± 1.9	0.0005	0.0035	−10.5 ± 2.3	0.0006	0.0042
rs12047830	−10.9 ± 2.7	0.0020	0.014	−7.5 ± 3.5	0.0518	0.3626
rs7419153	9.8 ± 2.1	0.0007	0.0049	8.1 ± 2.6	0.0089	0.0623

[£]*See description Supplementary Table S2; *linear regression of FEV_{1pp} (forced expiratory volume in 1 s percent-predicted) change with additive model adjusted on baseline; **Bonferroni adjustment.*

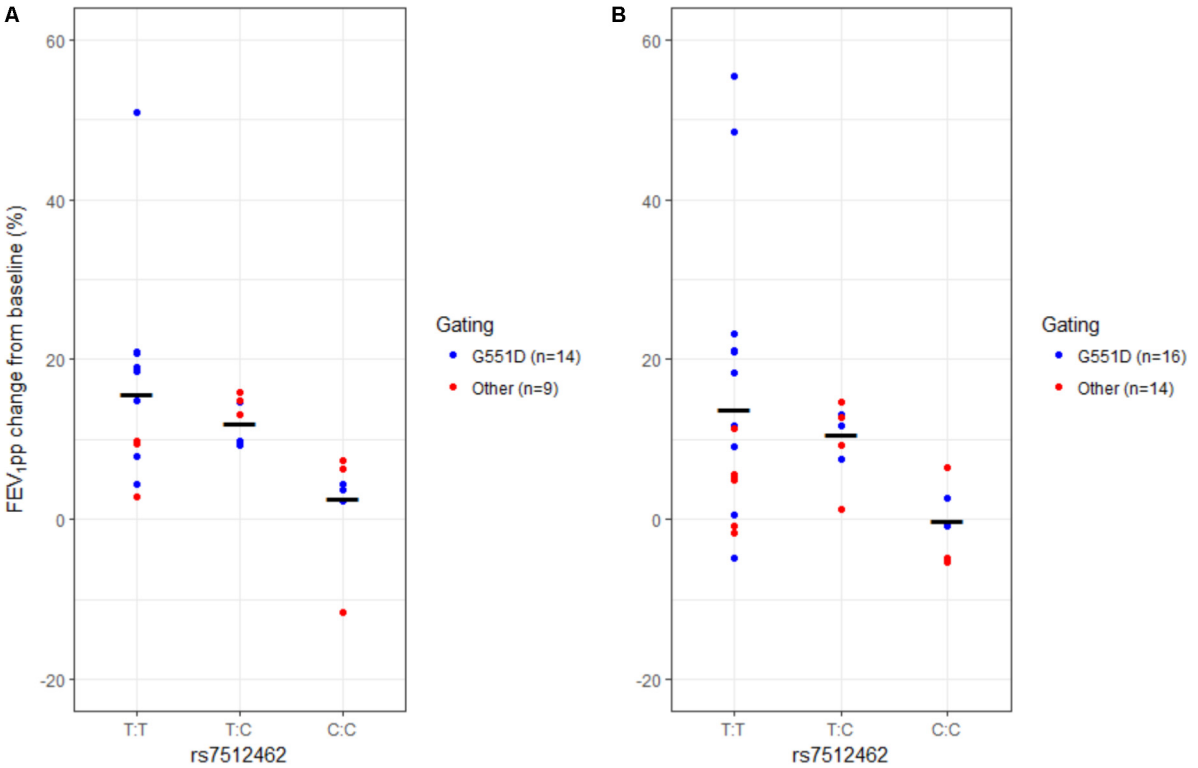

FIGURE 4 | FEV_{1pp} change from baseline in patients with ivacaftor treatment according to rs7512462 *SLC26A9* genotype. Individual change in patients with at least one G551D mutation (blue) and with other gating mutations (red) and model fitted mean change (horizontal bar) within the first 15–75 days of treatment **(A)** and over the 1st year of treatment **(B)**.

pharmacogenomics will become a major step toward functional personalized medicine (Corvol et al., 2016).

SLC26A9 Gene Modulates Ivacaftor Lung Response

We observed that *SLC26A9* variants were associated with the variability in lung responses to ivacaftor measured as FEV_{1pp} change over 1 year with treatment. Indeed, we have shown that, although the response varied between individuals, FEV_{1pp} improved after 15–75 days to 1 year on ivacaftor, in agreement with several clinical trials that led to the approbation of this drug (Ramsey et al., 2011; Davies et al., 2013, 2016; De Boeck et al., 2014; McKone et al., 2014). Further, we observed, as previously shown by Strug et al. (2016), that *SLC26A9* variants modulate this drug response. Focusing on *SLC26A9* rs7512462 in the patients carrying at least one G551D *CFTR* mutation, we observed that the CC genotype was associated with a decrease in FEV_{1pp} of −7.7%. Surprisingly, this effect was inverse to that observed in the pilot study of Strug et al. (2016) who showed an increase in FEV_{1pp} of approximately 8.5% for each additional C allele at rs7512462. Since rs7512462 is not presumed to be functional (Strug et al., 2016), it must be linked to the causal variants so that population-specific differences could explain this difference in direction. In fact, the North-American CF Gene Modifier consortium found significant population admixture in the North-American CF patients, with a large portion of patients reporting African–Caucasian, Mexican–Caucasian, and Indian–Caucasian ancestries (Li et al., 2011), whereby French patients are predominantly of Caucasian origin. Besides, it has been found that the pharmacogenetic response to drugs varied across ethnic groups, which might play a role in the differences observed here between French and Canadian cohorts (Corvol and Burchard, 2008). Nevertheless, Strug et al. (2016) observed in CF patients carrying at least one CFTR-G551D mutation, that the rs7512462 SNP in the *SLC26A9* gene explained 28% of the response variability to ivacaftor, a result similar to ours (22%) (Strug et al., 2016). We observed similar results when evaluating both G551D carriers and patients carrying other ivacaftor-approved gating mutations. There remains, however, a large part of interindividual variation besides the SNP status.

SLC26A9 Gene Is Not a Modifier of Lung Function in CF Patients

In this study, *SLC26A9* variants were not associated with variation in lung function of French patients with CF, regardless of their *CFTR* genotype (i.e., two copies of the F508del mutation and/or at least one gating mutation, the most frequent being G551D). These results are in agreement with previous, large international GWAS studies (Wright et al., 2011; Corvol et al.,

TABLE 5 | Change in FEV_{1pp} within 15–75 days and over 1 year on ivacaftor treatment according to *SLC26A9* variants in patients carrying at least one ivacaftor-approved *CFTR* gating mutation.

Period of evaluation	15–75 days after ivacaftor treatment start			Over the 1st year of ivacaftor treatment		
***SLC26A9* variants**[£]	**Gating[$]/other (*n* = 23)**			**Gating[$]/other (*n* = 30)**		
SNP	**FEV_{1pp} change***	***P*-value**	**Adjusted *P*-value****	**FEV_{1pp} change***	***P*-value***	**Adjusted *P*-value****
rs7512462	−5.9 ± 1.7	0.0031	0.0217	−5.2 ± 1.8	0.0042	0.0294
rs1874361	3.7 ± 1.3	0.0120	0.0840	3.9 ± 1.9	0.0479	0.3346
rs12741299	−2.8 ± 4.8	0.5656	1.0000	−2.7 ± 4.2	0.5270	1.0000
rs4077468	−7.0 ± 2.1	0.0033	0.0231	−7.3 ± 2.2	0.0030	0.0210
rs4077469	−7.8 ± 2.6	0.0070	0.0490	−8.2 ± 2.6	0.0040	0.0280
rs12047830	−6.3 ± 2.4	0.0138	0.0966	−6.6 ± 2.6	0.0161	0.1127
rs7419153	6.3 ± 2.6	0.0233	0.1631	7.9 ± 2.5	0.0038	0.0266

[£]*See description Supplementary Table S2; *linear regression of FEV_{1pp} (forced expiratory volume in one second percent-predicted) change with additive model adjusted on baseline; **Bonferroni adjustment;* [$]*Ivacaftor-approved CFTR gating mutations: G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, and S549R.*

2015). The latest and largest study, a meta-analysis of 6,365 French and North-American CF patients, identified five regions outside the *SLC26A9* locus that displayed significant association with variation in lung disease: the locus of the mucin genes *MUC4* and *MUC20*, of the solute carrier genes *SLC9A3* and *SLC6A14*, and of the HLA Class II region (Corvol et al., 2015). No association was observed between lung function and the *SLC26A9* gene. In the pilot study of Strug et al. (2016) *SLC26A9* rs7512462 also was not associated with lung function variation in CF patients who were homozygous for the F508del mutation. However, an association was observed for patients carrying at least one G551D variant, for whom the number of C alleles was positively associated with improved lung function (Strug et al., 2016). Another independent study of a smaller Brazilian CF cohort with various *CFTR* mutations also did not show an association of this variant with FEV_1 heterogeneity (Pereira et al., 2017). We tested other *SLC26A9* variants that had been previously shown in GWAS to contribute to the variability of other phenotypes, such as meconium ileus (rs4077468, rs4077469, rs7419153, rs12047830, rs12741299) (Sun et al., 2012) and CFRD (rs4077468, rs4077469, rs1874361) (Blackman et al., 2013). However, we found that none of these variants contributed to the lung function heterogeneity in our CF cohort.

Mechanisms of SLC26A9 Variants to Modulate Ivacaftor Lung Response

SLC26A9 is a highly conserved protein predominantly expressed in the lung (Lohi et al., 2002). It functions as a chloride channel with minimal bicarbonate conductance (Loriol et al., 2008; Bertrand et al., 2009), and may constitute an attractive alternative ion channel strategy to compensate for the CFTR defect (Mall and Galietta, 2015). Indeed, in human bronchial epithelial cells, it has been shown that SLC26A9 contributes to constitutive and cAMP-dependant chloride secretion (Bertrand et al., 2009; Avella et al., 2011). Physical interaction between SLC26A9 and CFTR has been shown in several studies (Bertrand et al., 2009; Chang et al., 2009; Avella et al., 2011), even if the consequences of this interaction [reviewed in (El Khouri and Toure, 2014)] are still controversial. According to these studies, SLC26A9 interaction with CFTR enhances CFTR activity (Bertrand et al., 2009; Avella et al., 2011). Reciprocally, CFTR has been shown to modulate SLC26A9 function (Bertrand et al., 2009). However, there has also been evidence showing that CFTR inhibits the activity of SLC26A9 (Chang et al., 2009; Ousingsawat et al., 2012). More recently, SLC26A9 membrane expression and activity was shown to be decreased by CFTR-F508del in co-expression experiments using HEK cells. Interestingly, the correction of F508del CFTR by VX-809 (lumacaftor) was also shown to restore SLC26A9 activity (Bertrand et al., 2017). Finally, a previous functional analysis of eight non-synonymous coding SNPs (p.Y70N, p.T1247N, p.I384T, p.R575W, p.606L, p.V622L, p.V744M, and p.H748R) revealed several functional modifications, including increased or decreased channel activity and altered protein expression that could modify disease (Chen et al., 2012).

The exact mechanism explaining how *SLC26A9* variants affect the ivacaftor responses that we observed in our patients is unknown (depicted in **Figure 5**) and requires future investigation. Based on previous reports, it is reasonable to hypothesize that the variants have an impact on SLC26A9-CFTR-G551D interactions, which could result in altered intracellular trafficking of CFTR-G551D and/or transporter activation.

Our study has several limitations, mainly related to the small sample size of the cohort treated with ivacaftor, due to the rarity of *CFTR* gating mutations (∼4% of individuals with CF). Moreover, baseline FEV_1 measurements at ivacaftor treatment initiation was missing for about one third of the patients (see flowchart), which reduced even more the size of the analyzed cohort. Nevertheless, this study highlights that the interindividual variability in the lung response to ivacaftor might genetically be associated with *SLC26A9* variants in French CF patients and confirms the key pleiotropic role of this gene in CF. To confirm these results, it will be important to extend this study to patients with CF from different countries worldwide. The elucidation of the biological mechanisms beyond this variability is also necessary and will require functional genetic studies. In

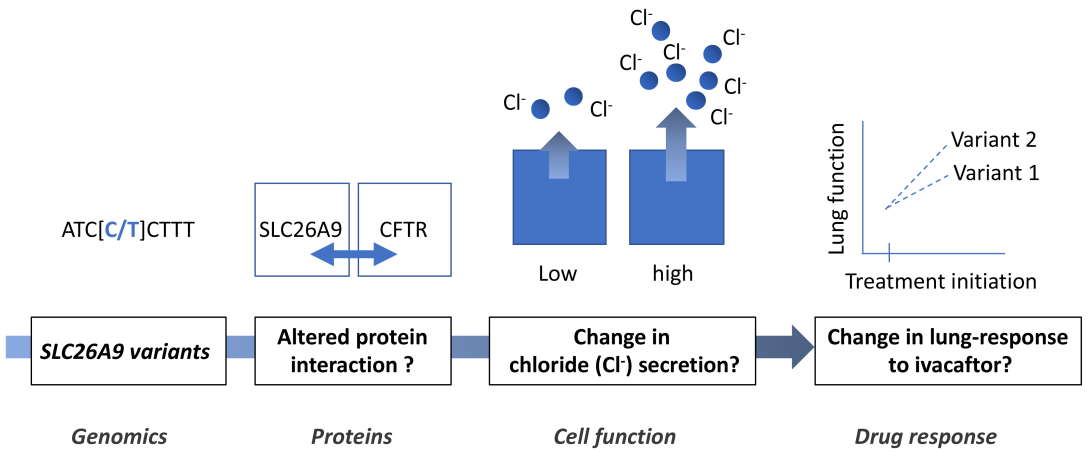

FIGURE 5 | Proposed mechanism of the modulation of ivacaftor lung response in CF patients by *SLC26A9* gene.

the exciting current era of curative treatment development in CF, pharmacogenomics will soon be an integral part of patient care, to modify treatment accordingly to provide the ultimate personalized medicine.

AUTHOR CONTRIBUTIONS

HC, P-YB, and LG designed the study and wrote the manuscript. P-YB, JM, and I-HD performed the data analysis. LS critically revised the manuscript. HC and JM participated in patient recruitment, sample collection, and phenotyping.

FUNDING

This study was supported by Institut National de la Santé et de la Recherche Médicale, Assistance Publique-Hôpitaux de Paris, Sorbonne Université, Agence Nationale de la Recherche (R09186DS), DGS, Association Vaincre La Mucoviscidose, Association Agir Informer Contre la Mucoviscidose, GIS-Institut des Maladies Rares, Cystic Fibrosis foundation (CORVOL16G0).

ACKNOWLEDGMENTS

We would like to thank the patients, care providers and clinic coordinators at CF Centres, as well as the Principal Investigators in the several contributing French CF Centres:

ABELY, M., American Memorial Hospital, Reims/BASSINET, L., Centre Hospitalier Intercommunal de Créteil, Créteil/BELLEGUIC, C., Hôpital Pontchaillou, Rennes/BESSACI, K., American Memorial Hospital, Reims/BILLON, Y., Hôpital de Brabois, Vandoeuvre les Nancy/BRÉMONT, F., Hôpital des Enfants de Toulouse, Toulouse/BROUARD, J., Centre Hospitalier Universitaire de Caen, Caen/BUI, S., Hôpital Des Enfants Groupe Pellegrin, Bordeaux/CAMARA, B., Hôpital de la Tronche, Grenoble/CAMPBELL, K., Centre Hospitalier Universitaire de Caen, Caen/CHIRON, R., Hôpital Arnaud de Villeneuve, Montpellier/COIRIER-DUET, E., Hôpital André Mignot, Le Chesnay/DALPHIN, J. C., CNRS-UFC, UMR 6249 Chrono-environnement, Hôpital Jean Minjoz, Besançon/DALPHIN, M. L., Centre Hospitalier Universitaire de Besançon, Besançon/DAVID, V., Hôpital Mère-Enfant, Nantes/DENEUVILLE, E., Hôpital Sud Annexe Pédiatrique, Rennes/DERELLE, J., Hôpital d'Enfants, Vandoeuvre les Nancy/DOMBLIDES, P., Hôpital Haut Lévêque, Pessac/DOMINIQUE, S., Centre Hospitalier Universitaire Charles Nicolle, Rouen/DUBUS, J. C., Hôpital d'Enfants de la Timone, Marseille/DUFEU, N., Hôpital Nord, Marseille/DURIEU, I., UCBL1, Groupe Hospitalier Lyon Sud, Hospices Civils de Lyon, Pierre Bénite/DURY, S., Hôpital Maison Blanche, Reims/EPAUD, R., Centre Hospitalier Intercommunal de Créteil, Créteil/FANTON, A., Hôpital d'Enfants du Bocage, Dijon/FAYON, M., Hôpital Des Enfants Groupe Pellegrin, Bordeaux/FLEURENCE, E., Hôpital d'Enfants, Saint-Denis de la Réunion/FOUCAUD, P., Hôpital André Mignot, Le Chesnay/GINIES, J.L., Centre Hospitalier Universitaire d'Angers, Angers/GRENET, D., Hôpital Foch, Suresnes/HUBERT, D., Hôpital Cochin, Paris/HUET, F., Hôpital d'Enfants du Bocage, Dijon/KESSLER, R., Hôpital Civil, Strasbourg/LABBÉ, A., Centre Hospitalier Estaing, Clermont-Ferrand/LANGUEPIN, J., Centre Hospitalier, Limoges/LE BOURGEOIS, M., Necker Hôpital d'Enfants Malades, Paris/LE ROUX, P., Hôpital Jacques Monod, Montivilliers/LEROY, S., CHU de Nice, Nice/LLERENA, C., Hôpital de la Tronche, Grenoble/LOEUILLE, G. A., Centre Hospitalier de Dunkerque, Dunkerque/MARCHAND, S., Hôpital Clocheville, Tours/MARGUET, C., Centre Hospitalier Universitaire Charles Nicolle, Rouen/MELY, L., Hôpital Renée Sabran, Giens/MUNCK, A., Hôpital Robert Debré, Paris/MURRIS-ESPIN, M., Hôpital Larrey, Toulouse/NOVE JOSSERAND, R., Groupe Hospitalier Lyon Sud, Hospices Civils de Lyon, Pierre Bénite/PIN, I., INSERM U823 Université Joseph Fourier, Hôpital de la Tronche, Grenoble/PREVOTAT, A., Hôpital Calmette, Lille/RAMES, C., Hôpital Nord, Amiens/RAULT, G.,

Centre de Perharidy, Roscoff/REIX, P., Hôpital Femme Mère Enfant, Bron/REMUS, N., Centre Hospitalier Intercommunal de Créteil, Créteil/RENOUIL, M., Groupe Hospitalier Sud Réunion, Saint-Pierre de la Réunion/REYNAUD-GAUBERT, M., Hôpital Nord, Marseille/SERMET-GAUDELUS, I., Necker Hôpital d'Enfants Malades, Paris/STREMLER, N., Hôpital d'Enfants de la Timone, Marseille/STORNI, V., Centre Hospitalier Bretagne Atlantique, Vannes/TASSET, C., Hôpital d'Enfants, Saint-Pierre de la Réunion/URBAN, T., Centre Hospitalier Universitaire d'Angers, Angers/VIGNERON, P., Centre Hospitalier Bretagne Sud, Lorient/WALLAERT, B., Hôpital Calmette, Lille/WEISS, L., Hôpital de Hautepierre, Strasbourg/WIZLA, N., Hôpital Jeanne de Flandre, Lille.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00828/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 29 January 2019
doi: 10.3389/fphar.2019.00003

TMEM16A in Cystic Fibrosis: Activating or Inhibiting?

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OPEN ACCESS

Edited by:
Nicoletta Pedemonte, Istituto Giannina Gaslini (IRCCS), Italy

Reviewed by:
Carlos A. Flores, Centro de Estudios Científicos, Chile
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Alessandra Livraghi-Butrico, University of North Carolina at Chapel Hill, United States

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *30 August 2018*
Accepted: *04 January 2019*
Published: *29 January 2019*

Citation:
Kunzelmann K, Ousingsawat J, Cabrita I, Doušová T, Bähr A, Janda M, Schreiber R and Benedetto R (2019) TMEM16A in Cystic Fibrosis: Activating or Inhibiting? Front. Pharmacol. 10:3. doi: 10.3389/fphar.2019.00003

The inflammatory airway disease cystic fibrosis (CF) is characterized by airway obstruction due to mucus hypersecretion, airway plugging, and bronchoconstriction. The cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is dysfunctional in CF, leading to defects in epithelial transport. Although CF pathogenesis is still disputed, activation of alternative Cl^- channels is assumed to improve lung function in CF. Two suitable non-CFTR Cl^- channels are present in the airway epithelium, the Ca^{2+} activated channel TMEM16A and SLC26A9. Activation of these channels is thought to be feasible to improve hydration of the airway mucus and to increase mucociliary clearance. Interestingly, both channels are upregulated during inflammatory lung disease. They are assumed to support fluid secretion, necessary to hydrate excess mucus and to maintain mucus clearance. During inflammation, however, TMEM16A is upregulated particularly in mucus producing cells, with only little expression in ciliated cells. Recently it was shown that knockout of TMEM16A in ciliated cells strongly compromises Cl^- conductance and attenuated mucus secretion, but does not lead to a CF-like lung disease and airway plugging. Along this line, activation of TMEM16A by denufosol, a stable purinergic ligand, failed to demonstrate any benefit to CF patients in earlier studies. It rather induced adverse effects such as cough. A number of studies suggest that TMEM16A is essential for mucus secretion and possibly also for mucus production. Evidence is now provided for a crucial role of TMEM16A in fusion of mucus-filled granules with the apical plasma membrane and cellular exocytosis. This is probably due to local Ca^{2+} signals facilitated by TMEM16A. Taken together, TMEM16A supports fluid secretion by ciliated airway epithelial cells, but also maintains excessive mucus secretion during inflammatory airway disease. Because TMEM16A also supports airway smooth muscle contraction, inhibition rather than activation of TMEM16A might be the appropriate treatment for CF lung disease, asthma and COPD. As a number of FDA-approved and well-tolerated drugs have been shown to inhibit TMEM16A, evaluation in clinical trials appears timely.

Keywords: TMEM16A, anoctamin 1, mucus secretion, cystic fibrosis, asthma, COPD, Ca^{2+} signaling

INTRODUCTION

The inflammatory airway disease cystic fibrosis (CF) is characterized by airway obstruction due to mucus hypersecretion, mucus plugging, and bronchoconstriction. The cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is dysfunctional in CF, leading to a loss of fluid secretion and probably impaired bicarbonate transport, along with Na^+ hyperabsorption (Boucher, 2007; Stoltz et al., 2015). Dehydration of the airway surface (periciliary) fluid layer (ASL) covering the airway epithelium is thought to be the crucial factor leading to abnormal rheological properties of the mucus and occlusion of smaller airways (Boucher, 2007). Independent of the underlying precise molecular defect, tremendous effort was put into identification of small molecules, and natural compounds that would correct the basic defect by restoring CFTR function (Amaral and Kunzelmann, 2007; Verkman and Galietta, 2009). This, however, turned out to be a long and stony path that has finally provided effective drugs that correct and potentiate mutant CFTR. Moreover, the search is on to identify compounds that inhibit salt hyperabsorption in CF, and to find molecules that activate alternative Cl^- secretory pathways. What has not been in the minds of CF researchers is to look for inhibitors of certain types of Cl^- channels as a treatment for inflammatory airway disease (Li et al., 2017).

STILL UNRESOLVED: THE PATHOGENESIS OF CF

Lack of appropriate Cl^- secretion due to defective CFTR was long regarded as the essential, if not only cause for CF lung disease. However, a pathogenic concept proposing Na^+ hyperabsorption relative to attenuated Cl^- secretion, leading to airway dehydration and mucus plugging, has also been put forward in a number of studies (Boucher et al., 1986; Grubb et al., 1994; Mall et al., 1998a, 2004). We detected enhanced Na^+ conductances in nasal *ex vivo* tissue and freshly isolated intestinal cells from CF patients (Mall et al., 1998a, 2000). Along this line, reduced ASL would lead to thickened airway mucus, airway plugging and impaired mucociliary clearance with subsequent chronic bacterial infections. Yet, this concept has been questioned by Welsh and collaborators as well as other investigators, who did not find evidence for Na^+ hyperabsorption. In contrast, reduced airway Na^+ absorption in CF was claimed, leading to salt accumulation in the ASL, which under normal conditions might be even hypotonic when compared with the interstitial fluid. Thus, hypertonic ASL was blamed to inactivate ß-defensins, thereby causing a predisposition toward bacterial infections (Zabner et al., 1998; Chen et al., 2010; Itani et al., 2011). In contrast, the Boucher team and others found neither evidence for a hypotonic ASL under normal conditions, nor any salt concentration (hypertonic ASL) in CF airways (Matsui et al., 1998). Given the fact that the airway epithelium is relatively leaky and has a large hydraulic conductivity, it appears somewhat unlikely that it maintains a large transepithelial osmotic gradient.

A similar controversy arose around the pH value of the ASL. It had been shown that CFTR is permeable for bicarbonate (HCO_3^-), or contributes to HCO_3^- transport as a Cl^- recycling pathway in a number of epithelial organs [reviewed in (Kunzelmann et al., 2017)]. To what extend HCO_3^- is conducted by CFTR or rather operates indirectly as a Cl^- recycling channel that drives HCO_3^- secretion by Cl^-/HCO_3^- exchangers, is still a matter of debate. At any rate, Smith and Welsh were among the first to show defective cAMP-induced bicarbonate secretion in airways of CF patients (Smith and Welsh, 1992), while others showed that CFTR is permeable for HCO_3^- (Poulsen et al., 1994; Tang et al., 2009). It should be noted that patch clamp and other types of experiments with isotonic concentrations of HCO_3^- are not trivial and may be compromised by pH fluctuations (Kunzelmann et al., 1991). Attenuated fluid/HCO_3^- secretion in CF airways was shown to have adverse effects on the biophysical properties of airway mucus (Trout et al., 1998). Quinton and others provided further evidence that bicarbonate transport is essential for proper mucus release and viscosity (Choi et al., 2001; Quinton, 2001). In fact, HCO_3^- transport is impaired in a number of different epithelial tissues derived from CF patients (Kunzelmann et al., 2017). Importantly, human lung pathology was brilliantly reproduced in a CF pig model. Using this CF pig model, reduced airway surface liquid pH, impaired bacterial killing, and mucus abnormalities were demonstrated (Pedersen et al., 1999; Stoltz et al., 2010; Pezzulo et al., 2012; Hoegger et al., 2014). Interestingly, Hoegger et al. demonstrated abnormal mucociliary transport in CF in submerged epithelia, which somewhat questions the role of surface dehydration in CF (Hoegger et al., 2014). In sharp contrast to these results, Schultz and coworkers found no evidence for acidic airway surface liquid pH in lungs of CF children, using a novel optical pH probe and a specialized bronchoscope (Schultz et al., 2017).

Stick and Schulz claimed that only a small fraction of infants diagnosed early with CF through the Australian AREST CF early surveillance program, present lower airway infection (Stick and Schultz, 2018). This may support the "inflammation first" concept, proposing that inflammation in CF lungs is present early in life and clearly before airway infection (Khan et al., 1995; Doring and Worlitzsch, 2000). Thus, CF epithelia appear to be in an inflammatory, pro-proliferative, and constantly remodeling state, even without any bacterial infection (Hajj et al., 2007; Rottner et al., 2007, 2009; Martins et al., 2011). CF epithelial cells were also shown to have a compromised anti-oxidant defense by superoxide dismutase (Rottner et al., 2011). Notably, some CFTR knockout mouse models do show signs of lung/airway inflammation even in the absence of mucus obstruction (Tirkos et al., 2006; Wilke et al., 2011). Moreover, airways of newborn CF pigs demonstrate developmental defects, such as cartilage abnormalities, muscle bundles, and smaller airways, which may support progression into a CF lung disease (Chen et al., 2010; Stoltz et al., 2010; Klymiuk et al., 2012). Considerable insight into epithelial ion transport and its defect in CF, was also obtained in studies with wt and transgenic mice, although mouse airways show differences in structure, distribution of submucosal glands and contribution of CFTR to Cl^- transport. While identical ion channels and transporters are expressed in human and mouse

airway, intestinal epithelium, and other epithelial organs, the course of lung disease is mild in CF mice and airway plugging is not observed (Wilke et al., 2011). Nevertheless, because of the available broad range of tools in mouse genetics, valuable insights into organ physiology and pathological changes in CF have been gained in the different mouse CF models.

IS ABNORMAL ION TRANSPORT RELEVANT IN CF OR ONLY AN EPIPHENOMENON?

Others argue that airway inflammation is second to the transport defect and to bacterial colonization (Ribeiro et al., 2005). At any rate, the most impressive clinical symptom in CF lung disease is accumulation of large amounts of airway mucus. Yet, are low pH, Na^+ hyperabsorption, or lack of Cl^- secretion are truly the cause for mucus plugging and obstruction of airways? If we compare the changes in absorptive and secretory ion transport present in ßENaC-overexpressing mice (Mall et al., 2004), with the changes in ion transport in kcne3 knockout mice (Preston et al., 2010), or mice lacking TMEM16A in ciliated epithelial cells (Benedetto et al., 2017), the contribution of ion transport appears less clear. In the three mouse models we find a shift toward enhanced net absorptive transport: (i) pronounced increase in Na^+ absorption with unchanged Cl^- secretion (Mall et al., 2004), (ii) milder increase in Na^+ absorption but strongly reduced Cl^- secretion (Preston et al., 2010), (iii) partial reduction in Na^+ absorption but pronounced inhibition of Cl^- secretion (Benedetto et al., 2017) (**Table 1**). Only the ßENaC mice develop a CF like lung phenotype, which may suggest that indeed Na^+ absorption by ENaC is rate limiting and determines the direction of net flux of ions and water through an otherwise relatively leaky airway epithelium (Cotton et al., 1983; Donaldson and Boucher, 2007). This may explains why loss of Cl^- secretion in the airways of adult *kcne3* and *tmem16A* knockout mice does not lead to a CF like lung phenotype.

As discussed below, basal airway mucus secretion is attenuated in mice lacking TMEM16A in ciliated epithelial cells. This leads to mucus accumulation in secretory epithelial cells (Benedetto et al., 2019) (**Figure 1**). The mechanism of crosstalk between the two cell types is currently unclear. However, it is likely that ciliated cells secrete a factor that is required to release mucus from club cells. This factor could be ATP, which is found in the airway surface liquid, and which is assumed to control basal mucus secretion. Evidence has been presented that TMEM16A supports vesicular/granular exocytosis and subsequent insertion of transmembrane proteins into the plasma membrane. Moreover, it was also shown to control paracrine release of inflammatory mediators (Benedetto et al., 2016, 2017, 2019; Cabrita et al., 2017). We would favor a pathogenic mechanism that initiates the disease by intrinsic inflammation caused by dyslocalization/dysfunction of CFTR, and in the absence of bacterial infection. Intrinsic inflammation is followed by upregulation of TMEM16A, particularly in mucus producing cells, with consecutive mucus hyperproduction/hypersecretion. Accordingly, inflammation and mucus hyperproduction/hypersecretion should be pharmacologically targeted. Along this line it appears noteworthy that treatment with the anti-inflammatory drug ibuprofen was able to rescue trafficking mutant F508del-CFTR (Carlile et al., 2015).

CORRECTING AND POTENTIATING CFTR

Pharmacological restoration of defective CFTR chloride transport has been the primary goal over the past decade and has led to considerable success in correcting the gating mutant G551D-CFTR by the potentiator compound VX-770 (ivacaftor, kalydeco) (Van Goor et al., 2009; Accurso et al., 2010). G551D accounts for only a small fraction (about 5%) of all CF cases (De Boeck et al., 2014). Thus, US-based Vertex developed the small molecule CFTR-corrector VX-809 (lumacaftor), a compound that is able to rescue a fraction of the class two mutant F508del-CFTR *in vitro* (Van Goor et al., 2011; Pranke et al., 2017). Large phase 3 studies demonstrated a moderate improvement in the percentage of predicted FEV1 between 2.6 and 4.0% (Wainwright et al., 2015). Studies with next generation compounds, e.g., VX-661 (Tezacaftor), reported improvement in FEV1 in the range of 7% (Rowe et al., 2017; Taylor-Cousar et al., 2017; Donaldson et al., 2018). Recent clinical trials reported great therapeutic success with triple combinations of the corrector VX661 with VX-445 or VX-659, together with the potentiator VX-770. The combinations increased the percentage of predicted FEV1 by more than 10% (Davies et al., 2018; Keating et al., 2018). Despite such great success in correcting mutant CFTR, critical voices were raised regarding a combinatorial drug treatment. For example, adverse effects of VX-770 were reported on VX-809-corrected F508del-CFTR in the combinatorial preparation Orkambi. Moreover, long-term effects of these drugs on CFTR expression could be negative (Cholon et al., 2014; Veit et al., 2014; Chin et al., 2018). Finally, the costs for treatment by these drugs are often prohibitive (Ferkol and Quinton, 2015). Thus, there is a need for alternative drug treatments.

A number of other strategies to correct biosynthesis of misfolded CFTR are currently under investigation Recent screening efforts identified the translation initiation factor 3a (eIF3a) as a potentially druggable central hub for the biogenesis of CFTR (Hutt et al., 2018). FDA-approved histone deacetylase (HDAC) inhibitors such as panobinostat (LBH-589) and romidepsin (FK-228) can help to correct misfolded CFTR, particularly in combination with other correctors such as VX809 (Angles et al., 2018). Promising results were also obtained with combinations of pharmacological chaperones with different sites of action, such as VX-809, RDR1, and MCG1516A (Carlile et al., 2018). All these recent results are rather encouraging, however, as not every CFTR mutation is accessible to such a CFTR-based therapy, activation of other airway epithelial Cl^- channels was proposed to compensate for defective CFTR (De Boeck and Amaral, 2016).

TABLE 1 | Ion transport assessed by the measurement of short circuit currents in different adult mouse models [wild type (wt) littermates vs. transgenic (trans) mice].

Mouse model	Amiloride-sensitive I_{sc} ($\mu A/cm^2$)			cAMP-activated I_{sc}			Ca^{2+}-activated I_{sc}			Phenotype
	wt	trans	%↓↑	wt	trans	%↓↑	wt	trans	%↓↑	
ßENaC overexpressing mouse (Mall et al., 2004)	22	59	↑168	25	27	↑11	79	84	↑6	CF-like lung disease
Kcne3-knockout mouse (Preston et al., 2010)	48	84	↑ 75	83	17	↓80	294	50	↓83	No lung phenotype
FOXJ1-Cre-TMEM16Aflox/flox mice (Benedetto et al., 2017)	104	49	↓ 47	99	58	↓42	176	74	↓58	No lung phenotype

Shown are approximate I_{sc} values. %↓↑, percent decrease or increase in unidirectional ion transport.

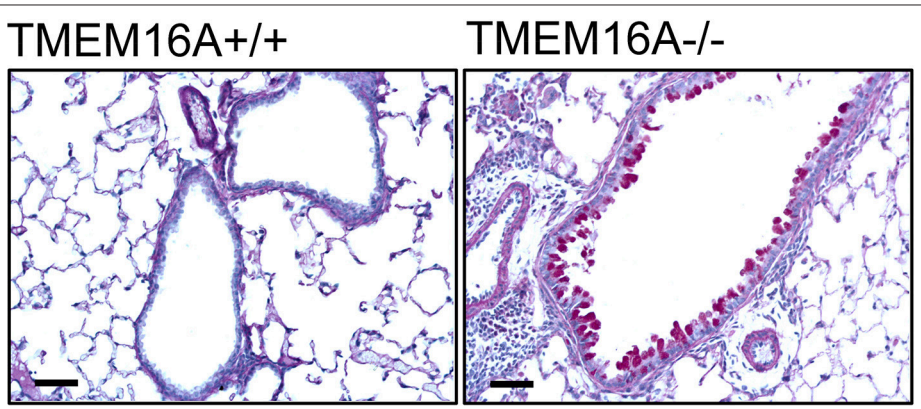


FIGURE 1 | Accumulation of mucus in airways of TMEM16A$^{-/-}$ mice. Mucus staining (PAS) in bronchi of wild type littermates (TMEM16Aflox/flox; TMEM16A$^{+/+}$) and mice with an airway epithelial-specific knockout of TMEM16A (FOXJ1-Cre-TMEM16Aflox/flox; TMEM16A$^{-/-}$). Strong accumulation of mucus in airway epithelial cells is observed in TMEM16A$^{-/-}$ mice. Bars indicate 20 μm (Benedetto et al., 2019).

AIRWAY CHLORIDE CHANNELS: SLC26A9

Apart from CFTR, there are two other major Cl^- channels present in human and mouse airways, namely SLC26A9 and TMEM16A. How much both Cl^- channels quantitatively contribute to production of the ASL and support mucociliary clearance is currently not known (**Figure 2**). SLC26 proteins typically operate as anion exchangers, but for SLC26A9 it was shown to function as a Cl^- channel (Mount and Romero, 2004; Ousingsawat et al., 2011b; Bertrand et al., 2017). In contrast to CFTR, SLC26A9 is spontaneously active, once inserted into the apical membrane of airway epithelial cells. However, it is also regulated by Cl^- feedback/WNK kinases and surprisingly is controlled by the Cl^- channel CFTR (Dorwart et al., 2007; Bertrand et al., 2009). SLC26A9 is likely to provide the basal Cl^- conductance that is found in airways in the absence of any secretagogue (Bertrand et al., 2017). Absence of basal Cl^- secretion in airways of CF patients carrying the type II mutation F508del-CFTR (De Boeck and Amaral, 2016), is probably due to the lack of expression of SLC26A9 in the apical membrane (**Figure 2B**) (El Khouri and Toure, 2014; Bertrand et al., 2017). SLC26A9 and CFTR form a complex with the help of PDZ scaffold proteins such as NHERF1 (**Figure 3**). When coexpressed with wtCFTR, SLC26A9 is co-trafficked together with CFTR from the ER to the plasma membrane. However, if coexpressed with F508del-CFTR, SLC26A9 will remain intracellularly and will be degraded (Bertrand et al., 2017). As shown recently, also CFTR and TMEM16A do interact via PDZ-domain proteins, and TMEM16A has an impact on plasma membrane expression of CFTR (Benedetto et al., 2017).

Furthermore, SLC26A9 may be regulated by the R domain of CFTR through STAS domain interaction (Chang et al., 2009). Indeed, SLC26A9 has been demonstrated to be a genetic modifier in CF (Sun et al., 2012; Miller et al., 2015), and the CFTR corrector VX-809 partially rescued SLC26A9, probably by facilitating trafficking of F508del-CFTR to the plasma membrane (Strug et al., 2016). Similar to TMEM16A, SLC26A9 is upregulated during airway inflammation and exposure of the airway cells to IL-13 (Anagnostopoulou et al., 2012). It will be interesting to learn more about the regulation of SLC26A9 expression, and whether SLC26A9 is upregulated in CF airways in ciliated or in mucus secreting cells. Taken together, SLC26A9 could potentially serve as an alternative Cl^- channel in CF, but compromised biosynthesis of CFTR carrying type two mutations need to be corrected. Identifying small molecules that would interfere with the formation of the F508del-CFTR/SLC26A9 complex could be an interesting therapeutic option in CF.

AIRWAY CHLORIDE CHANNELS: TMEM16A

TMEM16A is a Ca^{2+} activated Cl^- channel (CaCC) that belongs to a larger family of 10 paralogous proteins (TMEM16A-K), also

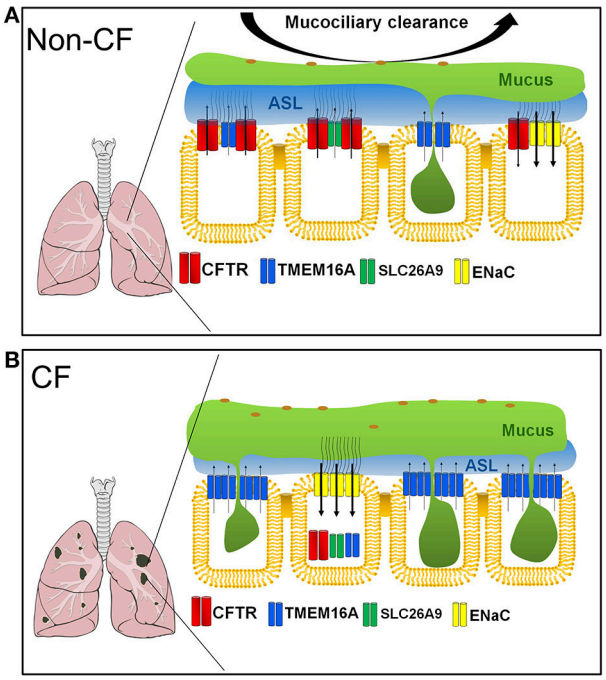


FIGURE 2 | Ion channels contributing to fluid/mucus balance in non-CF and CF airways. **(A)** In non-CF airways, CFTR is expressed in ciliated epithelial cells and ionocytes (not shown). Ca^{2+} activated Cl^- channels are sparsely expressed in both ciliated and secretory club and goblet cells. SLC26A9, TMEM16A and CFTR located in ciliated epithelial cells and probably in ionocytes are in charge of fluid secretion, while TMEM16A expressed in secretory club/goblet cells support mucus secretion. Epithelial Na^+ channels reabsorb Na^+ thereby causing fluid absorption. **(B)** In inflamed CF airways, CFTR is dysfunctional and often dislocated intracellularly together with SLC26A9 and TMEM16A in ciliated epithelial cells. TMEM16A is upregulated in secretory cells strongly contributing to mucus secretion. Na^+ absorption by ENaC is augmented. Inflammation and transport abnormalities lead to excessive mucus production/secretion, airway plugging, and reduced water secretion, strongly reducing mucociliary clearance.

called anoctamins (ANO1-ANO10) (Kunzelmann et al., 2011; Pedemonte and Galietta, 2014). The majority of these double-barreled channels are operating as phospholipid scramblases, i.e., they transport phospholipids from one site of the bilayer membrane to the other, once activated by a strong increase in intracellular Ca^{2+} (Suzuki et al., 2010). TMEM16A and B are solely CaCCs, whose structure and gating has been largely uncovered in cryo-EM studies (Paulino et al., 2017a,b). TMEM16A is typically localized in the apical plasma membrane of epithelial cells. However, it is also found to be expressed basolateral and in intracellular compartments (Schreiber et al., 2010, 2014). TMEM16F is a phospholipid scramblase that also conducts Cl^- and other ions (Yang et al., 2012; Grubb et al., 2013; Shimizu et al., 2013; Kunzelmann et al., 2014; Ousingsawat et al., 2015; Scudieri et al., 2015; Drumm et al., 2017; Schreiber et al., 2018). Although endogenous TMEM16 proteins are mostly localized intracellularly, overexpression of these proteins together with purinergic receptors allows partial trafficking to the plasma membrane (Tian et al., 2012a). TMEM16A is clearly the epithelial airway CaCC (Benedetto et al., 2017), but a number of other TMEM16 paralogues are also coexpressed in mouse airways and in human large and small bronchi, bronchiole and alveoli, such as TMEM16C, F, J (Kunzelmann et al., 2012). Particularly TMEM16F may participate as well in epithelial Cl^- transport.

UPREGULATION OF TMEM16A DURING INFLAMMATORY LUNG DISEASE: GOOD OR BAD?

TMEM16A is strongly upregulated during inflammation, a fact that was utilized to identify the molecular nature of CaCC (Galietta et al., 2002; Caputo et al., 2008). TMEM16A is strongly upregulated in CF and asthma, which parallels goblet cell metaplasia and mucus hypersecretion (Huang et al., 2012; Kondo et al., 2017), and is also upregulated by bacterial components (Caci et al., 2015). Upregulation of TMEM16A is predominant in mucus producing cells and to a much lesser degree in ciliated epithelial cells (Huang et al., 2012; Scudieri et al., 2012). Expression of TMEM16A is almost undetectable by immunocytochemistry in normal adult human and mouse airways; although CaCC is clearly present (Huang et al., 2009, 2012; Benedetto et al., 2017). While this may be explained by the limited sensitivity of the available antibodies, it also raises questions as to what degree other members of the TMEM16 family might participate in CaCC. As mentioned above, TMEM16C, F, and J are also expressed in mouse airway epithelium (Kunzelmann et al., 2012). On the other hand, knockout of TMEM16A completely abolished CaCC activated by purinergic stimulation (Benedetto et al., 2017). We will discuss below that TMEM16A has a strong impact on intracellular Ca^{2+} signals triggered by stimulation of G-protein coupled receptors (GPCRs), which then activate phospholipase C, increase inositol trisphosphate and intracellular Ca^{2+} (Kunzelmann et al., 2016).

As mentioned above, expression of TMEM16A in normal adult airways is hardly detectable by immunohistochemistry (Ousingsawat et al., 2009; Benedetto et al., 2017) (**Figure 4**). However, induction of airway inflammation in an ovalbumin asthma model induced a pronounced upregulation of TMEM16A in mucus producing club/goblet cells, but induced little expression in ciliated epithelial cells (Benedetto et al., 2019) (**Figure 4**). Ciliated epithelial cells, and particularly the recently identified ionocytes express CFTR and are in charge of fluid secretion (Montoro et al., 2018; Plasschaert et al., 2018). Expression of TMEM16A is low in ciliated cells when compared to mucus producing club and goblet cells. The contribution of TMEM16A to overall fluid secretion by the airway epithelium might therefore be limited, while it plays a central role for basal mucus secretion (Benedetto et al., 2019). This will be further outlined below.

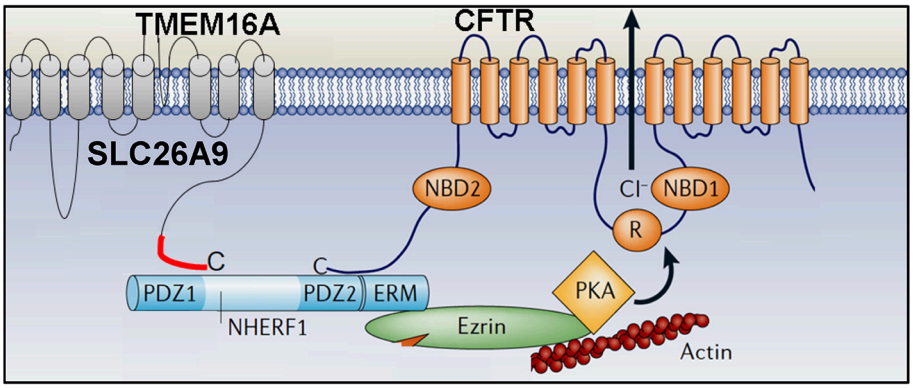


FIGURE 3 | Molecular and functional relationship of CFTR, SLC26A9 and TMEM16A. Both SLC26A9 and TMEM16A interact with CFTR via their C-terminal PDZ (PSD-95/Discs-large/ZO1) -binding domains with the help of the PDZ-protein NHERF1 (Na^+/H^+ exchanger regulatory factor 1).

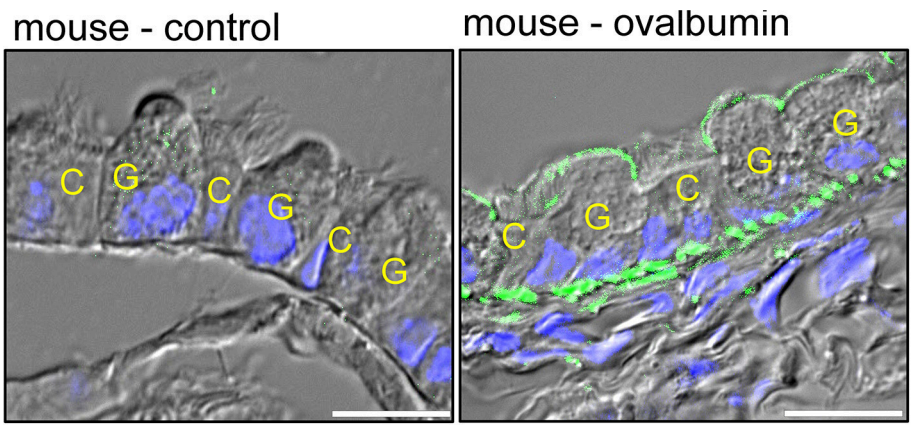


FIGURE 4 | Predominant expression of TMEM16A in mucus producing cells. Distal airways of a control mouse and an ovalbumin-sensitized (asthmatic) mouse. Expression of TMEM16A (green fluorescence) is hardly detectable in control airways, but is clearly detectable in mucus producing club/goblet cells (G) of asthmatic mice with inflamed airways. Little expression of TMEM16A is found in ciliated epithelial cells (C). Blue, DAPI staining of nuclei. Bars indicate 20 μm (Benedetto et al., 2019).

F508DEL-CFTR ATTENUATES EXPRESSION OF TMEM16A IN THE APICAL MEMBRANE

A functional coupling between TMEM16A and CFTR has been described in a number of previous publications (Kunzelmann et al., 1997; Wei et al., 1999; Ousingsawat et al., 2011a). Subsequent studies reported attenuated expression of TMEM16A in the apical membrane of airway epithelial cells by coexpressed F508del-CFTR (Ruffin et al., 2013; Benedetto et al., 2017). We demonstrated that TMEM16A and CFTR directly interact through PSD-95/Dlg/ZO-1 (PDZ) domain proteins, similar to SLC26A9 (**Figure 3**). The functional interaction between TMEM16A and CFTR is also demonstrated by a crosstalk of intracellular Ca^{2+} and cAMP-dependent signaling. This compartmentalized crosstalk is facilitated by exchange protein directly activated by cAMP (EPAC1) and Ca^{2+} -sensitive adenylate cyclase type 1 (ADCY1). Assembly of such a local signalosome was shown to depend on the number of phospholipase C coupled GPCRs (Lerias et al., 2018).

These studies suggest a significant overlap of cAMP- and Ca^{2+} activated Cl^- currents. Along this line, our early studies also showed that epithelial cAMP-dependent and Ca^{2+}-activated Cl^- currents cannot be easily separated based on apparently specific ion channel inhibitors (Kunzelmann et al., 1992; Benedetto et al., 2017; Lerias et al., 2018). Moreover, in both airway and intestinal epithelium, most of the Ca^{2+} activated Cl^- secretion is in fact through CFTR and not through TMEM16A Cl^- channels (Mall et al., 1998b; Namkung et al., 2010a; Billet and Hanrahan, 2013; Benedetto et al., 2017). The actual purinergic (ATP-activated) Cl^- secretion via TMEM16A is very transient, as TMEM16A deactivates quickly within 1–5 min after activation by ATP when examined in *Xenopus* oocytes (Faria et al., 2009), HEK293 cells (Tian et al., 2012b), mouse trachea (Kunzelmann et al., 2002), and human airways (Mall et al.,

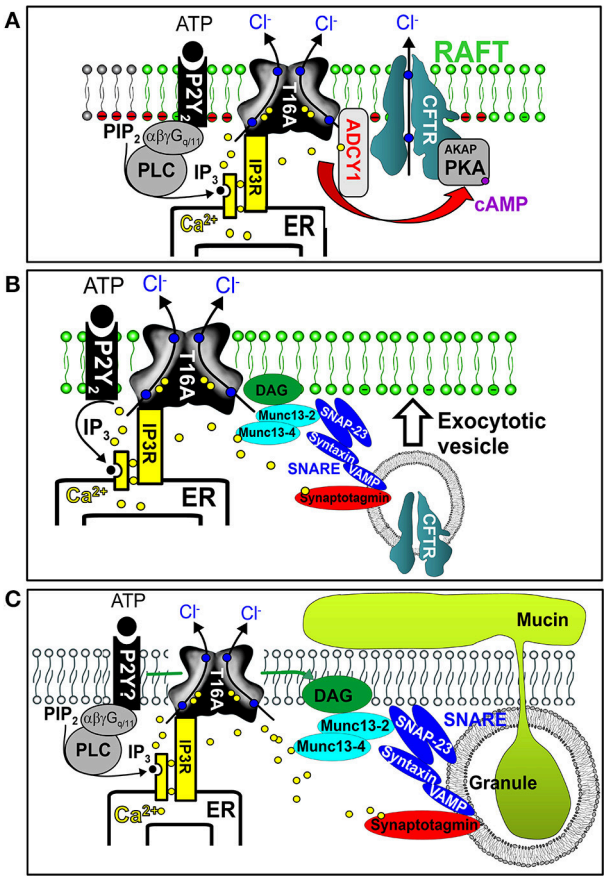


FIGURE 5 | Impact of TMEM16A on intracellular Ca^{2+}, exocytosis, and mucus secretion. **(A)** TMEM16A is colocalized with purinergic P2Y receptors and CFTR within the apical membrane. Stimulation of purinergic receptors leads to endoplasmic reticulum (ER) Ca^{2+} store release through IP3 receptors (IP3R). Ca^{2+} not only activates TMEM16A (T16A) but also stimulates adenylate cyclase type 1 (ADCY1) to produce cAMP and to activate CFTR via protein kinase A (PKA). TMEM16A binds to IP3 receptors and tethers the ER to the apical membrane, thereby facilitating effective compartmentalized Ca^{2+} signaling. **(B)** Effective apical Ca^{2+} signaling by TMEM16A leads to activation of the exocytic SNARE machinery insertion and improved expression of CFTR in the apical plasma membrane. **(C)** Effective apical Ca^{2+} signaling by TMEM16A leads to fusion of mucus containing granules, exocytosis, and release of mucus.

2003). Analysis of freshly isolated human nasal epithelial cells demonstrates ATP-induced steady-state secretion only in non-CF cells, but not in CF nasal cells. Thus, the direct contribution of TMEM16A to the epithelial secretory Cl^- transport is small. However, the non-transient steady component of purinergic Cl^- secretion that is produced by CFTR is essential for fluid secretion (Mall et al., 2003; Benedetto et al., 2017). The traffic mutant F508del is by far the most common mutation in CF that also compromises biosynthesis of TMEM16A. Therefore, the pro-secretory function of TMEM16A in CF is probably limited, and inhibition of TMEM16A may not much reduce Cl^- secretion in CF (Ruffin et al., 2013; Benedetto et al., 2017).

UPREGULATION OF TMEM16A IN AIRWAY SMOOTH MUSCLES

Upon induction of asthma, we also observed an upregulation of TMEM16A in mouse airway smooth muscle (ASM) cells (Benedetto et al., 2017; Miner et al., 2019) (**Figure 4**). This has already been described in a number of previous studies (Huang et al., 2009, 2012; Gallos et al., 2013; Danielsson et al., 2015). Inhibitors of TMEM16A were shown to induce hyperpolarization of ASM and airway relaxation (Yim et al., 2013; Danielsson et al., 2017; Miner et al., 2019). Airway inflammation is well-known to induce hyperresponsiveness of ASM (Brightling et al., 2002; Galli et al., 2008). Inflammatory mediators binding to GPCRs activate TMEM16A channels; depolarize the membrane voltage and cause airway contraction, a process that is upregulated in asthma (Wang et al., 2018). Expression of TMEM16A is not only upregulated in allergic asthma, but also in airway epithelial cells and probably ASM of CF patients (Caci et al., 2015). Moreover, the signaling cascade comprising GPCR - TMEM16A - intracellular Ca^{2+} is further augmented by inflammatory mediators and cholinergic stimuli.

TMEM16A CONTROLS Ca^{2+} SIGNALS, MEMBRANE EXOCYTOSIS AND MUCUS SECRETION

As pointed out above, membrane expression and activity of CFTR strongly relies on TMEM16A (Benedetto et al., 2017; Lerias et al., 2018). We showed that augmentation of apical Ca^{2+} signals in the presence of TMEM16A activates adenylate cyclase type 1, enhances local cAMP levels and boosts CFTR activity (**Figure 5A**). The enhanced plasma membrane expression of CFTR in the presence of TMEM16A may be caused by enhanced Ca^{2+} levels in the apical submembranous compartment, which triggers the exocytic machinery and membrane insertion of CFTR in ciliated epithelial cells and possibly ionocytes (Benedetto et al., 2017) (**Figure 5B**). A similar exocytic mechanism may apply to the process of mucus secretion by club and goblet cells. We found that ATP-induced mucus secretion by secretory cells is strongly compromised in the absence of TMEM16A. Without TMEM16A, intracellular Ca^{2+} concentrations in the apical pole of club and goblet cells are attenuated. These Ca^{2+} ions are required for basal and acute ATP-activated mucus release (Benedetto et al., 2019) (**Figure 5C**). ATP-dependent mucus secretion is characterized by Ca^{2+} dependent single granule docking to the apical membrane which requires Munc13 proteins and the SNARE (soluble N-ethylmaleimide–sensitive factor attachment protein receptor) machinery (Fahy and Dickey, 2010). Taken together, in healthy non-CF airways TMEM16A may support CFTR-driven fluid secretion in ciliated cells and possibly ionocytes, and supports basal mucus release by club and goblet cells. In inflammatory CF airway disease, the function of TMEM16A may be marginal in ciliated cells and ionocytes, but may be pronounced in secretory cells due to strong upregulation of expression.

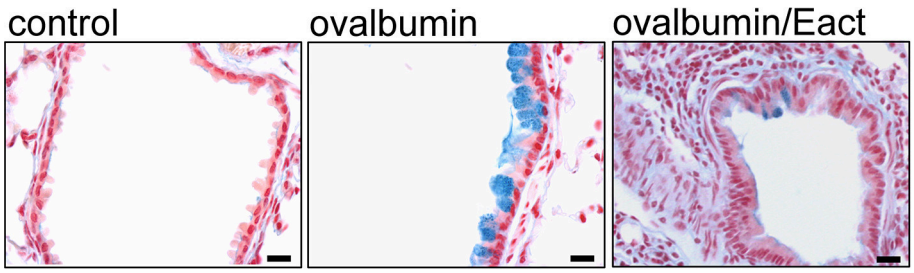


FIGURE 6 | Activation of TMEM16A leads to mucus release and airway contraction. Control mouse airways show very little mucus (alcian blue). In contrast, airways from ovalbumin-sensitized mice show pronounced mucus accumulation. Acute exposure of an ovalbumin-sensitized mouse to the known activator of TMEM16A, Eact (4.8 μg, tracheal instillation), induced a rapid mucus release and airway contraction (Benedetto et al., 2019). Bars indicate 20 μm.

According to this, pharmacological activation of TMEM16A in CF and asthma patients could have adverse effects on lung function due to its prosecretory effect on mucus release. Correspondingly, we found in OVA-sensitized asthmatic mice that activation of TMEM16A by the compound Eact (Namkung et al., 2011b) induced massive mucus release and a considerable expiratory stridor, suggesting airway contraction. Airway narrowing was confirmed by analysis of the airway cross section (Benedetto et al., 2019) (**Figure 6**). It may be argued that Eact raises intracellular Ca^{2+} and therefore induces adverse effects independent of TMEM16A. However, increase in intracellular Ca^{2+} by Eact is expected. As outlined below, activation of TMEM16A is known to increase intracellular Ca^{2+} (Cabrita et al., 2017). Activation of TMEM16A by Eact depolarizes the membrane voltage, which leads to an increase in intracellular Ca^{2+}. Notably, increase in intracellular Ca^{2+} by Eact was inhibited by 1 μM of the TMEM16A blocker niclosamide (Benedetto et al., 2019).

INHIBITORS AND ACTIVATORS OF TMEM16A

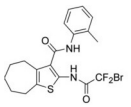
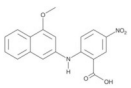
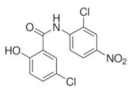
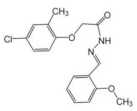
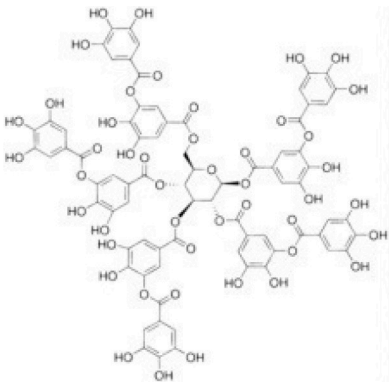
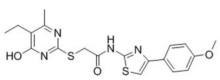
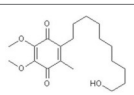
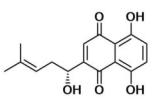
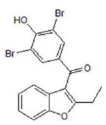
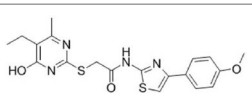
Meanwhile a larger number of inhibitors of TMEM16A has been identified, but there is only one published group of N-aroylaminothiazole "activators" (Eact) (Namkung et al., 2011b), apart from some herbal compounds like Ginsenoside Rb1 and Resveratrol, which apparently activate TMEM16A in a Ca^{2+} independent manner (Chai et al., 2017; Guo et al., 2017) (**Table 2**). Enterprise therapeutics (Sussex, UK; http://www.enterprisetherapeutics.com/) is currently working on potentiators of TMEM16A, but details on potentiating molecules are not yet available. Silurian pharmaceuticals (Oakland, US; http://www.silurianpharma.com/index.php) reported brevenal to activate Ca^{2+} activated Cl^- channels, possibly in a Ca^{2+} independent fashion. Denufusol was developed by Inspire pharmaceuticals (later taken over by Merck). Denufosol is a deoxycytidine-uridine dinucleotide with enhanced metabolic stability, to activate purinergic P2Y2 receptors which stimulate TMEM16A (Yerxa et al., 2002) and inhibit ENaC (Kunzelmann et al., 2002) (c.f. below). An interesting group of lipids was identified originally to uncouple GPCR-mediated Ca^{2+} increase from inactivation/desensitization of Ca^{2+} activated Cl^- channels. These D-myo-inositol 3,4,5,6-tetrakisphosphate [Ins(3,4,5,6)P4] (Vajanaphanich et al., 1994) were synthetically modified to result in the membrane permeable analog INO4995, which was shown to inhibit ENaC (Moody et al., 2005) and to activate TMEM16A (Tian et al., 2012b). INO4995 did not increase intracellular Ca^{2+}. It activated overexpressed TMEM16A directly, but potentiated ATP-dependent activation of TMEM16A expressed endogenously. Preliminary data suggested enhanced membrane localization of TMEM16A induced by INO4995 (Tian et al., 2012b) (**Table 3**).

BIOLOGICAL EFFECTS OF INHIBITORS AND ACTIVATORS OF TMEM16A

TMEM16A is broadly expressed in many epithelial and non-epithelial tissues. It is therefore expected that inhibition or activation of TMEM16A through systemic drug application might have a number of side effects. Apart from inhibiting mucus production and mucus secretion, TMEM16A-inhibitors will induce bronchorelaxation by blocking TMEM16A in airway smooth muscle (Huang et al., 2012; Gallos et al., 2013; Zhang et al., 2013; Danielsson et al., 2015; Wang et al., 2018; Miner et al., 2019). Another desired lung-specific effect of TMEM16A-inhibitors is the inhibition of release of inflammatory mediators (Knight, 2004; Danielsson et al., 2017; Benedetto et al., 2019).

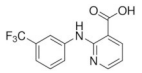
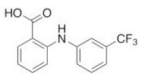
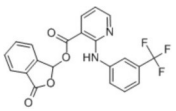
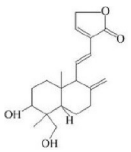
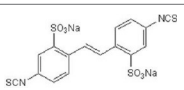
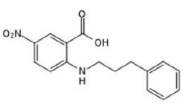
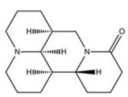
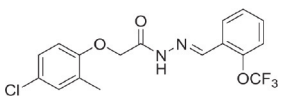
Some inhibitors of TMEM16A have additional beneficial effects, such as protection from reactive oxygen species (idebenone, Villalba et al., 2010), and general inhibition of inflammation (Knight, 2004; Schreiber et al., 2018). Many studies demonstrate inhibition of proliferation and anti-cancer effects by blocking TMEM16A (Wanitchakool et al., 2014; Wang et al., 2017). General anti-hypertensive effects are likely (Namkung et al., 2010b; Heinze et al., 2014), as well as inhibition of nociception, itching, and heat perception (Cho et al., 2012; Lee et al., 2014; Pusch and Zifarelli, 2014; Deba and Bessac, 2015). Inhibition of saliva production and dry mouth may occur mediation with TMEM16A-inhibitors (Ousingsawat et al., 2009; Catalan et al., 2015). Inhibition of TMEM16A may also attenuate intestinal contraction and abdominal peristalsis (Sanders et al., 2012; Singh et al., 2014), and therefore could have antidiarrheal effects (Tradtrantip et al., 2010; Namkung et al., 2011b; Jiang et al., 2016). Finally, inhibition of renal TMEM16A could

TABLE 2 | Inhibitors of TMEM16A.

Inhibitor	IC50 (μM)	Structural formula	References
10bm	0.03		Truong et al., 2017
Monna	0.08		Oh et al., 2013
Niclosamide	0.7		Miner et al., 2019
Ani9	0.1		Seo et al., 2016
Tannic acid	0.323		Namkung et al., 2010b
T16A-A01	1.1		Namkung et al., 2011a; Fedigan et al., 2017
Dichlorophen	5.49		Huang et al., 2012
Idebenone	5.52		Seo et al., 2015
Shikonin	6.5		Jiang et al., 2016
Benzbromarone	9.97		Huang et al., 2012
CaCC-A01	10		de La Fuente et al., 2007; Gianotti et al., 2016; Fedigan et al., 2017

(Continued)

TABLE 2 | Continued

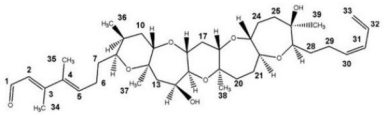
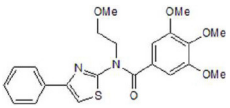
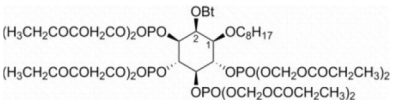
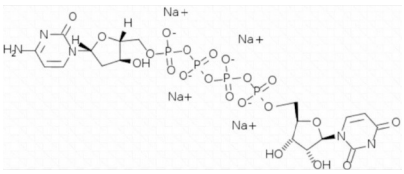
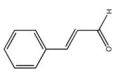
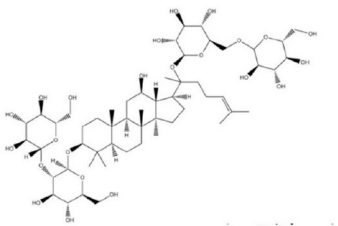
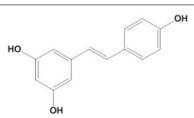
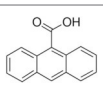
Inhibitor	IC50 (μM)	Structural formula	References
9-Phenanthrol	12		Burris et al., 2015)
Niflumic Acid	12		Hogg et al., 1994
Flufenamic acid	28		White and Aylwin, 1990
Talniflumate	–		Walker et al., 2006
A9C	58		Baron et al., 1991
Dehydroandrographolide	20–30		Sui et al., 2015
DIDS	10–100		Kubitz et al., 1992
NPPB	15–150		Kubitz et al., 1992; Yang et al., 2008
Rice bran extract			Sharm et al., 2017
Matrine	28 μM		Guo et al., 2018
(Ani9 derivative) 5f	22 nM		Seo et al., 2018

potentially lead to proteinuria and acidosis (Faria et al., 2014; Schenk et al., 2018). For activators of TMEM16A, opposite effects are possible, which is why local application via aerosol may be recommended for the treatment of CF lung disease. Nevertheless, oral application of a TMEM16A-blocker was shown to increase survival of CFTR-knockout mice (Walker et al., 2006).

ACTIVATING OR INHIBITING TMEM16A?

Under physiological conditions, airway mucus represents an innate defense mechanism against pathogens. It traps inhaled pathogens and particles and is part of the mucociliary clearance (Knowles and Boucher, 2002). However, mucus becomes a serious problem when hypersecreted in inflammatory lung diseases, such as asthma, COPD, and CF (Dunican et al., 2018). Despite many pathological findings and pathogenic mechanisms proposed for CF lung disease (c.f. above), the single most prominent finding is the excessive overproduction of highly viscous mucus with adhesive and cohesive properties in CF patients (Button et al., 2018). It causes airway obstruction and a reduced mucociliary clearance, and it thereby drives chronic inflammatory lung disease (Fahy and Dickey, 2010). Thus, inhibition of mucus production/secretion is likely to be the most

TABLE 3 | Activators of TMEM16A.

Activator	IC50 (μM)	Structural formula	References
Brevenal	–		http://www.silurianpharma.com/index.php
Eact	3		Namkung et al., 2011b
INO-4995	5		Tian et al., 2012b
Denufosol	10		Yerxa et al., 2002
Cinnamaldehyde	10		Huang et al., 2018
Ginsenoside Rb1	38.4		Guo et al., 2017
Resveratrol	47.9		Chai et al., 2017
A9C	100–1000		Ta et al., 2016

effective treatment of CF lung disease, normalizing the imbalance between excessive mucus secretion and reduced ASL (**Figure 7**). Our recent data show that TMEM16A and other TMEM16 proteins are essential for mucus production and basal secretion of mucus in airways and intestine (Benedetto et al., 2019). These findings open up a new avenue for the therapy of inflammatory airway diseases, and particularly for the treatment of airway mucus plugging and CF lung disease.

In contrast, activation of TMEM16A in CF to facilitate fluid secretion may be rather ineffective due to the reasons outlined above (**Figure 7A**). An earlier clinical trial was performed using stabilized dinucleotides (Denufosol) to induce purinergic Ca^{2+} dependent Cl^- secretion and to restore ASL with the goal of improving lung function in CF. However, the clinical trial failed to demonstrate any benefit of denufosol (Ratjen et al., 2012; Moss, 2013). As an adverse effect, the aerosol induced cough in 52% of all patients. It may be speculated that denufosol may have induced additional mucus secretion and even additional airway obstruction. Being strongly upregulated in inflammatory airway disease in secretory cells and in ASM, activation of TMEM16A could induce adverse effects by augmenting mucus secretion and bronchoconstriction (**Figure 7B**).

Inhibition of TMEM16A in CF may appear counterintuitive in the first place (assuming a potential inhibition of fluid secretion), however, the available data suggest otherwise. As outlined above, in CF TMEM16A membrane expression is

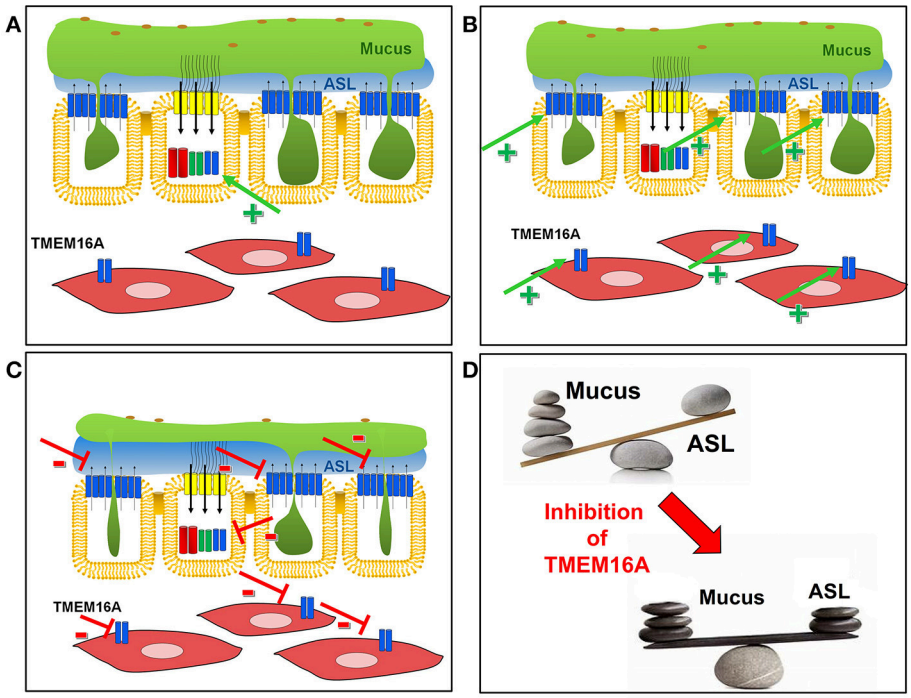


FIGURE 7 | Activation of inhibition of TMEM16A in airways of CF patients. **(A)** Activators of TMEM16A (green arrow, +) will find only few TMEM16A channels in the apical membrane of ciliated epithelial cells, which could enhance fluid secretion by ciliated epithelial cells. **(B)** In contrast, activators of TMEM16A will prominently activate TMEM16A strongly upregulated in mucus producing cells and airway smooth muscle cells during airway inflammation. This leads to hypersecretion of mucus along with airway contraction. **(C)** Inhibitors of TMEM16A will have an only limited (adverse) effect on TMEM16A channels expressed (fluid secreting) ciliated epithelial cells. In contrast, inhibition of TMEM16 channels in mucus producing cells and airway smooth muscle cells will inhibit mucus production/secretion and induce bronchodilation. **(D)** Inhibition of TMEM16 channels are likely to restore the balance between mucus and fluid in CF airways.

compromised (Ruffin et al., 2013; Benedetto et al., 2017), Cl^- secretion through CFTR is missing (Namkung et al., 2010a; Billet and Hanrahan, 2013; Benedetto et al., 2017; Lerias et al., 2018), and most TMEM16A is expressed in mucus secreting cells (Benedetto et al., 2019). Therefore, inhibition of TMEM16A may not substantially lower fluid secretion. In contrast, interfering with mucus and cytokine secretion by blocking upregulated TMEM16A is likely to improve mucociliary clearance and to improve lung function (**Figure 7C**). Along this line, talniflumate, the anti-inflammatory pro-drug of the common TMEM16A-inhibitor NFA that was originally developed by Argentinian Laboratorios Bago, increased survival of CF mice remarkably (Walker et al., 2006). Talniflumate had been further developed by the former company Genaera, as a mucoregulator for cystic fibrosis, chronic obstructive pulmonary disease, and asthma. Sadly, phase II trials have never been finished due to the shutdown of the company (Knight, 2004). In a large number of studies, mucus production, and secretion, as well as airway constriction were inhibited by niflumic acid and other inhibitors of TMEM16A (Kondo et al., 2012; Yim et al., 2013; Lin et al., 2015; Danielsson et al., 2017; Miner et al., 2019). The inhibitors niflumic acid, CaCCinhAO1, T16Ainh-A01, 17 benzbromarone, or niclosamide have been examined *in vivo* as well as *in vitro* in the low micromolecular range. Despite voltage dependence of TMEM16A-inhibition by NFA and other inhibitors, *in vivo* application to airway epithelial cells maintaining their intrinsic hyperpolarized membrane voltage demonstrated remarkable biological effects. Similar has been observed when TMEM16A inhibitors were applied to tracheal ring preparations *ex vivo*. Presumably TMEM16A is partially active in the airways, as low levels of ATP in the airway surface liquid maintain a basal activity of TMEM16A.

Because TMEM16A currents are blocked by niflumic acid, the published reports suggest that TMEM16A is in charge of both production and secretion of mucus. Niflumic acid, however, is a rather non-specific drug that inhibits a number of ion channels and blocks intracellular Ca^{2+} signals (Cabrita et al., 2017). Suppression of Ca^{2+} signals by niflumic acid is probably due to inhibition of TMEM16A. Other TMEM16A inhibitors such as CaCCinhAO1, T16Ainh-A01, benzbromarone, or niclosamide also inhibit intracellular Ca^{2+} and mucus release (de La Fuente et al., 2007; Kondo et al., 2017; Miner et al., 2019). It is important to note that other TMEM16 paralogues are also blocked by inhibitors of TMEM16A (Sirianant et al., 2015; Wanitchakool et al., 2016). Because several TMEM16 paralogues are expressed in airway epithelial cells, the possible contribution of other TMEM16 proteins to Ca^{2+} signaling and mucus production/secretion is currently unknown. Using different TMEM16 knockout mice and TMEM16 knockout cell lines, we found that most TMEM16 paralogues affect intracellular

Ca^{2+} signals (Kunzelmann et al., 2016; Cabrita et al., 2017). It is currently assumed that at least two TMEM16 members, TMEM16A and TMEM16F control mucus production and mucus secretion (Benedetto et al., 2019).

CONCLUSION

Despite significant progress in the development of CFTR–specific treatments for CF lung disease, it appears reasonable to search for alternative drug targets in CF. Potentiators and correctors of mutant CFTR show benefit in patients carrying the common F508del mutation. Improvement of lung function by the recent combinatorial drugs can be as high as 13%. Insight into mode of action of these compounds is still limited, and the costs for treatment may exclude some patients from therapy (Ferkol and Quinton, 2015). Moreover, a fraction of patients with particular CFTR mutations [type 1,5,6,7 mutations (De Boeck and Amaral, 2016)] will not respond to such a treatment.

Restoration of the mucus/liquid balance has been the driving force behind the search for novel openers of secretory Cl^- channels (SLC26A9 and TMEM16A) and basolateral pro-secretory K^+ channels, as well as for inhibitors of reabsorptive Na^+ channels. The counterintuitive idea of using inhibitors of TMEM16 channels is based on their role for mucus production and mucus secretion, which were uncovered only recently. Overwhelming mucus production and mucus plugging is the central problem in CF lung disease. Therefore, potent and well-tolerated TMEM16-inhibitors, which have FDA-approval for other diseases, should be further examined in preclinical and clinical studies to be use in CF lung disease and other inflammatory airway diseases.

ETHICS STATEMENT

All animals studies were approved by the local ethical board.

AUTHOR CONTRIBUTIONS

KK wrote manuscript, analyzed data, and designed experiments. RS, JO, and IC analyzed data, designed experiments, and performed experiments. TD, AB, and MJ wrote manuscript. RB wrote manuscript, analyzed data, designed experiments, and performed experiments.

ACKNOWLEDGMENTS

Supported by Cystic Fibrosis Trust, SRC 013, INOVCF, DFG KU756/14-1, DFG KU756/14-1, and Gilead Stiftung 2018: Inhibition of TMEM16A to reduce mucus plugging in CF.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 07 August 2018
doi: 10.3389/fphar.2018.00852

Anion-Transport Mechanism of a Triazole-Bearing Derivative of Prodigiosine: A Candidate for Cystic Fibrosis Therapy

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Cystic fibrosis (CF) is a genetic lethal disease, originated from the defective function of the CFTR protein, a chloride and bicarbonate permeable transmembrane channel. CF mutations affect CFTR protein through a variety of molecular mechanisms which result in different functional defects. Current therapeutic approaches are targeted to specific groups of patients that share a common functional defect. We seek to develop an innovative therapeutic approach for the treatment of CF using anionophores, small molecules that facilitate the transmembrane transport of anions. We have characterized the anion transport mechanism of a synthetic molecule based on the structure of prodigiosine, a red pigment produced by bacteria. Anionophore-driven chloride efflux from large unilamellar vesicles is consistent with activity of an uniporter carrier that facilitates the transport of anions through lipid membranes down the electrochemical gradient. There are no evidences of transport coupling with protons. The selectivity sequence of the prodigiosin inspired EH160 ionophore is formate > acetate > nitrate > chloride > bicarbonate. Sulfate, phosphate, aspartate, isothionate, and gluconate are not significantly transported by these anionophores. Protonation at acidic pH is important for the transport capacity of the anionophore. This prodigiosin derived ionophore induces anion transport in living cells. Its low toxicity and capacity to transport chloride and bicarbonate, when applied at low concentration, constitute a promising starting point for the development of drug candidates for CF therapy.

Keywords: cystic fibrosis, ionophore, ion transport, phospholipid vesicles, prodigiosin derivatives

OPEN ACCESS

Edited by:
Miquéias Lopes-Pacheco, Universidade de Lisboa, Portugal

Reviewed by:
Rainer Schreiber, University of Regensburg, Germany
Guido Veit, McGill University, Canada
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *16 April 2018*
Accepted: *13 July 2018*
Published: *07 August 2018*

Citation:
Cossu C, Fiore M, Baroni D, Capurro V, Caci E, Garcia-Valverde M, Quesada R and Moran O (2018) Anion-Transport Mechanism of a Triazole-Bearing Derivative of Prodigiosine: A Candidate for Cystic Fibrosis Therapy. Front. Pharmacol. 9:852. doi: 10.3389/fphar.2018.00852

INTRODUCTION

Cystic fibrosis (CF), the most common autosomal recessive lethal genetic disease in the Caucasian population (Strausbaugh and Davis, 2007), is caused by mutations on the gene coding for CFTR (cystic fibrosis transmembrane conductance regulator), an anion selective channel that transports chloride and bicarbonate in the apical membrane of epithelium. CFTR regulates salt and water transport across a variety of epithelium, and malfunction of this protein leads to defective mucus and airway surface liquid (ASL) properties, resulting in poor mucus clearance and bacterial infections on the airways (Berger et al., 1991; Saint-Criq and Gray, 2017). More than 1,500

mutations in CFTR, classified in six classes according to the effect of the mutation, lead to anion transport defects in epithelium by various mechanisms (failure to synthesize the protein, processing flaws, gating or conductance defects, reduced expression). In cell models, several small molecules, called correctors, increase CFTR defective expression at the cell membrane, and small molecules called potentiators to enhance the function of the CFTR channel (Zegarra-Moran and Galietta, 2017). While the potentiator ivacaftor (Kalydeco™) is successfully used for treatment in patients with gating mutations (Ramsey et al., 2011; De Boeck and Davies, 2017), the corrector lumacaftor, alone or in the combination with ivacaftor (Orkambi™) resulted on a limited clinical outcome (Clancy et al., 2012; Wainwright et al., 2015).

The discovery of small molecules capable to transport anions across lipid bilayers, named anionophores, has offered the opportunity of designing new substances to be used for pharmacological purposes (Davis et al., 2010; Valkenier et al., 2014; Hernando et al., 2018). The most striking among the potential applications of anionophores is the proposal to use these substances to substitute the defective anion transport in cystic fibrosis (CF). The idea of using anionophores to replace the defective CFTR protein has the advantage to become a general therapy for CF, that would be independent of the specific mutation. Different chemical structures have been described to transport anions across lipid bilayers, including prodigiosin and obatoclax derivatives (Seganish and Davis, 2005; Díaz de Greñu et al., 2011; García-Valverde et al., 2012; Gale et al., 2013), marine alkaloids such as tambjamines (Iglesias Hernández et al., 2012; Saggiomo et al., 2012; Hernando et al., 2014; Soto-Cerrato et al., 2015), steroid-based "cholapods" (Koulov et al., 2003; McNally et al., 2008; Valkenier et al., 2014), and calix[4]pyrrole derivatives (Gale et al., 2017), anion channel-forming peptides (Wallace et al., 2000; Broughman et al., 2004; Pajewski et al., 2006) and Calix[4]arene amides (Sidorov et al., 2002).

We have previously identified several triazole derivatives of prodigiosin with a significant anion transport capacity and relatively low toxicity (Hernando et al., 2018). There, we show that these compounds can transport chloride and bicarbonate across lipid bilayers, and the transport activity increases at acidic pH. Also evidences of anionophore-driven halides transport in cells is provided. Hence, to follow the search of "druggable" anionophores adequate for CF therapy, we have undertaken the analysis of the transmembrane anion transport mechanism of these molecules. Thus, for the functional characterization of this anionophore family, we chose of the most active substance of that series, EH160 (named **1b** in reference Hernando et al., 2018). Here, we have extended the data previously reported (Hernando et al., 2018), analysing the selectivity of the anionophore, demonstrating that the carrier has a good selectivity for physiologically relevant anions, such as chloride and bicarbonate. An important finding was that pH influence on the ionization state determines the anionophore activity, without any proton transport. We showed that this prodigiosin-inspired anionophore act as electro-neutral anion exchanger. To reinforce the concept that anionophores can induce chloride transport in mammalian cells, we repeated chloride efflux and iodide influx experiments in cells, similar to those reported elsewhere (Hernando et al., 2018).

MATERIALS AND METHODS

Synthesis of the Anionophore EH160

EH160 was synthesized as previously described (Hernando et al., 2018). In brief, it was prepared by acid-catalyzed condensation of 3-methoxy-5-(1-butyl-1H-1,2,3-triazol-4-yl)-1H-pyrrole-2-carbaldehyde and 2-methyl-3-pentyl-1H-pyrrole. The precursor aldehyde was prepared by standard click chemistry reaction between 1-azidobutane and the corresponding 5-ethynylpyrrole carbaldehyde. The compound is inspired by the structure of natural products prodiginines, replacing one of the pyrrole groups by a 1,2,3-triazole moiety. EH160 was fully characterized by mass spectroscopy and NMR (Hernando et al., 2018). Except when indicated, all chemicals were purchased from Sigma-Aldrich.

Large Unilamellar Vesicles

Asolectin large unilamellar vesicles (LUV) were made from phospholipids films (Baroni et al., 2014; Nicastro et al., 2016; Hernando et al., 2018). Soybean phospholipids (20 mg/ml) were dissolved in chloroform and lipid films were obtained by evaporation of the solvent under a gentle nitrogen flux; in order to remove all chloroform, films were further dried overnight in vacuum. The phospholipids were hydrated in chloride buffer (in mM: NaCl 450, 20 mM HEPES; pH 7.5, unless other composition was indicated), and vigorously vortex mixed and, to ensure equilibration, sonicated in 5 cycles of 1.5 min each, with 1 min rest, in ice. Liposomes were centrifuged at 2,000 g for 5–10 min to remove any titanium particles released by the sonicator tip. Large unilamellar vesicles (LUV) were then obtained by extrusion through polycarbonate filters mounted in a mini-extruder (Lipofast, Avestin, Mannheim, Germany). Samples were subjected to 19 passes through a single 100 nm mesh filter (MacDonald et al., 1991). External solution was exchanged twice on a Sephadex G25 column previously equilibrated with the external chloride-free solution: $NaNO_3$ 450 mM, 20 mM HEPES; pH 7.5 (unless other composition was indicated).

Chloride Efflux Measurements in LUV

To measure the efflux of chloride from LUV, chloride concentration was measured with an ion-sensitive electrode (Vernier, Beaverton, Oregon, USA) in a constantly stirred 3.5 ml LUV suspension. Data were acquired using a LabQuest mini interface (Vernier). Ionophores were dissolved in DMSO to a concentration of 10 mM. After an initial equilibration, chloride efflux was induced by a small volume (<1%) of ionophore. Control experiments where similar amounts of DMSO (without anionophores) were added demonstrated that these concentrations of DMSO do not induce any chloride efflux (see below). The measurement was concluded with the addition of the detergent polyoxyethylene 10 tridecyl ether (C13E10) to break off the bilayers and measure the maximum chloride encapsulated in the LUV. The time course of the chloride

concentration, *Cl*, in the experiment can be described by a single exponential function:

$$Cl^{-}(t) = Cl_0^{-} + \Delta Cl^{-}\left(1 - \exp\left[-tk\right]\right) \quad (1)$$

where Cl_0^- is the initial chloride concentration, ΔCl^- is the maximum change of the chloride concentration (after addition of detergent), and k is the rate constant of the process. The chloride efflux, J_{cl}, is defined as the time derivative of $Cl^-(t)$. Thus, deriving from equation (1), for $t = 0$, i.e., after the chloride gradient was changed, we obtain the initial chloride efflux-rate, J_0:

$$J_0 = \Delta Cl^{-} k \quad (2)$$

To compare different data sets, the chloride concentration was normalized to the maximum concentration change, ΔCl^-. Data are expressed as means ± s.e.m. Experiments were done at 25 ± 1°C. Every experimental condition was repeated at least three times.

Cell Preparation and Viability

The Human embryonic kidney (HEK) cell lines were grown in standard conditions, in Ham's F10 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS). Cell toxicity was evaluated by the trypan blue exclusion staining method (Louis and Siegel, 2011). Toxicity data is expressed as the half maximum toxic dose (TD_{50}).

Chloride Efflux in Cells

Cells at 80% confluence were detached from the bottom of the flask by soft scrapping, washed in chloride-free solution, and used immediately. For chloride efflux measurement, ~2×10^6 cells were suspended in 4 ml of buffer containing (in mM): 136 $NaNO_3$, 3 KNO_3, 2 $Ca(NO_3)_2$, 20 HEPES, 11 Glucose, pH 7.4. Ionophores were dissolved in DMSO to a concentration of 10 mM. Chloride concentration in the extracellular solution was continuously measured with a chloride-sensitive electrode. After an initial equilibration, chloride efflux was induced by a small volume (<1%) of ionophore. The measurement was concluded with the addition of the sodium dodecyl sulfate (SDS) to break off the membranes and measure the total chloride content in the cells. Experiments were done at 25 ± 1°C.

Iodine Influx in Cells

The activity of anionophores was determined in Fisher Rat Thyroid (FRT) cells expressing the halide-sensitive YFP protein as previously described (Caci et al., 2008). The assay is based in the fact that the fluorescence of the YFP protein is quenched to a greater extend by I^- than by Cl^- (Galietta et al., 2001). FRT cells stably transfected with a halide-sensitive yellow fluorescent protein (YFP-H148Q/I152L) were plated on 96-well micro-plates at a density of 40,000 cells/well in Coon's modified medium supplemented with 10% serum, 2 mM L-glutamine, 1 mg/ml penicillin, 100 µg/ml streptomycin, and 0.5 mg/ml hygromycin as selection agent for the YFP. Cells were maintained at 37°C in a 5% CO_2 /95% air atmosphere. Functional experiments were done 48 h after cell seeding. Cells were washed twice, with an external solution containing (in mM): NaCl 137, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.5, $CaCl_2$ 1 and $MgCl_2$ 0.5 (pH 7.3). The solution injected during the assay is similar but contained NaI 137 mM instead of NaCl (pH 7.3). To explore the effect of lowering the extracellular pH, the NaI solution was buffered at pH 6.9 with HEPES, or at pH 6.6 and 6.2 using MES. For the iodide influx assay, after washing, cells were incubated in 60 µl of the 137 mM NaCl-external solution, supplemented with the anionophore or with DMSO as control. A fluorescence baseline was recorded for 2 s after injection of 165 µl of NaI-external solution, so that the final concentration of NaI in the well is 100 mM. The iodide influx was observed as quenching of the YFP fluorescence.

RESULTS

Application of micromolar concentrations of anionophores to a LUV suspension induces a chloride efflux. In **Figure 1A** we show the time course of chloride efflux measured upon the application of 4 µM of prodigiosine and the triazole derivative EH160. The natural product, prodigiosine, is a more effective transporter, with a J_0 of 110.2 ± 1.2 µM/s. Instead, the triazole derivative EH160 induces a slower chloride efflux of 51.8 ± 0.5 µM/s. Application of DMSO does not induce a significant chloride efflux (J_0 of 0.2 ± 2.8 µM/s), confirming that the anion efflux was elicited by the anionophore and not by the solvent. Similar experiments were done using LUV formed with chemically defined phospholipids (palmitoyl-oleyl-phosphatidylcholine,

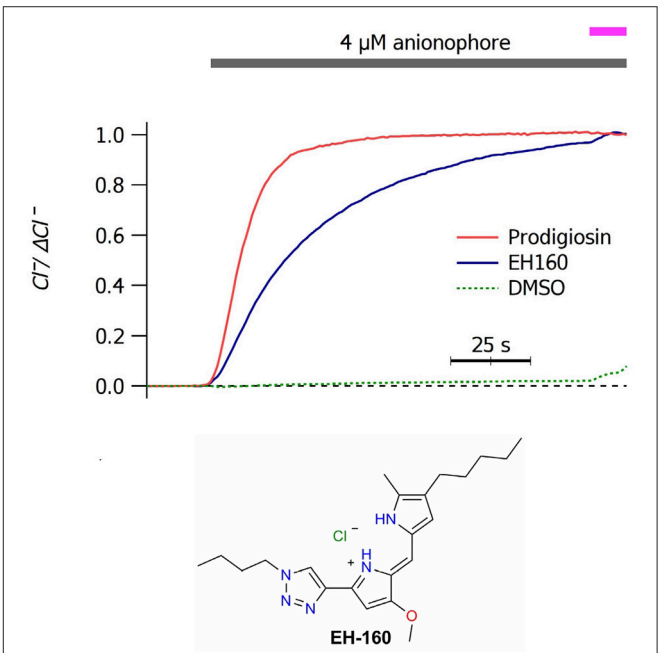

FIGURE 1 | Time course of the external concentration of chloride in a LUV suspension upon application of 4 µM of prodigiosin and EH160, as indicated by the upper bar. The magenta bar indicates the addition of detergent. A control trace was obtained after the application of DMSO. Data was normalized by the maximum chloride change, ΔCl^-. The chemical structure of EH160 is shown at the bottom.

POPC; palmitoyl-oleyl-phosphatidylethanolamine, POPE) and cholesterol (chol). Anionophore-driven chloride efflux measured in LUV composed by POPC:chol (19:1) (48.9 ± 0.4 μM/s), POPC:POPE (9:1) (52.3 ± 0.2 μM/s) and POPC:POPE:chol (8.1:0.9:1) (47.1 ± 0.9 μM/s), was very similar to that measured in asolectin LUV, indicating that the lipid composition is not critical for the anionophore transport (data not shown). Similarly, substitution of sodium by potassium in the internal or in the external solutions does not affect the measured chloride efflux, confirming that EH160 is an anion selective carrier.

The chloride efflux rate depends on the concentration of anionophore (see **Figure 2A**). To evaluate the concentration to induce half of the maximum rate, EC_{50}, was estimated plotting the initial efflux rate, J_0, against the anionophore concentration, [EH160] (**Figure 2B**). Data was fitted with the Equation (3):

$$J_0 = \frac{J_{max}}{1 + \frac{EC_{50}}{[EH160]}} \quad (3)$$

where *Jmax* is the maximum chloride efflux initial rate. The time course of the traces obtained at EH160 concentration higher than 10 μM was often quite variable. We interpreted these data as a destabilization of the LUV bilayers. Thus, to remove these possible outliers, we fitted iteratively the data, removing data points that lie beyond the 95% confidence prediction interval at each iteration, until no outliers remain. After this procedure, the doses-response fit yielded a maximum initial chloride efflux of 87.3 ± 6.4 μM/s, and an EC_{50} of 5.64 ± 1.28 μM.

Selectivity of the Anionophore

To evaluate the selectivity of the anionophores, we measured the chloride efflux from LUV with an internal concentration of 450 mM chloride, and the external solution containing an isomolar concentration of different anions. Measurements were done in the presence of 10 mM HEPES to adjust the pH at 7.5 in both compartments. As no differences in the chloride efflux were observed substituting sodium by potassium, neither, in the internal solution nor in the external solutions, the cationic ion was used indifferently in this series of experiments. The time course of the chloride efflux measured with different external anions is shown in **Figure 3A**.

As a first approach, we could hypothesize that the anionophore interchanges the anions from both sides of the bilayer, and the chloride efflux is proportional to the efficiency of the counter-anion influx. Thus, the estimation of the initial rate of the chloride efflux should represent the permeability of the external anion. From these data (**Figure 3B**), we can assert that the anionophore EH160 has a consistent transport rate for small organic anions, as acetate and formate, and in less extend for inorganic anions, as nitrate and bicarbonate. Conversely, transport of bigger, more hydrophilic anions, as aspartate, gluconate, phosphate, sulfate, and isethionate, is more than ten-fold reduced.

However, in this experimental design it is not possible to use external chloride to compare the relative permeability of this anion with other anions. To overcome this limitation we designed a series of experiments in which a fraction of the internal chloride was substituted by nitrate or bicarbonate. Thus, the second anion will compete with chloride for binding the anionophore, and therefore, the chloride efflux will be modified accordingly. The initial chloride efflux rates, measured for different combinations of chloride/nitrate and chloride/bicarbonate at the internal side are shown in **Figures 3C,D**, respectively. The reduction of the chloride efflux, as the concentration of the second anion increases, occurs because the carrier binds bicarbonate or nitrate instead of chloride, resembling a competitive inhibition of an enzyme. Hence, the continuous lines in **Figures 3C,D** represent the best fits of data with:

$$\frac{J_0\left(Cl^-, anion\right)}{J_0\left(Cl^-, 0\right)} = \frac{Cl^-}{K_{Cl}\left(1 + \frac{anion}{K_{anion}}\right) + Cl^-} \quad (4)$$

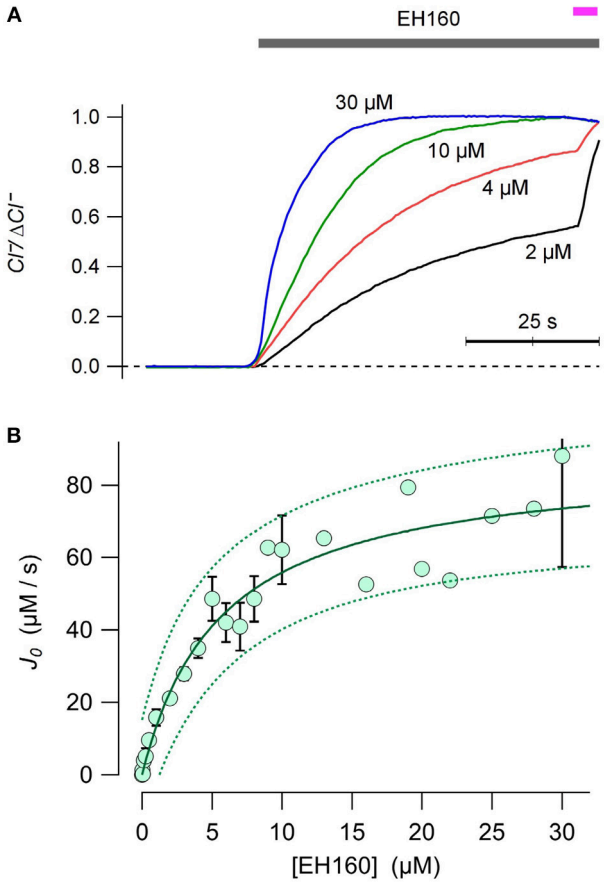

FIGURE 2 | (A) Time course of external concentration of chloride elicited by various concentrations of EH160, as shown near each trace. Application of the anionophore is indicated by the upper horizontal bar, and the addition of detergent is indicated by the magenta bar. Data was normalized by the maximum anion change, ΔCl^-. **(B)** The initial chloride efflux rate, J_0 is plotted against the anionophore concentration. When the measurement was repeated more than once, data represent the average and the bar is the standard error of the mean. The continuous line is the best fit of data with equation 3, yielding the concentration for the half of the maximum effect, EC_{50} = 5.6 μM, and a maximum chloride initial efflux rate of 87.3 μmoles/s. Broken lines represent the prediction interval for 95% confidence.

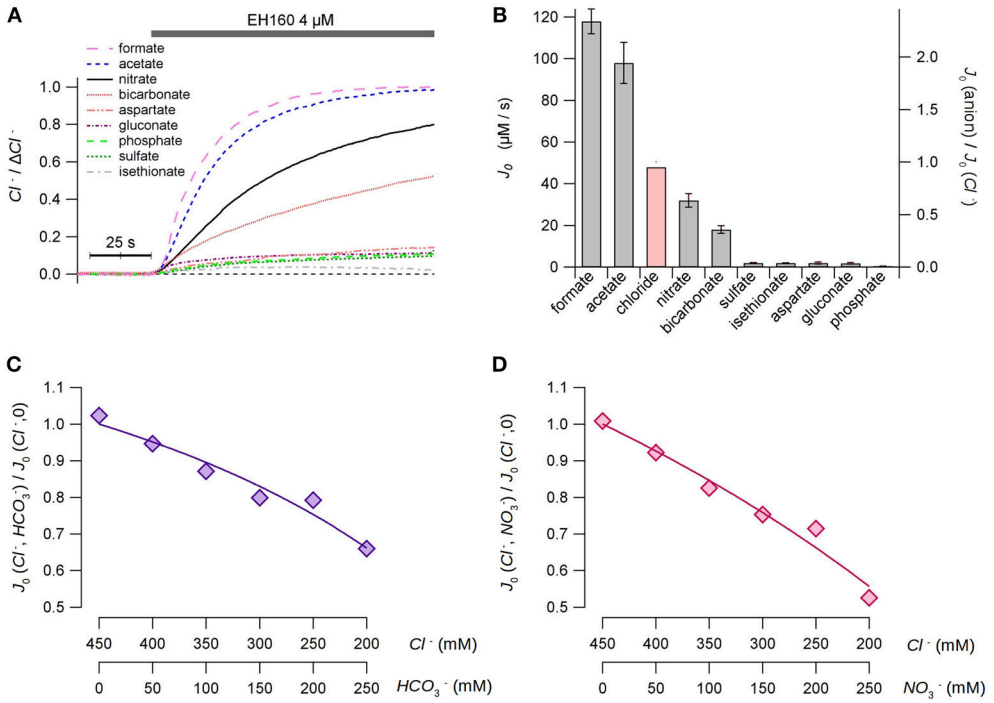

FIGURE 3 | Permeability of the anionophore EH160 to different anions. **(A)** Time course of the external concentration of chloride measured in LUV with 450 mM internal chloride and variable external iso-osmotic anions, as indicated in the figure. The application of the anionophore is indicated in the upper bar. Data was normalized by the maximum anion change, ΔCl^-. **(B)** The initial chloride efflux, J_0, measured for different external anions. The red bar is the estimated chloride efflux in the presence of external chloride, calculated from the apparent dissociation constants of the anionophore for chloride, bicarbonate and nitrate estimates in the competition experiments. Data represent the average (± s.e.m.) of, at least, three different measurements. Panels **(C,D)** show the results of the bicarbonate/chloride and nitrate/chloride competition experiments, respectively. The concentrations of anions are shown in the bottom axes of the figures. The continuous lines represent the best fit of data with Equation (4).

where K_{cl} and K_{anion} are the apparent dissociation constants of chloride and the second anion (nitrate or bicarbonate), respectively. The average chloride apparent dissociation constant is $K_{cl} = 3.17 \pm 0.48$ mM, and the apparent dissociation constants for nitrate and bicarbonate are $K_{NO_3} = 4.79 \pm 0.67$ mM and $K_{HCO_3} = 8.18 \pm 0.71$ mM. The higher affinity of chloride (lower apparent dissociation constant) clearly reflects a higher permeability of this ion. Thus, we can assume that the ratio of the apparent dissociation constants $K_{NO_3}/K_{HCO_3} = 0.59$ is proportional to the bicarbonate:nitrate permeability ratio. This concept is confirmed by the ratio of the initial efflux rates of 0.57, measured with bicarbonate and nitrate in the external solution (**Figure 3A**). These data allow to estimate the hypothetical chloride initial efflux rate when the external solution is chloride, yielding a value of 48 μM/s. Scaling data to the theoretical chloride initial efflux rate it is possible to estimate the relative permeability of all assayed ions with respect of chloride, as shown in the right axis in **Figure 3B**.

It is intriguing to notice that when nominally impermeable anions, such as sulfate and gluconate, are in the external solution, a tiny chloride efflux induced by anionophores is still measurable, although it is very small (chloride efflux of 1.96 and 1.75 μM/s for sulfate and gluconate, respectively). As previously reported (Hernando et al., 2018), in these cases, after the initial chloride efflux, the system seems to stop transport chloride, and no further changes in the external chloride concentration are measured by the ion sensitive electrode (see **Figure 4**). The trace in **Figure 4** show the time course of the external chloride concentration upon the addition of 8 μM EH160 in a suspension of LUV with the internal solution containing 450 mM chloride and the external solution of 450 μM gluconate. The initial chloride efflux is 3.4 μM/s, and the flux is arrested when the external chloride concentration is $0.11 \times \Delta Cl$. The successive addition of a permeable anion to the external solution, to a final concentration of 225 mM of nitrate, induces again a chloride efflux. The restarted efflux, $J_0 = 6.2$ μM/s, is larger than the efflux obtained with only gluconate in the external solution, even after the 11% reduction of the chloride gradient and the dilution of the anionophore. In the presence of external impermeable anions, the initial chloride efflux occurs until the net charge displacement, occurring when the chloride ions move outside from the LUV, is balanced by the potential difference because of the asymmetrical distribution of permeable ions.

Thus, to avoid the charge accumulation and be independent of the potential difference, we "shunted" the LUV bilayer with cationic carriers. The addition of valinomycin (0.5 ng/ml), a

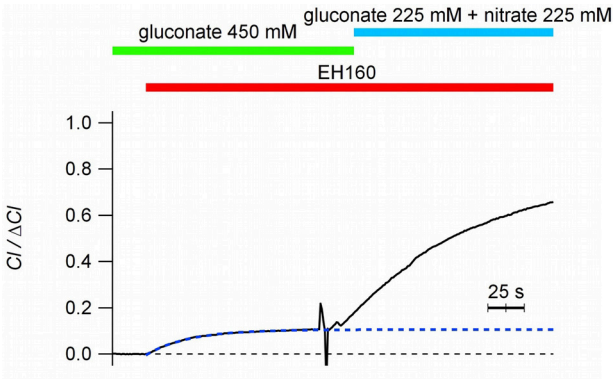

FIGURE 4 | Time course of the external concentration of chloride induced by the anionophore EH160 with different external anions, as indicated in the figure. When gluconate is in the external solution, chloride efflux is small, and is arrested without reaching the equilibria of concentrations (internal Cl = 49.5 mM; external Cl = 0.099 mM). Further addition of nitrate, a permeable anion, restores the normal anionophore-induced chloride efflux. Data was normalized by the maximum anion change, ΔCl^-.

specific potassium transporter, does not induce any chloride transport in the vesicles (**Figure 5A**), and does not modify the typical chloride efflux induced by the anionophore when the external ion is permeable. Differently, in the presence of an impermeable anion, as sulfate, addition of valinomycin restore the chloride efflux (**Figure 5B**). The same result, reactivation of the chloride efflux halted by an impermeable anion, gluconate outside, is obtained by the addition of 25 μg/ml of carbonyl cyanide-4-phenylhydrazone (FCCP), a proton ionophore (**Figure 5C**). The application of valinomycin before the addition of EH160 serves to condition the system to produce a chloride efflux in the presence of an external impermeable anion, as aspartate (**Figure 5A**, green trace).

Interestingly, in the presence of external impermeable anions, the time course of anionophore-driven chloride efflux favored by valinomycin is not exponential, but linear, as shown by the regression lines represented in blue in **Figure 5**; there, the correlation coefficient for a linear regression is $r > 0.999$, confirming the linearity of the traces. The exponential shape of the chloride efflux is due to the depletion of the anion from the LUV, that reduce the chloride gradient, and according to the Fick law, will reduce the anion flux. Conversely, the linear time course of the chloride efflux reflects a constant chloride gradient during the experiment.

This paradox may occur because, in the presence of the cation ionophore, the efflux of chloride driven by EH160 is accompanied with the facilitated efflux of potassium ions by valinomycin [or protons by cyanide-4-(trifluoromethoxy)phenylhydrazone, FCCP], thus maintaining the electro-neutrality of the process and resulting in a net solute loss from the LUV, with the consequent osmotic water withdrawal. The consequence is the maintenance of the concentration of the solutes, leaving essentially unaltered the ionic gradients.

To examine whether the anionophore-driven transport is affected by the electric field, we measured the chloride

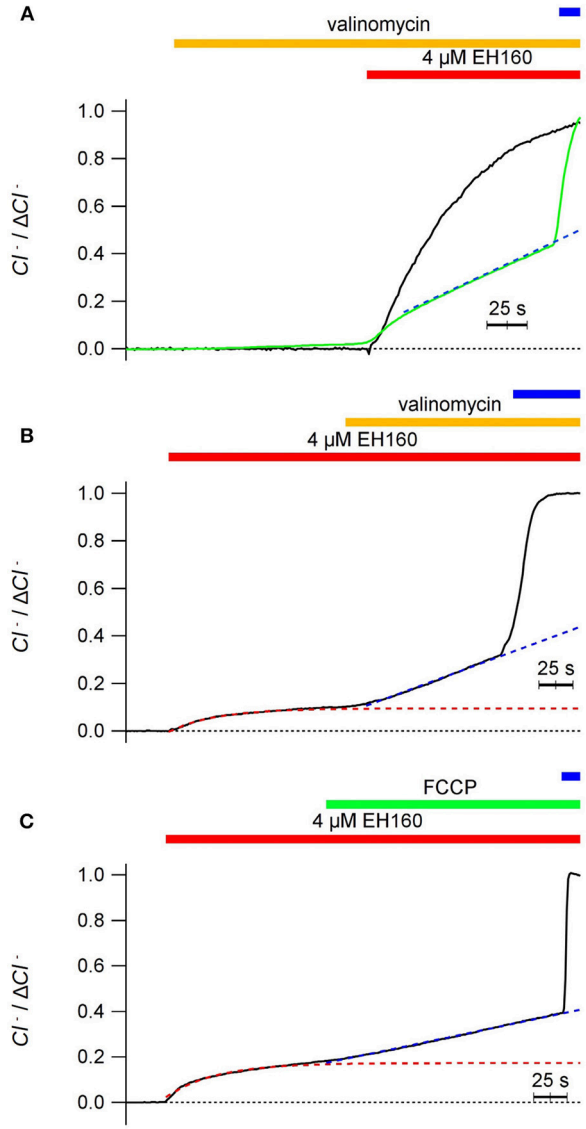

FIGURE 5 | Time course of the external concentration of chloride, revealing the anion efflux induced by the anionophore EH160 recorded in LUV shunted with a second ionophore. In all cases LUV had 450 mM internal potassium chloride and the application of 4 μM of EH160 is indicated by the horizontal (red) bar over the traces. Application of 0.5 ng/ml of valinomycin or 0.25 μg/ml of FCCP is indicated by the middle horizontal bar. The upper horizontal bar indicate the addition of detergent. Data was normalized by the maximum anion change, ΔCl^-. In panel **(A)**, the anionophore was added after the application of valinomicyn in preparations with the external solution containing 450 mM potassium nitrate (black trace) and 450 mM potassium aspartate (green trace); the external solution in panel **(B)** was 300 mM of potassium sulfate, and in panel **(C)** was 450 mM potassium gluconate. The blue broken lines are the lineal fitting of the traces after application of the cation ionophores. The red broken lines in panels **(B,C)** are the exponential fitting of the traces before the application of the cation ionophores.

efflux at different electric potential differences. To impose a membrane potential difference, we prepared LUV with different combinations of sodium chloride and potassium chloride inside, and sodium nitrate and potassium nitrate outside. The anion

gradient was always the same (450 mM chloride inside, and 450 mM nitrate outside). Because valinomycin is permeable to potassium, but does not transport sodium, the bilayer potential difference, according to the Nernst equation, depends on the potassium concentration at both sides. The initial chloride efflux, J_0, measured at potential differences between 50 to −150 mV (reference at the external side) resulted virtually independent from the voltage (**Figure 6**), indicating the EH160 activity is not voltage dependent.

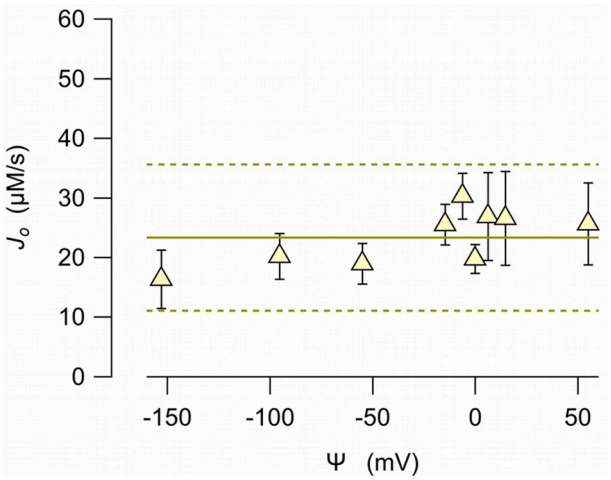

FIGURE 6 | EH160-driven chloride initial efflux, J_0, measured from LUV with 450 mM chloride in the internal solution, and 450 mM nitrate in the external solution. Different potassium concentrations at either side of the bilayer resulted in a potential difference, Ψ, by the presence of valinomycin (0.5 ng/ml). Symbols correspond to the mean values of, at least, three experiments, and the bars are the standard error of the mean. The continuous line is the average of all data, and the broken lines are the prediction limits with 95% confidence.

pH Dependence of EH160 Anionophore Activity

We have previously reported that the transport efficacy of EH160 in LUV strongly depends on pH (Hernando et al., 2018). In LUV with the same values of pH in the internal and external buffers, the rate of the chloride efflux carried by EH160 is faster at acidic pH than at alkaline (**Figure 7A**). This pH dependency of the anionophore activity is similar when the internal pH in the LUV is kept constant at 7.0, and the external pH is varied from 5.0 to 9.0 (**Figure 7B**). Conversely, when the external pH is kept fix at 7.0, variations of the internal pH in LUV from 5.0 to 9.0 does not determine any significant variation of the anionophore induced chloride efflux (**Figure 7C**).

An interesting feature of these observations is that the chloride efflux seems to be independent of the proton gradient. If the anionophore co-transports anions and protons, in absence of an active transport mechanism, it is expected that the gradient of both ligands should determine the transport rate. On the contrary, the chloride efflux in absence of a pH gradient (**Figure 7A**) varies in the same manner as observed in the experiments where a pH gradient was imposed varying the external pH (**Figure 7B**). Moreover, imposing a pH gradient varying the internal LUV pH, but maintaining the external pH constant does not modify the chloride efflux (**Figure 7C**). We conclude that the anionophore chloride transport is independent of the pH gradient, but depends only on the external pH. These data is consistent with the influence of pH in the ionization state at which the anionophore is incorporated into the membrane, but does not affect the ionic transport itself. To test this hypothesis, we compared the pH dependency of the initial chloride efflux, J_0, with the titration curve of the EH160. The titration curve was constructed plotting the ratio of absorbance measured at 502 and 474 nm of 20 μM EH160 against the pH of solvent buffer (**Figure 7D**, blue circles). These data were fitted with the Henderson–Hasselbach equation, yielding a pK_A of 6.47 ± 0.05. This titration curve can be superimposed with the plot of J_0 vs. the external pH (**Figure 7D**, pink squares), confirming that the pH dependence of the anionophore-driven chloride efflux corresponds to the ionization state of EH160. Noteworthy, in mammalian cells the maximum quenching rate of the YFP fluorescence measured at different extracellular pH can be easily superimposed to the EH160 titration curve (**Figure 7D**, green diamonds), further confirming the similarity of the behavior of EH160 in plasma membranes and in model bilayers. Remarkably, the pK_A values estimated for the measurement of chloride efflux in LUV, 6.66 ± 0.07, and influx of iodide in cells, 6.54 ± 0.19, are not significantly different from the pK_A yielded from the EH160 titration.

To further reinforce the idea that anion transport is not coupled with proton (or hydroxide) transport, we measured the EH160 induced chloride efflux in LUV with different pH gradients, to observe the effect of the collapse of the gradient with the proton carrier FCCP. In these experiments, 0.25 μg/ml of FCCP were added to the external solution before the application of the anionophore (**Figure 7E**). The collapse of the pH gradient, induced putative proton transport induced by the FCCP, does not produce any significant chloride efflux; then, application of the anionophore induced a measurable chloride efflux corresponding to the external pH. Similarly, a chloride efflux, correspondent to the external pH, is observed upon application of EH160, but further application of FCCP does not modify the chloride efflux time course (**Figure 7F**). Application of FCCP will plausibly equilibrate the pH to values near to the external pH, thus dissipating the pH gradient. Similar results were obtained in experiments where the pH gradient was dissipated with nigericin, that is another proton transporter (data not shown). Thus, the lack of effect of this action on the chloride efflux rules out the coupling of anion transport and proton transport for EH160, confirming the independence between the anion transport and the proton gradient present.

Anionophore-Driven Transport in Mammalian Cells

Since the ultimate objective of the characterization and further optimization of the anionophores is to open the possibility to

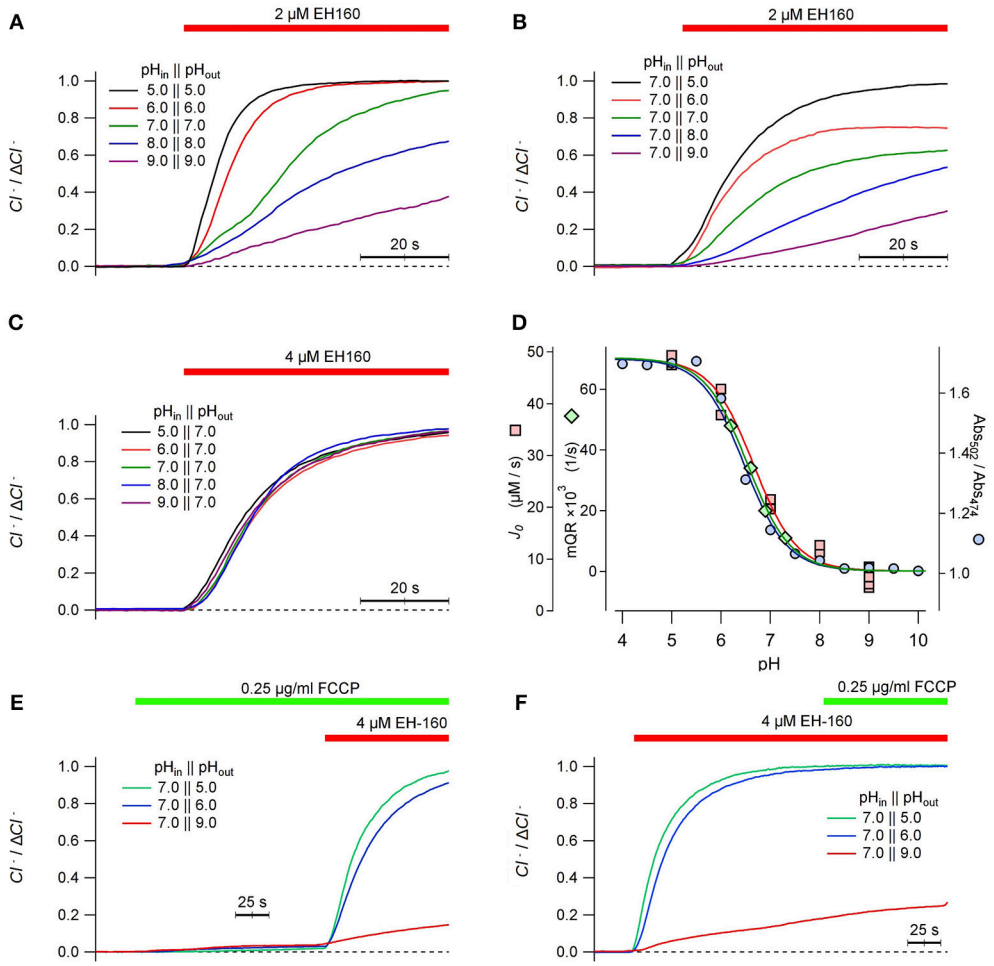

FIGURE 7 | Time course of the external concentration of chloride induced by the anionophore EH-160 was measured in LUV with 450 mM internal chloride and 450 mM external ions, in symmetric pH buffer **(A)** and asymmetrical pH buffer, varying either the external pH **(B)** and the internal pH **(C)**, as indicated in the figure. The application of the anionophore is indicated by the horizontal bar. **(D)** The initial influx rate measured in LUV, J_0, at different external pH is represented by squares; the maximum YFP-quenching rate, mQR, representing the iodide influx in FRT cells measured at different extracellular pH values, is pictured by diamonds. The ionic state of EH160, expressed as the ratios of absorbances at 502 and 474 nm (circles) is also plotted against the pH. The three continuous lines are the corresponding best fits of data with the Henderson-Hasselbach equation, normalized by the maximum and the minimum asymptotes. The time course of the external concentration of chloride measured in LUV treated with the proton ionophore FCCP prior the application of EH-160 **(E)**, or applying the proton ionophore FCCP after the induction of chloride efflux by the anionophore **(F)**, as shown by the horizontal bars.

use them in cells as therapeutic agents, we report the proof of concept that this class of carriers is able to transport anions in mammalian cells. Therefore, we have repeated the measurements of anionophore-driven iodine influx and chloride efflux in mammalian cells. The toxicity of EH-160 ($TD_{50} = 7.1 \pm 1.1\,\mu M$) is reduced respect to that of prodigiosin ($TD_{50} = 2.9 \pm 2.4\,\mu M$). **Figure 8A** shows the time course of the iodide quenching of the YFP fluorescence, representing the influx of the halide into the FRT cells. The iodide influx is clearly dependent of the concentration of EH160. Similarly, a chloride efflux was observed in HEK cells upon the application of the anionophore at different concentrations (**Figure 8C**). Quantification of the chloride efflux under these conditions was difficult because the removal of chloride from the external solution modifies the membrane potential and activates endogenous mechanism to maintain the cell homoeostasis that partially hidden the signal of anionophore-driven transport. It is, however, clear that EH160 induces halide transport in living mammalian cells.

Interestingly, the transport capacity of the anionophore also depends on the extracellular pH. Using both methods to display the anion transport in cells, the fluorescent probe to measure the iodide influx (**Figure 8B**), and the use of ISE to evaluate the chloride efflux (**Figure 8D**), we observed that the efficacy of transport increases as the extracellular pH is more acidic, similarly as observed for the measurements in LUV. We conclude that the transport properties of the triazole derivative of prodigiosine in mammalian cells are similar to those observed in LUV.

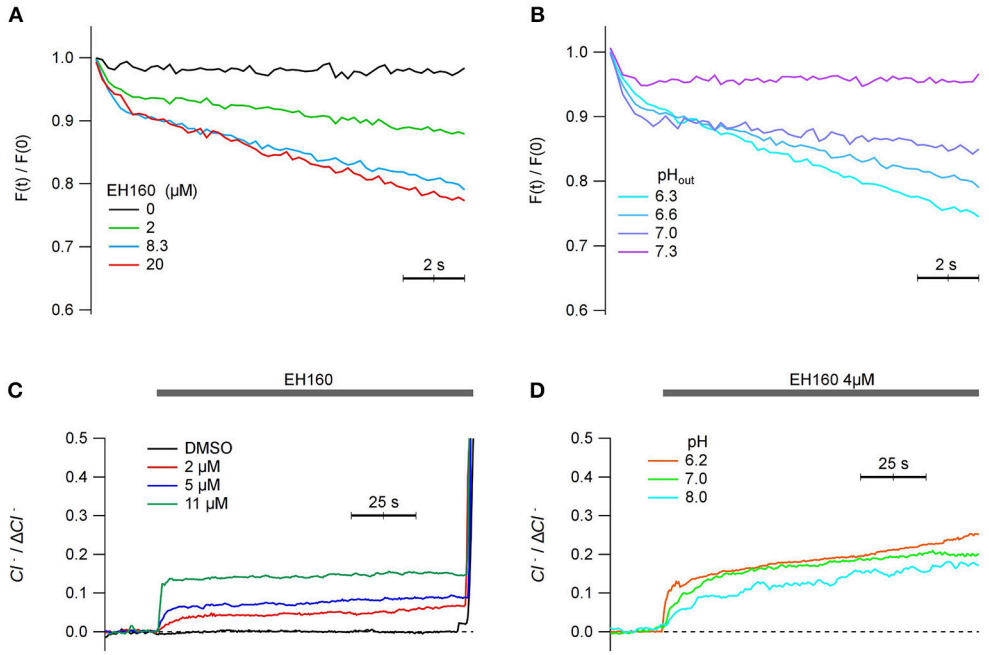

FIGURE 8 | Anionophore-driven transport in mammalian cells. **(A,B)** show the time course of the iodide-sensitive YFP fluorescence, normalized by the initial fluorescence [F(t)/F(0)], measured in FRT cells after the addition of 100 mM iodide in the external solution. The decrease of the fluorescence, due to the quenching of YFP fluorescence by iodide, represents the influx of the halide. Each trace is the average of three measurements. **(A)** Traces obtained with the treatment of cells with different concentrations of EH160, at pH 6.6. **(B)** Different efficacy of 8.2 μM EH160 at different extracellular pH. **(C,D)** represents the time course of the external concentration of chloride on HEK cells perfused with different EH160 concentrations **(C)** and at different pH **(D)**. Data was normalized by the maximum anion change, ΔCl^-.

DISCUSSION

We have examined the properties of a prodigiosin-inspired anionophore to characterize its anion transport properties. A detectable chloride efflux was measured in LUV for the 1,2,3-triazole heterocycle assayed, EH160 at micromolar concentrations (**Figure 1A**). At equimolar concentrations, the natural product prodigiosin showed a considerable higher potency than EH160. Notably, the application of the anionophore solvent, DMSO, does not induce any chloride efflux in LUV (**Figure 1A**), confirming that the results reflect the anion transport driven by the anionophore. The dose-response curves for anionophores shown in **Figure 2** further confirm that the examined molecules are responsible for the observed chloride efflux. These data indicated that EH160 in LUV exerts the half of its maximum activity at 5.6 μM (**Figure 2B**). This value is two orders of magnitude higher than that we reported before (Hernando et al., 2018). This discrepancy is due to the different method employed to calculate this EC_{50} value. In our previous work the dose response curves were empirically constructed plotting the amount of chloride transported after a time interval (300 s). This is an arbitrary time interval and this measurement is useful to compare the potency of series of compounds. Here we have obtained this EC_{50} value fitting the initial chloride flux calculated according to Equations (1, 2). This is thus an absolute measurement of the potency of the compound in the assayed conditions. The main advantage of the triazole derivatives is that they exhibit a reduced toxicity while retaining a remarkably high transport activity. Prodigiosine is a highly toxic compound ($TD_{50} = 2.9\,\mu M$), as reported in numerous studies (Manderville, 2001). Although it is likely that cytotoxicity account for some of the intriguing pharmacological properties of this compound it jeopardizes its potential application as CFTR replacement therapy. The TD_{50} of EH160 is 7.1 μM, that is, indeed, no very different of the EC_{50} of 5.4 μM. Although the difference is small, it should be possible to optimize the anionophores to obtain less toxic substances. In any case, to apply these transporters for therapeutic uses, one have to consider also the efficacy of the anionophores. The efficacy (maximum quenching rate) of the iodide influx driven by the anionophore EH160 is similar to the CFTR activated by applying 20 μM forskolin and 10 μM genistein in FRT cells (Hernando et al., 2018). Since the CFTR activity of the CFTR transfected in the FRT cells is more than 13-fold greater that expected in human bronchial cells (Taddei et al., 2004; Moran et al., 2005; Kreindler et al., 2009; Melani et al., 2010; Gianotti et al., 2013, 2016).Thus, to induce an anion transport equivalent to that expected in bronchial cells, it would be necessary to apply $<0.5\,\mu M$ of EH160, significantly increasing the width of the therapeutic window.

Experiments were done with a very simple system, unilamellar vesicles, to avoid the contribution of other anion transport

mechanisms present in cells. Another advantage to use such artificial system is the possibility to create large anion gradients, up to 450 mM, that improves the resolution of the measurements. Noteworthy, the phospholipid composition seems not to modify significantly the transport capacity of the anionophores. We cannot exclude, however, that mayor modifications in surface charge or membrane viscosity could induce differences on chloride efflux. We have also assayed the two most biological relevant cations, sodium and potassium, and could not find any difference on chloride efflux. Thus, we concluded that these cations very unlikely contribute to anionophore-driven chloride transport.

To better explain the EH160-driven transport we propose the model presented in **Figure 9**, where the carrier incorporated in the bilayer has three states, the free carrier, $\mathbf{TH^+}$, the carrier bound to chloride, **THCl**, and the carrier bound to a second anion, **THA**, for example nitrate, bicarbonate or gluconate. The binding to the carrier occurs in the aqueous solution-bilayer interface with the first order equilibrium constants $\mathbf{K_{Cl}}$ and $\mathbf{K_A}$ for chloride and the second ion, respectively. The internal solution (**in**) has a high concentration of chloride and there is no second anion; on the other hand, the concentration of the second anion is high in the external solution (**out**), meanwhile chloride is virtually absent. Thus, when the anionophore is on the internal face of the bilayer, the binding of chloride and the release of the second anion are favored, while the unbinding of chloride and the binding of the second anion takes place on the outer face of the bilayer. In this manner, as the anionophore diffuses across the bilayer, a chloride efflux, as a result of the exchange with the second anion, is established.

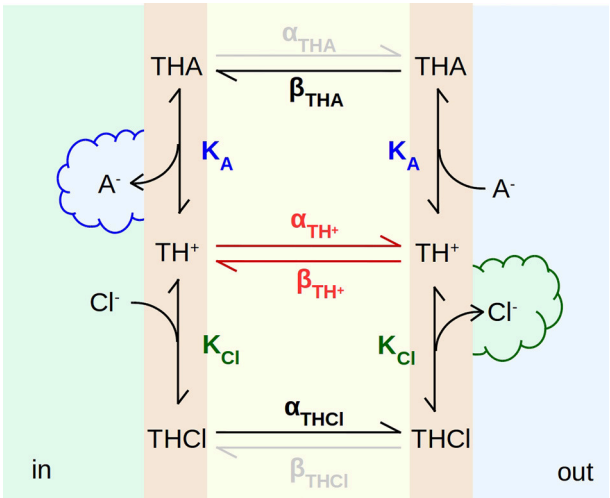

FIGURE 9 | Scheme of the mechanism of transport of anions across a bilayer driven by the anionophore EH160. $\mathbf{TH^+}$, **TCl**, and **TA** are anion-free, chloride-bound, and any other anion-bound forms of the EH160 transporter, respectively. These three carrier forms are located in the aqueous solution-bilayer interface, where they can reversibly bind anions, with a binding equilibrium constant, $\mathbf{K_{Cl}}$ and $\mathbf{K_A}$, for chloride and the second anion, respectively. Each anionophore form can diffuse across the hydrophobic region of the bilayer with a rate constant **α** and **β**. The high chloride concentration at the internal side **(in)**, and the high concentration of a second permeable anion (nitrate or bicarbonate) at the outer side **(out)**, will produce a chloride efflux.

These three anionophore forms diffuse across the hydrocarbon chains with rates of **α** and **β**. If we assume that the bilayer is symmetric and homogeneous, for a given carrier state, rate constants **α** and **β** are equivalent. However, the diffusion of the carrier will be determined by the binding state of the anionophore significantly. The diffusion rates for the free carrier, $\mathbf{TH^+}$, are probably lower than for the chloride bound form, **THCl**, or the **THA** form where $\mathbf{A^-}$ is a permeable anion as bicarbonate or nitrate. It is possible to speculate that the energy barrier to cross the hydrophobic region of the membrane is higher for the $\mathbf{TH^+}$ form, bearing a the positive net charge (see **Figure 1**), than the complexes **THCl** and **THA**. Complexed anions form a tightly bound ion par with the carrier which is overall neutral, and undergoes the diffusion across the hydrophobic environment easily. Thus, the permeability of different anions will depend on both, the binding equilibrium constant, and the diffusion rate of the anion-EH160 complex.

Comparing with permeable ions (chloride, nitrate, bicarbonate), the impermeable anions, such as sulfate, phosphate or gluconate, are characterized by a more stable interaction with water, with a more negative hydration enthalpy and Gibbs energy (Marcus, 1987, 1991). The structural study and theoretical analysis of synthetic prodigiosines have shown that anions bind the molecule in a groove formed by the three N–H groups (Díaz de Greñu et al., 2011; García-Valverde et al., 2012; Hernando et al., 2018). A similar binding of anions was also described for analogs of the marine alkaloids tambjamines (Iglesias Hernández et al., 2012; Hernando et al., 2014). Accordingly, the difficulty to remove the hydration water from these impermeable anions, and their larger molecular volumes (Marcus, 1993) will contribute to reduce the affinity of these anions for the carrier, and therefore the form **THA** is virtually absent, abolishing the flux of these anions across the bilayer. However, as long as electroneutrality conditions are maintained, shunting the bilayer with a cation carrier, the chloride flux is appropriately restored (**Figure 5**). Albeit, the chloride efflux occurring in absence of counter-anion influx (see **Figure 5**), when the **THA** state is not present, demonstrates that the free, unbounded anionophore $\mathbf{TH^+}$, is able to diffuse across the bilayer. At this point we could delineate the transport mechanism of EH160 as a molecular carrier embedded in the lipid bilayer. It should bind an anion at the bilayer interface at one side, cross the bilayer, and release the anion at the other side; the same mechanism occurs when the second ion is transported in the opposite direction, closing the transport cycle.

Interestingly, in the presence of external impermeable anions, the time course of anionophore-driven chloride efflux favored by valinomycin is not exponential, as described by equation 1, but becomes linear (**Figures 5B,C**). The exponential shape of the chloride efflux curve is due to the depletion of the anion from the LUV, that reduces the chloride gradient. According to the Fick law, this results in the reduction of

the anion flux (observed as the derivative of the time course). Hypothetically the linear time course of the chloride efflux is due to a shrinking volume of LUV, as the efflux of a chloride ion should be accompanied with the efflux of a potassium ion mediated by valinomycin (or protons through FCCP), to maintain the electroneutrality. The result should be a net solute lost, with the consequent water osmotic withdrawal. These movements, within the measuring time interval, would result in the maintenance of the concentration of the solutes, and the consequent maintenance of the ionic gradients.

In the model depicted in **Figure 9** the movement of anions facilitated by the EH160 carrier has been illustrated. According to this scheme, EH160 is a reversible uniporter, and the direction of the flux is driven by the chemical gradient. Conversely, due to the fact that the measurements of the effect of prodigiosine and its derivatives in cells have shown that they induce cellular death accompanied with modifications of intracellular pH levels (Ohkuma et al., 1998; Castillo-Avila et al., 2005; Seganish and Davis, 2005; Díaz de Greñu et al., 2011; Gale et al., 2013; Cheung et al., 2018), it has been suggested that this class of anionophores are H^+/Cl^--symporters. However, we have seen that the pH influence of the EH160-transport activity is independent of the H^+ gradient (**Figures 7**, **8**), but just on the ionization state of the carrier (**Figure 7D**). Indeed, there is no modification of the chloride efflux when the proton gradient is dissipated using proton carriers like FCCP (**Figures 7E,F**) or nigericin (data not shown). This is consistent with the conformational analysis showing that the anion binding is favored by the protonated form of a model prodigiosine (García-Valverde et al., 2012). Therefore, amount of an active protonated carrier in the bilayer will be determined by the pH in the external solution, which has a several orders of magnitude larger volume, and will dominate the equilibria in the three-compartment system formed by the internal space, the external space and the bilayer.

To estimate the chloride exchange rate in LUV we have to take into account the concentration of the anionophore in the bilayer membrane based on the concentration of EH160 in the aqueous solution. The average radius of the LUV, 47 nm, and the bilayer thickness of 3.75 nm, were obtained by small angle x-ray scattering (Baroni et al., 2014). It follows that the bilayer volume per vesicle is 3.39×10^{-16} cm^3. From the final external chloride concentration, $\Delta Cl \approx 0.9$ mM, and the total volume of the assay, 3.5 cm^3 we can estimate the total number of vesicles 1.61×10^{13}, and the total bilayer volume in the sample, 1.55×10^{-3} cm^3. To estimate the concentration of the anionophore in the bilayer, we used the water/*n*-octanol partition coefficient as calculated by the computational chemistry suite Marvin Sketch (https://www.chemaxon.com). It allows to calculate the partition coefficient of ionized and non-ionized species from the molecular structure (Viswanadhan et al., 1989), taking into account the ionization at a given pH, and the effect of the counter ion concentration. Thus, the *n*-octanol/buffer partition coefficient for EH160 in 450 mM NaCl (or KCl) at pH 7.5 is $P_{octanol/buffer} = 68.1$ (logP = 1.83). Hence, for an anionophore concentration of 1 μM, we expect a bilayer anionophore concentration of 6.62×10^{-8} moles cm^3, that corresponds to 6.18×10^{13} molecules of EH160 in the bilayers. The initial chloride efflux expected for 1 μM EH160 is 1.31×10^{-5} M/s, that corresponds to a transport rate of 7.45×10^{-22} moles of chloride/s per anionophore molecule, that represents 449 chloride ions/s per anionophore molecule. It is important to highlight that the efflux, and consequently the transport rate, depends on the chloride gradient, that for LUV experiments is ∼450 mM. In contrast, in mammalian cells, we could assume that the chloride gradient is ∼120 mM (≥30 mM intracellular and 150 mM extracellular), therefore, the transport rate should be scaled accordingly, resulting in 120 chloride ions/s per anionophore molecule. These values are similar to the exchange rates reported for other natural ion carriers in membranes, like the bacterial sugar transporters 2×10^2 (Waygood and Steeves, 1980), or the sodium calcium exchangers 5×10^3 (Baazov et al., 1999), but significantly lower than those characteristic of ion channel transport (6×10^6-12×10^7; Hille, 2001). A detailed calculation of the chloride turnover is presented as **Supplementary Material**.

We assayed the anionophores in a cellular model to assess whether anionophores could transport halides across the plasma membrane. By using an iodide-sensitive YFP to monitor the intracellular iodide concentration, we could demonstrate that, as described for other small organic molecules such as calix[4]pyrroles (Ko et al., 2014), tambjamines (Soto-Cerrato et al., 2015), ortho-phenylene bis-ureas (Dias et al., 2018), and bis-(p-nitrophenyl)ureidodecalins (Li et al., 2016), EH160 is able to transport this ion through the cell membrane (**Figures 8A,B**). The advantage of the iodide influx measurements is that the ion gradient driving the flux is well-controlled in the experiment. On the other hand, measurements of the chloride efflux revealed difficult because cells regulate the intracellular chloride concentration maintaining it low: in epithelium chloride concentration is ≤30 mM; in an experiment, when the extracellular chloride is removed, the effective gradient is 15-fold smaller that used in LUV experiments, and the efflux must be proportionally smaller. On the other hand, the cell homoeostasis implies a series of mechanisms that transport different ions, including several chloride and bicarbonate transporters, that may conceal a proper estimation of the anionophore- driven chloride efflux. Nevertheless, we could demonstrate that anionophores do induce chloride efflux in mammalian cells, with general characteristics similar to those observed in LUV bilayers (**Figures 8C,D**). Chloride efflux was observed when substituting the extracellular chloride by nitrate.

Here we have complemented the data previously reported (Hernando et al., 2018), designing a series of experiments useful to understand the transport mechanism of triazol derivatives of prodigiosine. These experiments demonstrate that these anionophores could be used to promote chloride and bicarbonate transport in cells, i.e., are good candidates to replace the defective or missing CFTR in an attempt to design a new cystic fibrosis therapy, as proposed for other anion transporters (Shen et al., 2012; Valkenier et al., 2014; Li et al., 2016, 2017; Liu et al., 2016; Dias et al., 2018). The analysis of anionophore-induced anion transport in cells needs, in any case, to be extended, studying the anionophore-induced ion transport in epithelial

models, where the polarization of cells plays a fundamental role on the directionality of ion transport, to find the best suited compounds to become candidates for cystic fibrosis therapy. Preliminary experiments on other prodigiosine and tambjamine derivatives have shown that the properties of the anionophore EH160 could be extended to other analogous compounds, opening the possibility to design molecules optimized for clinical development. This proof of concept represents an encouraging promise for future developments toward a mutant-independent cystic fibrosis therapy.

AUTHOR CONTRIBUTIONS

OM and RQ planned the study. OM, RQ, and EC designed the experiments and analyzed data. RQ and MG-V synthesized the anionophores. CC performed the experiment on vesicles under the supervision of OM. VC, DB, and MF performed the experiments on cells under the supervision of OM and EC. OM and RQ wrote the manuscript.

FUNDING

This work has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 667079 and Consejería de Educación de la Junta de Castilla y León (Project BU092U16).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00852/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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PERSPECTIVE
published: 27 November 2018
doi: 10.3389/fphar.2018.01381

Gene Therapy for Cystic Fibrosis Lung Disease: Overcoming the Barriers to Translation to the Clinic

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Cystic fibrosis (CF) is a progressive, chronic and debilitating genetic disease caused by mutations in the CF Transmembrane-Conductance Regulator (*CFTR*) gene. Unrelenting airway disease begins in infancy and produces a steady deterioration in quality of life, ultimately leading to premature death. While life expectancy has improved, current treatments for CF are neither preventive nor curative. Since the discovery of *CFTR* the vision of correcting the underlying genetic defect – not just treating the symptoms – has been developed to where it is poised to become a transformative technology. Addition of a properly functioning *CFTR* gene into defective airway cells is the only biologically rational way to prevent or treat CF airway disease for *all CFTR* mutation classes. While new gene editing approaches hold exciting promise, airway gene-addition therapy remains the most encouraging therapeutic approach for CF. However, early work has not yet progressed to large-scale clinical trials. For clinical trials to begin in earnest the field must demonstrate that gene therapies are safe in CF lungs; can provide clear health benefits and alter the course of lung disease; can be repeatedly dosed to boost effect; and can be scaled effectively from small animal models into human-sized lungs. Demonstrating the durability of these effects demands relevant CF animal models and accurate and reliable techniques to measure benefit. In this review, illustrated with data from our own studies, we outline recent technological developments and discuss these key questions that we believe must be answered to progress CF airway gene-addition therapies to clinical trials.

Keywords: Cystic fibrosis, rat, mouse, airway, lung, genetic therapies, gene-addition, lobe-targeted delivery

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *03 August 2018*
Accepted: *09 November 2018*
Published: *27 November 2018*

Citation:
Donnelley M and Parsons DW (2018) Gene Therapy for Cystic Fibrosis Lung Disease: Overcoming the Barriers to Translation to the Clinic. Front. Pharmacol. 9:1381. doi: 10.3389/fphar.2018.01381

INTRODUCTION

Cystic fibrosis (CF) is an insidious disease that slowly smothers the health and potential of too many young lives. It is the most common fatal genetic disease in the developed world; 1 in 25–30 people with Caucasian ancestry carry a single defective copy of the *CFTR* gene (Cystic Fibrosis Foundation, 2018) and have no symptoms; 1 in ∼3000 babies are born with mutations on both alleles, resulting in the disease. CF is a multisystem disease that affects many organs, producing a life expectancy of approximately 40 years (Australian Cystic Fibrosis Data Registry, 2016). Premature death usually results from lung disease, after a lifetime struggling to deal with progressive respiratory failure.

New mutation-specific modulator and corrector pharmaceuticals such as Kalydeco (ivacaftor), Orkambi (lumacaftor/ ivacaftor), and Symdeko (tezacaftor/ ivacaftor) have given substantial benefit to some people with CF. Assuming a patient has the right *CFTR* mutation class, these personalized medicines can increase the presence and/or function of the CFTR protein in the cell, improve lung function, and slow lung disease progression. However, they have been neither preventive nor curative since they do not address the *CFTR* gene defect itself, and some have limited effectiveness. Importantly, the cost of these once-daily medications is prohibitive and likely to be unsustainable, with the health systems in several countries declining to recommend Orkambi for governmental financial support due to poor cost-benefit.

Since CF is a recessive genetic disorder, addition of a single copy of the properly functioning *CFTR* gene into affected CF airway cells is recognized as the only rational and feasible way to prevent or treat CF airway disease (Griesenbach et al., 2016). Gene therapy would provide proper cellular function, regardless of the person's *CFTR* mutation class. The vision for a child born with CF is that treatment with a proven *CFTR* gene-addition therapy at birth would prevent that child ever developing CF lung disease. For those already living with CF, the same therapy would halt progression of their lung disease.

Gene therapies for a range of inherited diseases have now reached the clinic in China (Kim et al., 2008), Europe (Touchot and Flume, 2017), and most recently in the United States where Luxturna, a gene-addition treatment for retinal dystrophy (Russell et al., 2017) was approved by the FDA in Dec 2017 as a prescription medicine (Morrison, 2018). To 2017, there have been almost 2600 gene therapy trials, including 36 for CF (Ginn et al., 2018). Gene therapy is now a therapeutic reality for some genetic diseases, but the challenge for the CF field is to convert the extensive preclinical developments into an effective and safe treatment option for people with CF.

DEVELOPMENTS IN AIRWAY GENE TRANSFER TECHNIQUES

Although gene therapy has the potential to be efficacious for CF, a range of challenges have been identified. Here we describe those challenges and their solutions.

Vector Designs Affect Efficacy

A 12-month, monthly repeat-dose Phase II *CFTR* non-viral (liposome) gene-transfer clinical trial showed significant, albeit modest and transient, lung function benefits in CF patients (Alton et al., 2015). That study confirmed that a *CFTR* gene therapy can correct human CF lung disease, is likely to be safe, have low immunogenicity, and be amenable to repeated dosing. However, due to the poor efficiency and transient response, that group has since concentrated their development efforts on a more efficient lentiviral (LV) gene vector (Alton et al., 2017). Recently, the delivery of *CFTR* mRNA using nanoparticles has been shown to improve chloride channel function in CF mice for 2 weeks, with a superior response compared to liposomal delivery (Robinson et al., 2018). However, the short duration of action may be a barrier to adoption.

Initial CF gene-addition research used adeno- (Ad) and adeno-associated viruses (AAV) as the gene vectors, but these failed the clinical transition process for CF lung disease due to significant side-effects, and/or lack of efficacy and duration (Moss et al., 2007). AAV also has a DNA packaging capacity limit that requires truncation of the CFTR gene, and AAV vector genomes remain largely episomal (not integrated), meaning that gene transfer to terminally differentiated airway cells is transient and rapidly lost with airway cell turnover (Karda et al., 2016). Despite these challenges [well described by Guggino and Cebotaru (2017)] AAV vector development is continuing, including the assessment of alternative serotypes (Steines et al., 2016; Guggino et al., 2017; Duncan et al., 2018). Interestingly, an integrating *piggyBac*/Ad *CFTR* vector was recently shown to phenotypically correct CF pig airways, with the possibility that it could produce extended gene expression (Cooney et al., 2018).

Lentiviral vectors are now one of the lead CF airway gene-addition vectors for therapeutic development because they transduce dividing and non-dividing cells, can be pseudotyped to target specific cell types by altering surface receptor recognition elements, integrate into the host cell chromosome providing lasting benefit, and generate little immune response. LV vectors have been effective in a range of preclinical studies (Copreni et al., 2004; Cmielewski et al., 2014a; Cooney et al., 2016; Alton et al., 2017). Supported by the clinical successes in effectiveness and safety within *ex vivo* CAR-T cell therapies in cancer treatment (Milone and O'Doherty, 2018), we believe that LV vectors are currently the *CFTR* delivery vehicle of choice. The remainder of this article focuses specifically on the barriers of translating LV gene vectors for CF to the clinic.

Physical and Biological Barriers of the Airway Surface Restrict Gene Delivery

The airway epithelium has evolved to protect cells against foreign invaders such as viruses and bacteria. The normal physical host defenses at the airway surface play a leading role in limiting the efficiency of any type of gene transfer into the airway epithelium (Kim et al., 2016). Mucus that covers the epithelial surface traps vector particles, which are then removed from the airway by mucociliary clearance (MCC) efficiently preventing vector particles from reaching vector-relevant receptors on the cell membrane surface (Castellani and Conese, 2010). This situation is exacerbated in CF due to the increased volume and viscosity of the mucus (Duncan et al., 2016). Finally, for vectors designed to target the airway stem cells, known to be a subpopulation of airway basal cells at the deep-lying epithelial basement membrane (Rock et al., 2009), access past the epithelial tight junctions is also needed. These tight junctions separate the luminal surface from the deeper layers, acting as a normal defense against pathogenic particles.

Gene vectors must be able to penetrate the CF mucus barrier and reach the underlying target epithelium and target cells. One

recognized approach that enables gene vector particles to reach the relevant airway-surface cell vesicular stomatitis virus (VSV-G) receptors and the basal stem cells, is conditioning the airway epithelium with the compound lysophosphatidylcholine (LPC) prior to gene vector delivery. LPC is a normal component of lung surfactant, and its primary role is to transiently permeabilize airway tight-junctions, and it may also help to solubilize surface mucus and enable greater vector access to airway cells. *In vivo* acute and long-term reporter gene studies have shown the effectiveness of LPC conditioning for enhancing gene expression in mice (Limberis et al., 2002; Stocker et al., 2009; Cmielewski et al., 2014a, 2017). In addition, successful gene expression using the LacZ reporter gene has been achieved in short-term (7 day) studies, transducing the conducting airways in sheep (Liu et al., 2010) (a large lung model), marmosets (Farrow et al., 2013) (a primate model), ferrets (Cmielewski et al., 2014b), and rats (McIntyre et al., 2018).

Reporter gene expression is predominantly in ciliated cells, but the LV vector also reaches the alveolar space where type I and II cells and macrophages are transduced. LPC airway conditioning has enabled transduction of endogenous adult airway stem cells (Farrow et al., 2018), confirming the mechanistic basis for stem-cell-based durable *in vivo* gene expression. These studies showed the potential for life-long replenishment of therapeutic benefit via the emergence of cell lineages that were corrected *in situ* by the purposely transduced airway stem cells. LV vectors can also robustly transduce human airway basal cells *in vitro* (Farrow et al., 2018), and human air liquid interface (ALI) cultures, supporting applicability to human airways.

While much understanding can be gleaned from reporter gene studies, the gene transfer methods must also be demonstrated to effectively alter a CF airway, by delivering the *CFTR* gene. Previous studies have shown that LV vectors can partially correct CFTR function in CF mouse nasal airways (the only site of measurable CF airway functional pathophysiology in mice) (Limberis et al., 2002; Stocker et al., 2009; Cmielewski et al., 2014a). Improvement was significant and extended for at least 12 months after the single *CFTR* vector dose, validating the strength and persistence of benefit possible with a LV vector.

The efficacy of LPC enhancement is also not limited to LV vectors. Helper adenoviral vector studies in pigs have showed strong and extensively distributed reporter gene transfer, with no systemic toxicity or infection reported from use of the combined LPC-hAdv vector aerosol (Cao et al., 2013). However, the key question from these studies remains: can a *CFTR* gene-addition process improve the course of disease in a CF lung?

Suitability of CF Animal Models for CFTR Gene Therapy Research

A range of CF animal models have been developed, with the mouse, pig, ferret and rat the most well characterized (Lavelle et al., 2016; McCarron et al., 2018). Small animal models are ideal for developing gene-addition techniques for CF for cost and handling reasons, and the gene vector volume required to treat the lungs is modest facilitating large well-powered studies. Although many CF mouse models have been developed, the *CFTR* Null mouse does not exhibit lung disease, and the β-ENaC mouse is not suitable for gene-addition therapy development because its lungs contain functional CFTR. The pig (Meyerholz, 2016) and ferret (Sun et al., 2014) models recapitulate human lung disease but exhibit severe gut disease (Meyerholz et al., 2010; Sun et al., 2010) that necessitates intensive husbandry requirements with associated high costs. Lung gene vector dosing requirements prohibit extensive testing in the pig.

Our group recently used CRISPR/Cas9 gene-editing to create colonies of Phe508del (Class II) and 512X CF (Class I) rats (McIntyre et al., 2017). Hallmark CF pathophysiology is consistent with the United States CF rat (Tuggle et al., 2014; Birket et al., 2018), with frequent severe gut obstruction and increased gut motility, as well as poorly developed vas deferens, seminal vesicles & epididymis. As in humans, the phenotype of these two mutations is different. Both have higher mortality than wild-type, with the 512X rats having higher pre- and post-weaning mortality than the Phe508del. Both have a lower average body weight compared to wild-type, although this effect appears to be more pronounced in male animals.

Nasal-airway potential difference (PD) measurements have confirmed altered CFTR airway function in both models. The 512X rats have no response, or a weak response to a chloride-free environment that is consistent with classically defective CFTR function. The Phe508del animals are also significantly different to heterozygote/normal rats, but with an intermediate response consistent with the presence of residual CFTR function seen in the human Phe508del mutation. Since our treatment focus is CF *lung* disease, we are investigating methods of performing PD measurements in the trachea and/or deeper airways, as is possible in humans (Davies et al., 2005), as a method for tracking functional CFTR changes in the CF rat lung.

Improvements in Airway and Lung Function Must Be Measured

Measuring the effects of airway gene therapies on lung and airway health has been a major challenge for all CF research groups, with CFTR channel function typically assessed using transepithelial potential difference measurements, along with Ussing chamber and halide assays. However, these techniques all have limitations, particularly for *in vivo* use in animal models. Fortunately, Phase Contrast X-ray Imaging (PCXI) based approaches have recently been demonstrated to be able to directly measure airway and lung health *in vivo*.

Phase Contrast X-ray Imaging utilizes X-ray refraction to achieve high spatial and temporal resolution as well as excellent airway and lung soft tissue contrast, and has the potential to dramatically reduce radiation doses (Kitchen et al., 2017). Using PCXI it is now possible to measure changes in airway surface liquid (ASL) depth following treatment (Siu et al., 2008; Morgan et al., 2013, 2016), as well as the clearance of micron-sized marker particles on the airway surface by mucociliary clearance (MCC) (Donnelley et al., 2012a,b, 2014, 2017; Gradl et al., 2018). ASL depth and MCC are the two key airway physiological parameters that must show rapid beneficial changes if a CF airway gene therapy is to be considered effective.

Phase Contrast X-ray Imaging -based analyses have now also been developed to provide quantitative measurement of lung function, taking the focus away from the requirement to use qualitative assessment of structure (i.e., via CT imaging) to *infer* function. Structural changes that occur with CF lung disease alter the flow of air in the lung and the regional patterns of lung motion, whether by obstruction that increases airway resistance, or by changes to the lung parenchyma that alter the mechanical properties of the tissue. It follows that abnormal motion of lung regions during respiration is an accurate indicator of disease (Fouras et al., 2012). Lung motion can be tracked by combining velocimetry techniques with the enhanced airway contrast offered by PCXI. This technique can perform pinpoint spirometry – a method referred to as 4DxV – and was developed and validated in β-ENaC mice at the SPring-8 synchrotron (Stahr et al., 2016). It allows local ventilation to be quantitatively measured at every point in the lung, enabling *local* treatment effects to be assessed. The 4DxV imaging methods can now be used in rat lungs at the Imaging and Medical Beamline at the Australian Synchrotron (Murrie et al., 2015), with commercially available turnkey systems now available (4Dx, Melbourne, VIC, Australia).

With long-lived and easily maintained CF rats now available, and the ability to measure changes in airway surface and lung health in only those lung regions that are treated, it is now possible to quantify the short- and long-term effects of contained, local-region dosing of a gene therapy to the lungs of a living CF animal model, without the need for post-mortem lung analyses.

Scalable Vector Production and Precision Delivery Capabilities Are Essential

Translating this gene-addition technology to humans requires larger gene vector volumes, but there are a range of challenges associated with upscaling LV vector production (McCarron et al., 2016). Common methods currently employ adherent cell cultures using cell-factories (Rout-Pitt et al., 2018), however, packed-bed bioreactor approaches can now also yield unconcentrated titres of 10^5–10^6 TU/ml. Once concentrated and purified for *in vivo* use titres in our laboratory are 10^8–10^9 TU/ml. Commercially available, suspension-based production methods such as the LV-MAX™ LV Production System (Gibco) have the potential for scalable production in stirred-tank bioreactors and are animal component-free, important characteristics for establishing LV manufacturing methods for future clinical use (McCarron et al., 2016). In our laboratory unconcentrated LV-MAX™ titres are already greater than 10^6 TU/ml and are easily scalable to human-sized lung doses by using larger bioreactor vessels. However, until stable packaging cell lines can be used, a major barrier to transient transfection systems remains the production of large quantities of plasmid DNA.

To translate successful preclinical developments into humans it is important to determine whether viral gene therapy techniques developed in small animal models are readily translatable to a human-sized lung. Although intended to be beneficial, these LV gene-addition therapies are deliberately designed to induce permanent genetic alterations. It is the authors' opinion that the first LV CF gene vector trials in humans will be accompanied by a heightened level of caution compared to new transient-effect daily pharmaceuticals. We propose that the first lung gene-addition trials with viral vectors in humans must be performed bronchoscopically, to enable precise gene delivery and to examine the gene vector effects in a specific limited region of the lung. This method offers an "exit strategy" – by wedge or lobe excision – should a treatment unexpectedly produce unresolvable or unacceptable serious adverse events. However, until recently this strategy could not be tested in small animal models because bronchoscopic delivery was not possible.

The ability to accurately and precisely deliver fluid doses to a small region of the lung has been almost impossible in small animals such as rats and mice due to the small size of their airways. However, the first reliable bronchoscopic dosing technique that can target pre-selected regions of the rat lung was recently reported (McIntyre et al., 2018), and is based on a 1.1 mm diameter rigid sialendoscope normally used for human salivary duct procedures. This miniature bronchoscope system has light, video vision, two access channels, and can be used to dose fluid into at least the fourth-generation branches of the rat airway in ∼200 gram rats. That study showed that rats lungs are amenable to reporter gene delivery and expression at a similar level and with similar cell type distributions across nasal and lung regions as found in mouse studies. *LacZ* expression was present in ciliated and goblet / secretory cells, the two most relevant cell populations on the conducting-airway surface epithelium. However, unlike nasal or tracheal delivery, bronchoscopic delivery limited transduction to only the treated lung lobe/region.

Together, bronchoscopic dosing and bioreactor production methods allow the developments in small animal models to be easily scaled to the levels required for translational to human clinical trials.

THE MAIN CHALLENGES FOR THE FIELD

An effective gene-addition therapy for CF lung disease requires accurate compound delivery to the target location, high levels of transduction, and effective CFTR protein expression in the cells relevant to CF disease. Reliable and relevant measurements of the benefit to the treated region, as well as the whole lung, are essential. However, several challenges remain before genetic therapies for CF lung disease can be translated to the clinic.

Gene Therapy Efficacy Should Be Validated in a CF Lung

The ability of a viral gene-addition vector to adequately modify CF lung disease health and progression has never been demonstrated, but it is the authors' opinion that this is essential prior to human clinical trials. Certainly, the effectiveness of *CFTR* gene-addition *per se* has been validated clinically by the UK CF Gene Therapy Consortium (Alton et al., 2015), and that group has undertaken extensive preparation for clinical trials of lung *CFTR* gene-addition using a SIV LV vector. Although the consortium

has not yet reported *in vivo* CFTR functional improvements in a CF animal model, they have approval for human trials (Alton et al., 2017). Similarly, the Iowa United States CF gene therapy research group have used uninfected neonatal CF pigs to examine LV-*CFTR* gene transfer efficacy, but the absence of suitable outcome measures (as described in the previous section) meant that the CF pigs were necessarily humanely killed 2 weeks later for lung-tissue analysis (Cooney et al., 2016). The ability to perform localized delivery of LV-*CFTR* into a diseased CF rat lung, combined with non-invasive PCXI based assessments is expected to enable the first-ever *in vivo* long-duration testing of LV gene addition therapy for CF lung disease. Future long-term studies should also assess survival differences produced by *CFTR* gene-addition, to estimate the likely human therapeutic benefit.

The Safety Profile of LPC and LV Must Be Demonstrated in a CF Lung

The power of gene therapy was illustrated following clinical trials using first generation γ-retroviral vectors, which successfully treated genetic disorders such as SCID-X1. However, they resulted in serious adverse events such as acute lymphoblastic leukemia due to viral promoters that upregulated proto-oncogenes lying close to the integration site (Hacein-Bey-Abina et al., 2008; Howe et al., 2008). LV vectors were subsequently redesigned to improve their safety profile. Those modifications included reducing the amount of native HIV genome in the vector and modifying the viral long terminal repeat (LTR) to remove endogenous viral promoter sequences to render the vectors replication deficient (Yu et al., 1986; Dull et al., 1998). Finally, the viral components required for replication were separated onto separate plasmids and delivered *in trans*. Nonetheless, comprehensive integration site analyses remain essential for vectors destined for clinical trials.

The safety of airway conditioning compounds must also be established. LPC action is transient, dose-controllable, and well-tolerated in *in vivo* animal studies. Despite demonstrating that LPC enables effective gene-addition and is well-tolerated in multiple animal species, it is not known whether the desired transient tight-junction permeabilization in a CF airway, where infective bacteria are normally present, may bring unacceptable risks. Potentially allowing those pathogens access to normally protected sub-surface regions, raises the possibility of additional local or even systemic infections to occur. The theoretical dangers of altering airway barrier function, even transiently, in the infective milieu of a CF lung are obvious. However, LPC has a convincing preclinical history of safe use, so the potential of airway conditioning and gene-addition therapy in humans with CF must not simply be dismissed as too risky in the absence of any evidence. The rule *primum nil nocere,* first do no harm (Smith, 2005), which drives much of the bioethics of medicine, it is not an absolute. For example, acceptance of the harm, pain, and risk from myeloablative conditioning is accepted as an essential conditioning procedure for bone marrow transplantation. These important questions related to the acceptability of airway / lung conditioning are yet to be answered.

Methods to Increase Gene Expression Levels Must Be Developed

The ability to achieve reporter or *CFTR* gene transfer using LV vectors has previously been established, with *in situ* transduction of resident airway basal cells that replenish the airway with transgene-expressing daughter cells the likely basis for this long-term expression. However, in most mice gene expression eventually wanes, and in some reduces to zero after only 6-months (Cmielewski et al., 2014a). This data, and the inherent uncertainties of translating *in vivo* mouse data to a human disease, suggests that it may be beneficial or necessary to produce higher levels of gene expression through initial multi-dosing, or by repeat-dosing if gene expression wanes. However, a host immune response directed against the gene transfer agent may block initial gene expression and/or prevent expression arising from repeated doses. Few studies have examined strategies for effectively re-administering LV vectors to the airways.

Feline immunodeficiency virus (FIV) and simian immunodeficiency virus (SIV) LV vectors have been successfully re-administered to the airways without loss of effectiveness, suggesting LV vectors may evade adaptive immune responses (Sinn et al., 2008; Griesenbach et al., 2012). These vectors were pseudotyped with the GP64 and F/HN envelopes, respectively, which both target receptors located on the apical surface of the airway, so they are unlikely to be able to reach or transduce the basal stem cells located on the basolateral airway surface. The SIV study showed repeat dosing was feasible but did not increase transgene expression. These studies also highlighted that expression levels are highly dependent on the dose timing and the choice of transgene delivered at each dose. The knowledge that patients will continue to benefit from additional doses will revolutionize CF airway gene therapy and pathways to clinical trials.

Dosing Methods Need to Be Optimized in Human-Sized Lungs

The ability to dose human sized lungs must also be established early. Human lungs are substantially larger than in small animal models. They are also differently developed and branched, and cell type distributions and proportions along the conducting airways where CF pathophysiology begins are different. Confirming the feasibility and effectiveness of LV gene-addition techniques in human-sized lungs, such as in pigs or sheep, along with the necessary vector production requirements, is of vital importance.

CONCLUSION

While expensive daily drug-based options for treating the downstream effects of the *CFTR* gene defect are emerging, gene-addition therapies are the only approach with the immediate potential to prevent or halt CF lung disease. Although treatment

for pancreatic insufficiency, CF related diabetes and other CF associated pathologies will still be required, by dealing with the fundamental gene defect at its source within airway cells, airway gene therapy can be expected to transform the treatment options for CF lung disease.

A range of proven LV gene vectors and adequate novel vector production techniques have been developed. LPC and LV gene-addition techniques have already been validated in multiple animal models. The production of new animal models such as the CF rat will enable significant advances in CF translational capability, with bronchoscopic delivery techniques able to dose individual lobes of the rat lung. Innovative new non-invasive PCXI-based measurements of airway and lung function can now be used to complement standard measurement techniques.

It is the authors' opinions that key questions that must be answered before airway gene therapy can be translated to the clinic involve clearly demonstrating long-term efficacy and the safety of LPC and LV delivery in a CF lung, the ability to re-dose to boost *CFTR* gene expression levels, and the ability to translate these techniques into human-sized lungs. Together the new capabilities described here will allow these key questions that will enable the translation of airway gene therapy to humans to be answered.

AUTHOR CONTRIBUTIONS

DP and MD contributed to the conception and design of the work and wrote the manuscript.

FUNDING

MD was supported by a Robinson Research Institute Career Development Fellowship. Studies supported by NHMRC Project Grants GNT1079712, GNT1098127, and GNT1160011, grants from the United States CF Foundation, a Fay Fuller Foundation Discovery Grant, CF South Australia, the Women's and Children's Hospital Foundation, and the Cure 4 CF Foundation.

ACKNOWLEDGMENTS

X-ray imaging research was undertaken on the Imaging and Medical Beamline at the Australian Synchrotron, beamline 20XU at the SPring-8 Synchrotron, and at the Munich Compact Light Source. The authors thank Kaye Morgan, Patricia Cmielewski, Nigel Farrow, Juliette Delhove, Alexandra McCarron, Nathan Rout-Pitt and Chantelle McIntyre for their assistance with the studies and their helpful contributions to this paper.

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Conflict of Interest Statement: DP is a current shareholder of 4Dx Ltd. He has no financial benefit relationships with 4Dx Ltd.

The handling Editor declared a past collaboration with one of the authors DP.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 08 October 2018
doi: 10.3389/fphar.2018.01113

Emerging microRNA Therapeutic Approaches for Cystic Fibrosis

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Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator *(CFTR)* gene and remains the most common life-shortening diseases affecting the exocrine organs. The absence of this channel results in an imbalance of ion concentrations across the cell membrane and results in more abnormal secretion and mucus plugging in the gastrointestinal tract and in the lungs of CF patients. The direct introduction of fully functional CFTR by gene therapy has long been pursued as a therapeutical option to restore CFTR function independent of the specific CFTR mutation, but the different clinical trials failed to propose persuasive evidence of this strategy. The last ten years has led to the development of new pharmacotherapies which can activate CFTR function in a mutation-specific manner. Although approximately 2,000 different disease-associated mutations have been identified, a single codon deletion, F508del, is by far the most common and is present on at least one allele in approximately 70% of the patients in CF populations. This strategy is limited by chemistry, the knowledge on CFTR and the heterogenicity of the patients. New research efforts in CF aim to develop other therapeutical approaches to combine different strategies. Targeting RNA appears as a new and an important opportunity to modulate dysregulated biological processes. Abnormal miRNA activity has been linked to numerous diseases, and over the last decade, the critical role of miRNA in regulating biological processes has fostered interest in how miRNA binds to and interacts explicitly with the target protein. Herein, this review describes the different strategies to identify dysregulated miRNA opens up a new concept and new opportunities to correct CFTR deficiency. This review describes therapeutic applications of antisense techniques currently under investigation in CF.

Keywords: cystic fibrosis, miRNA, therapy, oligonucleotides, CFTR (Cystic Fibrosis Transmembrane conductance Regulator), ANO 1 channel

OPEN ACCESS

Edited by:
Nicoletta Pedemonte, Istituto Giannina Gaslini (IRCCS), Italy

Reviewed by:
Oscar Moran, Istituto di Biofisica (IBF), Italy
John Philip Winpenny, University of East Anglia, United Kingdom

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *13 July 2018*
Accepted: *10 September 2018*
Published: *08 October 2018*

Citation:
Bardin P, Sonneville F, Corvol H and Tabary O (2018) Emerging microRNA Therapeutic Approaches for Cystic Fibrosis. Front. Pharmacol. 9:1113. doi: 10.3389/fphar.2018.01113

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal genetic disease in the Caucasian population. Since the gene responsible for the disease was identified in 1989 (Riordan et al., 1989), numerous discoveries have led to an improvement in patients' longevity and quality of life. It is a complex disease, and many advances are still needed to understand the mechanisms of the pathology, to cure patients.

Cystic fibrosis affects about 70,000 people worldwide (in the homozygous or heterozygous composite state). It is induced by the recessive mutation of the gene that encodes a chloride channel called CFTR for Cystic Fibrosis Transmembrane conductance Regulator (Riordan et al., 1989).

The *CFTR* gene is located on the long arm of chromosome 7 (7q31) and encoded for the CFTR protein composed of 1,480 amino acids and belongs to the family of ABC transporters (ATP-binding cassette transporters) whose central role is the active transport of various substrates, such as amino acids, peptides, proteins, and ions through the plasma membrane. The CFTR protein participates directly in the regulation of the transport of chloride ions at the cell membrane, as well as in the transport of bicarbonate ions, which are involved in the regulation of the pH of the airway surface liquid covering the pulmonary epithelium (Jun et al., 2016).

From a pathophysiological point of view, mutations cause alterations in the tissues or organs integrating the exocrine glands, such as the skin, pancreas, lungs, intestines, and reproductive systems. Currently, the leading cause of morbidity and mortality is related to damage to the lungs. They are characterized by an accumulation of thick, viscous mucus, recurrent infections, and chronic inflammation, causing an impairment of mucociliary clearance. Inflammation could appear before any infection; the secondary infection would only exacerbate the uncontrollable inflammation (Jacquot et al., 2008). In the long term, these various symptoms lead to the deterioration of the pulmonary epithelium with, as a result, repair mechanisms being activated at the root of the appearance of areas of epithelial rearrangement. Alongside the peeling and remodeling of the epithelium, subepithelial fibrosis develops, leading to impaired respiratory function.

To date, more than 2,000 mutations of the CFTR gene have been identified, the most common (found in nearly 70% of patients) being the F508del mutation of the protein (deletion of a phenylalanine at position 508). These mutations are divided into six classes according to their degree of severity in CF disease and the mechanism that disrupts CFTR function and induced a tremendous phenotypic variability of CF patients (Corvol et al., 2016). CFTR mutation classes were created according to their consequences on CFTR function mutations interfering with protein synthesis (class I), mutations affecting protein maturation (class II), mutations altering channel regulation (class III), mutations affecting chloride conductance (class IV), mutations reducing the level of normally functioning CFTR at the apical membrane (class V), and mutations decreasing the stability of CFTR present at the plasma membrane (class VI) (Fanen et al., 2014). The high number of these mutations and their effects on the functionality of the protein make an unique therapeutic approach complicated[1].

CYSTIC FIBROSIS AND THERAPEUTIC APPROACHES

At the moment, there is no cure for CF. The increase in life expectancy observed in recent years is in fact mainly due to better management of the disease with symptomatic treatments (physical therapy, mucolytic agents, anti-inflammatories, and antibiotics). New pharmacological therapeutic approaches (Vertex Pharmaceuticals) have been proposed to patients for less than a decade. They help to restore the defective protein to the cell membrane (correctors) and/or to potentiate its activity (potentiators). The first small molecule that has demonstrated efficacy is ivacaftor. Preclinical and different clinical studies have shown that ivacaftor corrects CFTR-mediated chloride transport in most class III mutations, class IV mutations, and some other residual function mutations (Bessonova et al., 2018). A subsequent series of clinical trials have shown that ivacaftor has a high level of efficacy in class III mutations, particularly in patients with G551D (Gly551Asp) mutation even in very young children (Rosenfeld et al., 2018). In all clinical studies, compared with placebo, treatment with ivacaftor improved lung function (FEV1) by around 10%, reduced sweat chloride concentration by around 60 mmol/L, improved quality of life, and reduced the frequency of pulmonary exacerbations. The second small molecule strategy has been to target patients who are homozygous for the F508del mutation with a combination of a corrector drug to restore trafficking of CFTR and a potentiator to make it functional even if the activity of the channel remained low. The different clinical trials have demonstrated an improvement of 2.8 to 3.3% in FEV1 read out, that reflect a small effect for a high cost that induces a debate in the different national institute for health (Gulland, 2016). Due to the heterogeneity of the mutations that affect the *CFTR* gene from patient to patient (Veit et al., 2016), these treatments are not applicable to everyone and therefore have a highly variable benefit, depending on the class mutation (Gulland, 2016). Unfortunately, at present, there is no treatment that would treat all patients and in particular patients with a class I mutation. These drug classes, however, are a frame of reference in the field, and new molecules based on the same strategy are still under development. However, other strategies are proposed at the same time. They improve mucociliary clearance, decrease inflammation or fight infection[2]. One of the new therapeutic strategies proposed in the literature is based on the stimulation of an alternative pathway to that involving CFTR for the transfer of chloride ions, such as that induced by the chloride channel ANO1 (Anoctamin-1, also called TMEM16A [transmembrane protein 16A]) (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008; Sonneville et al., 2017), which could also participate in the activation of CFTR in the cell membrane (Benedetto et al., 2017). Another proposed emerging approach is based on modulating the expression of the microRNAs that regulate target RNAs such as CFTR.

miRNAs: CFTR AND OTHER ASSOCIATED CHANNEL PROTEINS

MicroRNAs (miRNAs) are defined as small, non-coding, 21–23 nucleotide endogenous RNAs able of suppressing the expression of their target genes. miRNAs can be classified on their genomic location and gene structure, and in the miRBase database, more than 28,000 miRNAs are referenced (Kozomara and Griffiths-Jones, 2014). Almost half of the known

[1] www.genet.sickkids.on.ca/cftr

[2] https://www.cff.org/Trials/Pipeline

miRNA genes are located in intergenic regions. Intronic miRNA, are located in the introns of annotated genes, including both coding and non-coding genes. Intergenic and intronic miRNAs gene may exist as a single gene or a cluster of genes under the control of their own promoters. Mixed miRNA are found overlapping across an exon and an intron of non-coding genes (Wang and Cai, 2017; **Figure 1**).

They participate in the regulation of many cellular processes, and the impairment of their expression may be associated with some pathologies (Foster et al., 2013). miRNAs are produced by transcription and maturation of their mRNA, ultimately with the release of a mature miRNA that will be incorporated into a ribonucleoprotein complex called miRISC (miRNA-induced silencing complex; **Figure 2**). Traditionally, the regulation exerted by miRNAs on their target gene is specific, through the pairing of the mature miRNA with the 3′-UTR sequence of the target mRNA, called miRNA response element (MRE) (**Figure 3**). The activity of the miRNAs depends on a long sequence of only 6 nucleotides (the "seed" sequence). An MRE motif can, therefore, be found on different mRNAs, and a mRNA can contain a repetition of the same MRE motif or other motifs. miRNAs play a dominant role in the complex multiple-gene expression regulation networks (Sonneville et al., 2015). When talking about a therapeutic approach, it is essential to take into account the emergence of isomiR, the 3′ and 5′ sequence variants of canonical miRNAs, as well as the wrong annotations that can be found in the database. The data collected by sequencing also need to be systematically verified, as described previously for isomiR-34/449, involved in ciliogenesis (Mercey et al., 2017). Today, it is estimated that more than 60% of the genes encoding a protein are regulated by miRNAs (Friedman et al., 2009). Modulating the activity of a single miRNA as a result of the inhibition of its function or overexpression can thereby have a significant biological impact.

Many studies have focused on the potentially regulatory miRNAs of *CFTR* expression in CF. Gillen et al. (2011) were the first to identify miRNAs related to CFTR activity, several of them directly regulated its expression by binding to the 3′-UTR end of its mRNA. The regulation of *CFTR* mRNA expression differs according to the cell type being studied: it is, in fact, tissue-specific, and time-dependent, which makes the development of miRNA-targeting therapies meant to restore the function of CFTR complicated (**Table 1**). Thus, Viart et al. (2015) showed that *CFTR* regulation was different before and after birth, confirming previous results CFTR channel is strongly expressed but it falls after birth at the pulmonary level (Marcorelles et al., 2007). In fact, miR-101 negatively regulates *CFTR* expression in adult lung cell lines, but it has no effect on fetal bronchial epithelial cells. It, therefore, seems to play a crucial role, which evolves over time (Viart et al., 2015). The combination of this miRNA with miR-494 makes it possible to remarkably suppress the expression of CFTR in human renal embryos. *In silico* analyzes have shown that these two miRNAs can target genes concomitantly and thus influence the severity of CF patients' pathology (Megiorni et al., 2011). The direct link between miRNA and *CFTR* has subsequently been confirmed by other teams who have shown that miR-145, miR-223, and miR-494, alone or together synergistically, directly regulate *CFTR* expression. This is also the case for miR-509-3p and miR-494, which induce stronger repression of its expression (Oglesby et al., 2013; Ramachandran et al., 2013). In addition to being able to interact directly with *CFTR*, some miRNAs can also act on intermediate actors of its biosynthesis, such as the transcription factor *SIN3A* (SIN3 transcription regulator family member A), repressed by miR-138 (Ramachandran et al., 2012; **Table 1**). They showed that the overexpression of miR-138 contributes to increase the expression and the number of CFTR channels on the cell surface of secretory epithelia but also the activity of the channel, in healthy subjects and CF patient with F508del mutations even if the mutated channel is less active than the wild-type. All these results were mainly performed on mutated F508del CFTR protein and should be also performed on other class mutations. The implication of miRNAs in CFTR expression is also controlled by the highly variable, and compelling information on phenotypic variability and lack of genotype-phenotype correlation among patients with the same mutation in the CFTR gene. Thus, three polymorphisms have been identified in the miR-99b/let-7e/miR-125a cluster that modulates the expression of these miRNAs and may be associated with patient phenotypes (Endale Ahanda et al., 2015). In addition, polymorphisms present in the 3′-UTR region of CFTR mediate the binding of miR-509-3p and cause a decrease in CFTR expression (Amato et al., 2013).

Expression of miRNAs can also regulate the expression of other channels or others proteins whose activity is modulated by CFTR (**Table 2**), such as the cotransporter NKCC1 ($Na^+-K^+-2Cl^-$-cotransport protein) (Gillen et al., 2011) or the sodium channel ENaC (epithelial sodium channel) by miR-183, which is able to target the three subunit of ENaC (Kim et al., 2017). Recently, an increase in the expression of miR-9 was at the root of a decrease in expression of the chloride channel, ANO1, which modulated mucus hydration and chloride efflux activity (Sonneville et al., 2017).

miRNAs: OBSTRUCTION, INFECTION, AND INFLAMMATION

In recent years, studies showing the involvement of miRNAs in the regulation of other targets involved in various aspects of the pathology, such as inflammation, airway obstruction or infection, have opened up new ways of investigation. In fact, miRNAs are able to modulate the expression of genes involved in airway obstruction and the production of mucus, such as miR-146a modulating *MUC5AC*, which encodes one of the main pulmonary mucins implicated in the obstruction of the airway. The data highlight a negative feedback role for miR-146a in the control of MUC5AC production from airway epithelial cells stimulated by neutrophil elastase, which may be associated with the inactivation of MAP kinase and NF-κB signaling, the primary pathways implicated in CF airway inflammation (Zhong et al., 2011). The expression of miR-101 in the airways is therefore increased in the lungs of patients with CF, with an airway obstruction characterized by low levels of

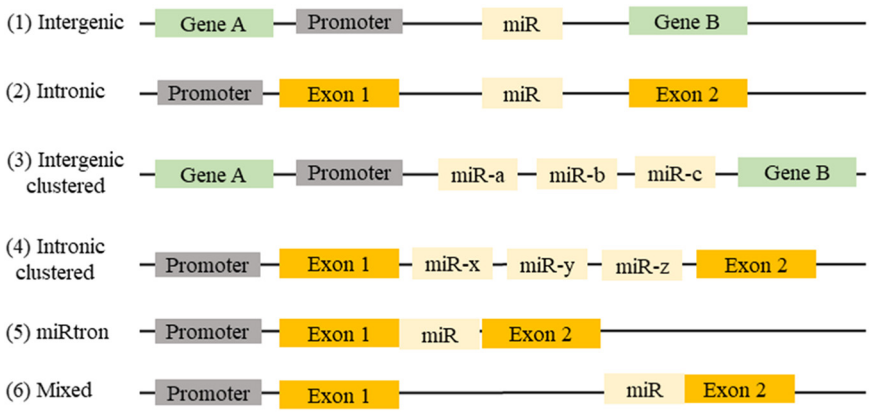

FIGURE 1 | Location of miRNAs gene in the genome: (1) miRNAs can be found between two genes (intergenic) or (2) in a gene (intronic). (3) They may be present in a single miRNA gene or (4) in a cluster of miRNA genes. (5) Sometimes intronic miRNAs may exist between two exons (miRtron) or (6) overlapping an exon and an intron of non-coding genes (mixed).

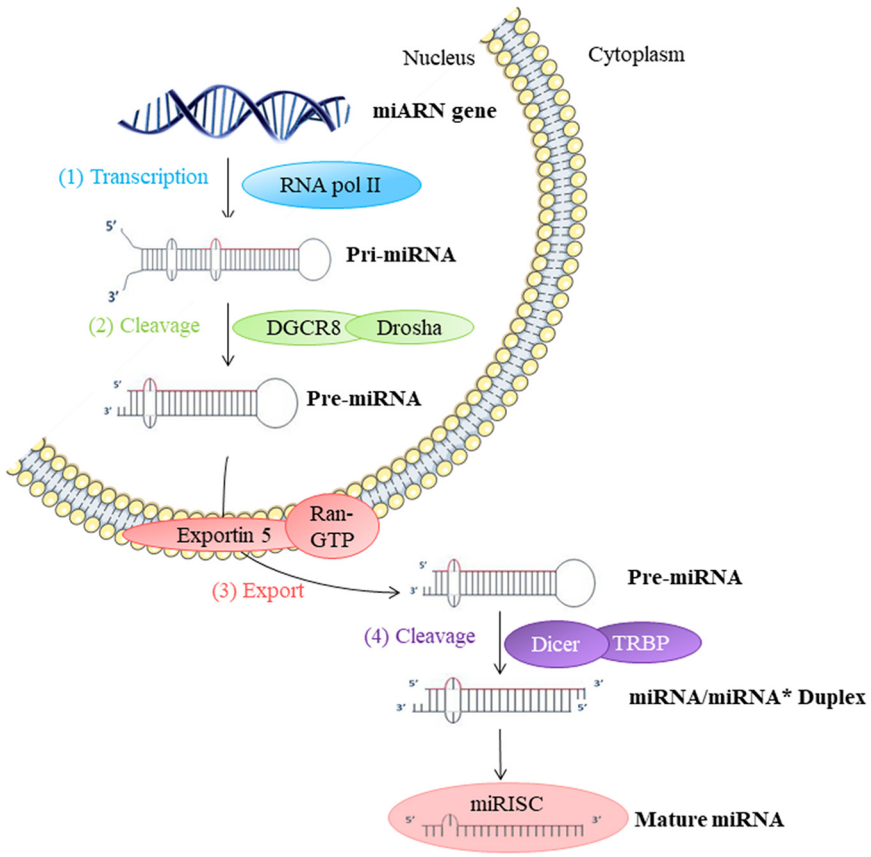

FIGURE 2 | Biosynthesis of miRNAs: (1) The biosynthesis begins in the nucleus by transcription of miRNA genes by RNA polymerase II (Pol II). (2) Long transcripts, pri-miRNA, are cleaved by Drosha and DGCR8 protein creating pre-miRNA with hairpin structure. (3) Exportin 5 transfers pre-miRNA into the cytoplasm. (4) Pre-miRNA is cleaved by Dicer into miRNA duplex in mature single-strand miRNA form. This miRNA mature form is incorporated into a miRISC ribonucleoprotein complex. This complex can then act directly on the mRNA in the cell where it is synthesized or out of the cell.

CFTR (Hassan et al., 2012) expression. miRNA expression is also influenced by external factors, like pathogenic bacteria, such as *Haemophilus influenza, Staphylococcus aureus,* or *Pseudomonas aeruginosa* (Oglesby et al., 2013; Ramachandran et al., 2013). The presence of pathogens in the light of CF airway surface epithelium contributes to modulate miR expression and consequently CFTR

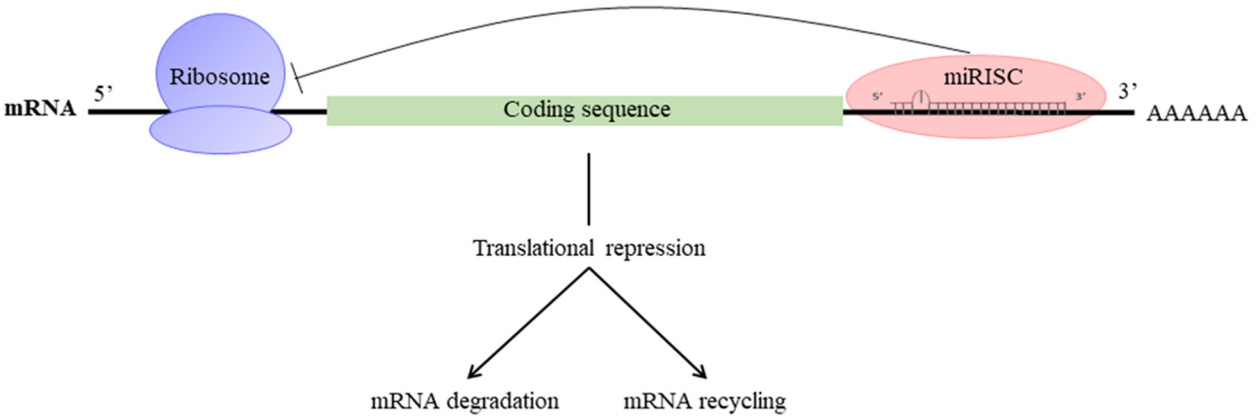

FIGURE 3 | Mechanism of action of miRNAs: miRISC complex attenuates mRNA translation and leads to the destabilization of mRNA by deadenylation and/or inhibition of translation.

TABLE 1 | Table showing deregulated miRNAs targeting directly or indirectly CFTR in the context of cystic fibrosis.

Target	miRNA	Models	Reference	Year
CFTR mRNA	miR-494, miR-384, miR-376b, miR-1246, miR-145, miR-331-3p et miR-939	Caco-2	Gillen et al., 2011	2011
	miR-600, miR-494, miR-384, miR-1290, miR-1246, miR-145, miR-1827, miR-331-3p et miR-939	PANC-1		
	miR-600, miR-494, miR-607 et miR-384	16HBE14o-		
	miR-101, miR-494	HEK293	Megiorni et al., 2011	
	miR-101, miR-144	16HBE14o-	Hassan et al., 2012	2012
	miR-145, miR-223, miR-494	Bronchial brushing, 16HBE14o-, CFBE41o-, HEK293	Oglesby et al., 2013	2013
	miR-509-3p, miR-494	Differentiated primary cell cultures, Calu-3	Ramachandran et al., 2013	
	miR-145, miR-384, miR-101, miR-600	A549	Viart et al., 2015	2015
	miR-505, miR-943, miR-377, miR-384, miR-101, miR-600	Beas-2B		
	miR-600	HBEpiC		
	groupe miR-17-92	Human macrophages	Tazi et al., 2016	2016
	miR-145-5p	Calu-3	Fabbri et al., 2017	2017
	miR-200b	Calu-3, 16HBE14o-	Bartoszewska et al., 2017	
	miR-145	CF and non-CF differentiated primary cell cultures	Lutful Kabir et al., 2017	
CFTR mRNA through SIN3A mRNA	miR-138	Differentiated primary cell cultures, Calu-3, HEK293, HeLa	Ramachandran et al., 2012	2012

expression. In an infectious diseases context (e.g., in the presence of *P. aeruginosa*), miR-93 expression, which is decreased, is associated with stabilization of IL-8 mRNA, thereby contributing to the maintenance of the inflammatory state. Altogether, the different works highlighted some complexes regulation and needed further investigation (Fabbri et al., 2014).

In the airways, inflammatory processes controlled by NF-κB are also partially regulated by miRNAs. The miR-199a-3p expression in the airway, which is decreased in CF context, is associated with an NF-κB hyperactivation. In fact, miR-199a-3p directly regulates IKKβ, which acts on the NF-κB signaling pathway and therefore on IL-8 secretion, the main cytokine dysregulated in the airways of CF patients (Bardin et al., 2018). Moreover, miR-509-3p, miR-494, and miR-126 directly target NF-κB and have shown that CFTR expression and function decreases when NF-κB is functional (McKiernan et al., 2013; Ramachandran et al., 2013). The two miR-93 and miR-17 regulate the production of IL-8 in bronchial epithelial cells (Fabbri et al., 2014; Oglesby et al., 2015). Bhattacharyya et al. (2011) therefore showed that miR-155 expression was

TABLE 2 | Table showing deregulated miRNAs direct/indirect targeting others targets in the context of cystic fibrosis.

miRNA	Target	Models	Reference	Year
miR-126	TOM1 Target of Myb1 Membrane Trafficking Protein	Bronchial brushing, 16HBE14o-, CFBE41o-, HEK293	Oglesby et al., 2010	2010
miR-155	SHIP1 (indirect: IL-8) SH-2 containing inositol 5′ polyphosphatase 1	IB-3, S9	Bhattacharyya et al., 2011	2011
miR-101, miR-1246, miR-494 et miR-384	SLC12A2 Solute Carrier family 12 Member 2	PANC-1	Gillen et al., 2011	
miR-449	Notch1 Notch homolog 1	Differentiated primary cell cultures	Marcet et al., 2011	
miR-146a	MUC5AC Mucin 5 AC	16HBE14o-	Zhong et al., 2011	
miR-145	SMAD3 Mothers Against Decapentaplegic homolog 3	Nasal epithelium cells, HEK293	Megiorni et al., 2013	2013
miR-31	IRF1 (indirect: Cathepsin 5) Interferon Regulatory Factor 1	Differentiated primary cell cultures	Weldon et al., 2014	2014
miR-93	IL-8 Interleukin 8	IB3-1, CuFi-1, NuLi-1	Fabbri et al., 2014	
miR-17	IL-8	Bronchial brushing, 16HBE14o-, CFBE41o-, HEK293	Oglesby et al., 2015	2015
miR-221	ATF6 Activating Transcription Factor 6			
miR-199a-5p	CAV1 Caveolin 1	Human and murine macrophages from lungs	Zhang et al., 2015	
miR-155	RPTOR Regulatory Associated Protein of mTOR complex	IB3-1, S9	Tsuchiya et al., 2016	2016
miR-199a-5p	TβRII TGF beta receptor II	Stellar hepatic cells	Chen et al., 2016	
miR-1343	TGF-β receptor	A549, 16HBE14o-, Caco-2	Stolzenburg et al., 2016	
miR-145	TGF-β Transforming growth factor beta	Primary cells from CF and non-CF patients	Lutful Kabir et al., 2017	
miR-183	SCNN1α,β,γ Sodium Channel Epithelial 1 alpha, beta, gamma subunit	CFBE41o-	Kim et al., 2017	2017
miR-9	ANO1 Anoctamin 1 (TMEM16A)	16HBE14o-, CFBE41o-	Sonneville et al., 2017	
miR-199a-3p	IKKβ Inhibitor of Kappa light polypeptide gene enhancer in beta cells, kinase beta	CFBE41o-	Bardin et al., 2018	2018

associated with CFTR activity. This miRNA can directly regulate SHIP1 (SH-2 containing inositol 5′ polyphosphatase 1) and indirectly alter the expression of IL-8 through activation of the signaling pathway involving PI3K/Akt (phosphatidylinositol-3 kinase/protein kinase B) and inhibition of the MAPKs (mitogen-activated protein kinases) (Bhattacharyya et al., 2011). The origin of the deregulation of miR-155 expression in CF cells is mediated by TTP (tristetraprolin) and KSRP (KH-type splicing regulatory protein) known to regulate miRNA biogenesis. The origin of this miRNAs dysregulation needs to be assessed. Some miRNAs may participate in the remodeling of the pulmonary epithelium and have a significant role in the development of the pathophysiology of the disease. Expression of miR-449 in the respiratory epithelium is essential to inducing direct inhibition of the Notch pathway and modulation of that involving the small GTPases, two events necessary for the production of motile cilia the beating of which allows mucus to be evacuated (Marcet et al., 2011; Chevalier et al., 2015). The remodeling of the pulmonary epithelium can also be caused by deregulation of TGF-β (transforming growth factor-beta) pathway, both in the expression of its receptors (TGF-βR1 and R2), and in signaling intermediates such as RPTOR (regulatory associated of mTOR complex 1) resulting in increased fibrosis and CTGF (connective tissue growth factor) or SMAD proteins, respectively through miR-1343, miR-155 and miR-145, thereby affecting fibrotic markers, cell migration and epithelial-mesenchymal transition (Stolzenburg et al., 2016; Tsuchiya et al., 2016; Fabbri et al., 2017). The deregulation of miR-31 in the airways of patients with CF contributes to pulmonary inflammation by increasing the activity of cathepsin 5, which causes antimicrobial proteins to deteriorate (Weldon et al., 2014). As for miR-221, it controls the transcription factor *ATF6* (activating transcription factor 6), which is involved in inflammation caused by endoplasmic reticulum stress (Oglesby et al., 2015). At last, miR-199a-5p regulates caveolin 1 (CAV1), a protein involved in the resolution of inflammation processes, targeting the PI3K/Akt/CAV1 axis (Zhang et al., 2015) and the TGF-β pathway (Lino Cardenas et al., 2013). Furthermore, it has been shown that Celecoxib, which modulates the Akt/miR-199a-5p/CAV1 axis, improves pulmonary hyper-inflammation caused by macrophages in patients.

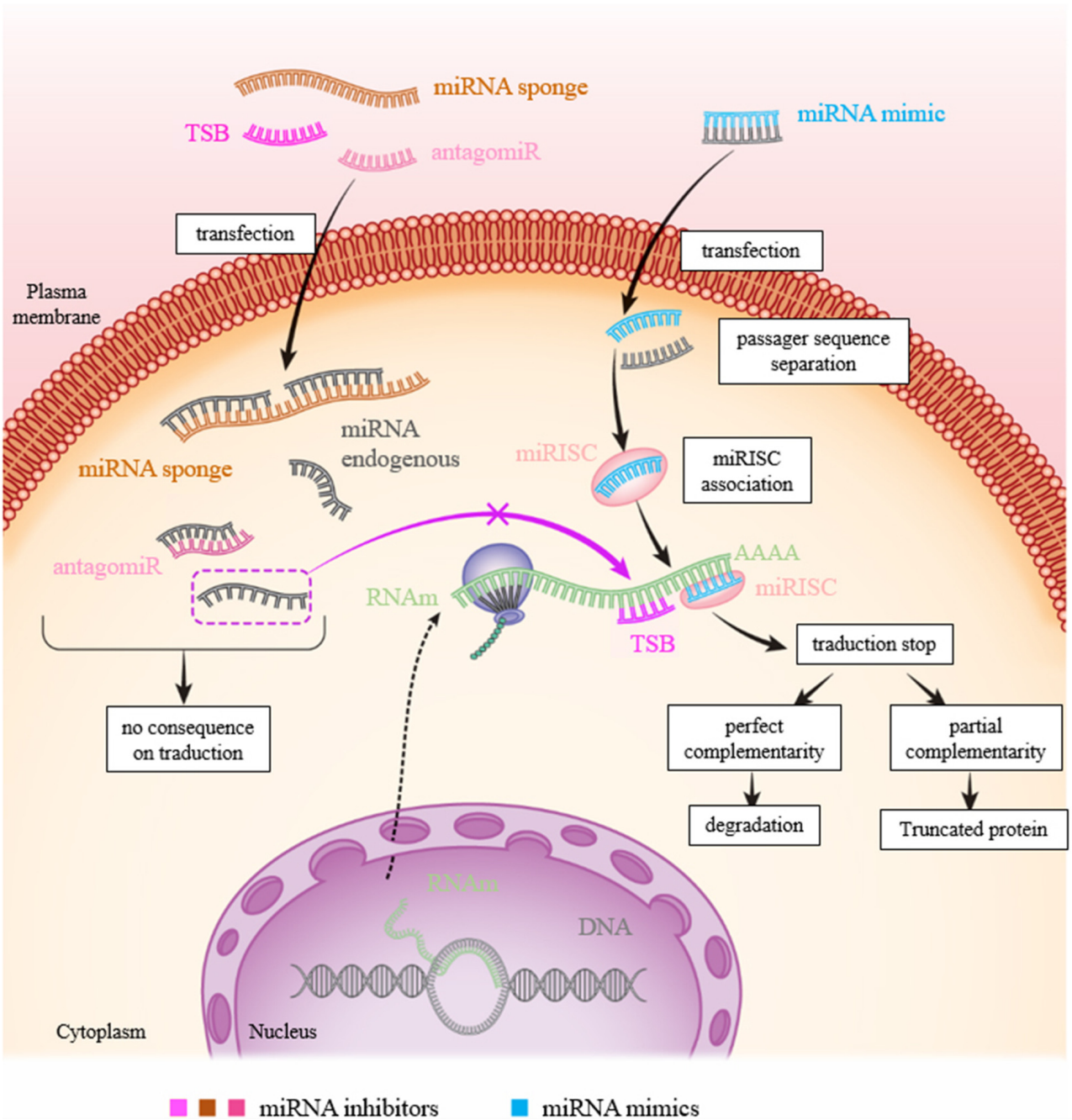

FIGURE 4 | Figure showing the various approaches to inhibit or mimic a miRNA. In physiological condition: the miRNA will bind to the 3′-UTR of its target to inhibit translation resulting to RNA degradation and/or mRNA recycling. To inhibit a miR action, there are three possibilities: transfection of (1) miRNA inhibitor (antagomiR): the miRNA inhibitor binds to the latter by complementarity and prevents its action on its target; (2) TSB: the TSB specifically binds to the 3′-UTR of the target mRNA at the miRNA binding site, the miRNA can then no longer attach to it and exert its action; and (3) mRNA sponge: the miRNA is saturated by the miRNA sponge which contains many miRNA complementary sequences, the miRNA can then no longer attach itself to its target. To increase the expression of a miR, transfection of a mimic of this miR is sufficient to inhibit translation of mRNA.

miRNAs: THERAPEUTIC STRATEGIES

MicroRNAs (miRNAs) are now considered as potential therapeutic targets. Two approaches have been followed for developing therapies based on their use: antagonists also called antisense oligonucleotides (ASOs) including inhibitors, miR sponges and target site blocker [TSB]) and miRNA mimics (**Figure 4**).

miRNA mimics are RNAs mimicking endogenous molecules and helping amplify their function. They are used to restore a loss

of function. The purpose of this so-called "miRNA replacement therapy" approach is to reintroduce miRNAs whose expression is reduced in a pathological context. The proof of concept of this miRNA replacement therapy has been demonstrated with the use of miRNA tumor suppressors that stimulate anti-oncogenic signaling pathways and lead to the eradication of tumor cells (Wiggins et al., 2010). Let-7 and miR-34 mimics are currently in the clinical development phase to target a broad spectrum of tumors. The first molecule aimed at increasing the expression of miR-34 through the use of a mimic (MRX34) entered phase I clinical trials in 2013, as part of studies on multiple solid tumors (Bouchie, 2013). The main limitation of this approach is the difficulty formulating the mimic for its delivery to the target cells. One of the existing strategies to remedy, this is their coupling to nanoparticles. In the clinical trial using MRX34, the mimic is encapsulated in a liposome delivery system to facilitate its uptake by the target cells (Bouchie, 2013). An alternative approach uses delivery through miRNA expression vectors using adenovirus infection as previously described in a cancer therapy approach (Kota and Balasubramanian, 2010).

As for miRNA antagonists, they are used to inhibit endogenous miRNAs that show a-gain-of function in a pathological context. These therapies are similar to using siRNA (small interfering RNA), even through the regulatory systems involved are different. The miRNA antagonist or antagomiR (also called antimiR) binds to the mature miRNA targets with a powerful affinity. The duplex thus formed will then deteriorate. The main disadvantage of this type of therapy is that a miRNA can regulate the expression of several genes. Inhibition of a miRNA, which is nonspecific for a particular gene, can thus result in many side effects (**Figure 4**). TSB are antisense oligonucleotides designed to bind perfectly to the region of the 3′-UTR complementary to the miRNA.

Recent developments on miRNAs have accelerated the evolution of methods and chemical modifications that make it possible to inhibit miRNAs in a stable manner, and to optimize their delivery. These are locked nucleic acids (LNA), peptide nucleic acids (PNA), phosphorothioate groups, miRNA sponges and nanoparticles (McKiernan et al., 2013; Nguyen and Chang, 2017). Phosphorothioate oligonucleotides increase the resistance to 3′-exonuclease hydrolysis and bind more promiscuously and with higher affinity to proteins than antisense oligonucleotides with phosphodiester linkages, which are present in natural DNA and RNA (Gilar et al., 1998; Crooke et al., 2017).

The base constituting the LNA is a nucleic acid analog in which the ribose ring is chemically modified with through the introduction of a methylene bridge. This chemical modification provides the molecule with great thermodynamic stability and prevents its deterioration by nucleases by reinforcing its affinity for its target (Lindow and Kauppinen, 2012).

miRNA sponges are constructs, or RNAs, which have several binding sites for a miRNA of interest, which makes it possible to limit its availability and therefore its action on associated targets (**Figure 4**). In melanoma, TYRP1 (tyrosinase-related protein 1) RNA acts like a sponge which, when associated with miR-16, limits its tumor suppressor activity. Inhibition of the targets with a binding blocker allows miR-16 to act on its target RAB17 and prevents cell proliferation (Gilot et al., 2017). This type of approach using the properties of miRNA sponges and LNA could be considered for therapeutic purposes.

The primary route of administration for oligonucleotides for systemic applications is by parenteral injection, including intravenous (IV) or subcutaneous injection (SCI). Following systemic administration, phosphorothioate-modified single-stranded ASOs rapidly transfer from the blood into tissues (minutes to hours). Following SC administration, ASOs are rapidly absorbed from the injection site into the circulation with peak plasma concentrations consistently reached within 3 to 4 h, followed by a much slower terminal elimination phase (half-life of up to several weeks). Cell uptake is predominantly mediated by endocytosis. The substitution of one non-bridging oxygen with the more hydrophobic sulfur atom, as phosphorothioate, increases both plasma stability and plasma protein binding and thus, ultimately, tissue bioavailability (Yu et al., 2013).

Pharmacokinetic properties of ASOs are similar across species and gender that facilitate drug development (Yu et al., 2007). In the case with all second-generation ASOs and for all animal species evaluated, ASOs distributes broadly into most tissues with the exception of the central nervous system after systemic administration. The major systemic tissues of distribution include liver, kidney, bone marrow, adipocytes (cell body but not lipid fraction), and lymph nodes (Geary et al., 2015). Once intracellular, ASOs exhibit long half-lives (2–4 weeks) and prolonged activity in suppressing or altering expression of their target RNA. Few data are available about the oral route of administration, because this strategy requires profound modification of the formulation for the delivery. An *in vivo* study have compared IV and oral administration of an ASO and demonstrated that the tissue distribution profile was similar following both routes of administration, with highest concentrations observed in the kidney followed by the liver, lymph nodes and spleen (Raoof et al., 2004). In the case of CF, inhaled ASOs could be considered because the lung remains the primary target and to dismissed side effects but we currently have no setback on this approach, which has only been carried out by two teams targeting chloride ion channels with modified activity in CF (Crosby et al., 2017; Sonneville et al., 2017).

The first clinical antagonist developed in clinical studies, miravirsen (SPC3649), is an antimiR-122, which targets and sequesters liver-specific miR-122. MiR-122 binds to the 5′-UTR of the hepatitis C virus (HCV) mRNA that it stabilizes, causing the virus to accumulate. Miravirsen has the advantage of being coupled to an LNA base (making it resistant to nucleases) and having phosphorothioate linkages. Miravirsen was initially tested in mice and African green monkeys (Elmen et al., 2008). Its action was then studied in chimpanzees chronically infected with HCV, and a prolonged decrease in viral replication, without any evidence of viral resistance or side effects, was observed in the treated animals. In 2011–2012, a phase IIa study was conducted in patients with chronic hepatitis C who received five SCIs per week of miravirsen for a period of 29 days. This

clinical study showed a prolonged, dose-dependent decrease in viral RNA levels and cholesterol levels and a change in the expression of about 100 genes in the liver. Viral RNA levels became undetectable in five patients, with, however, a rebound in the virus's expression in some of them (van der Ree et al., 2014). The variability of responses observed in patients suggests, however, that viral and/or host factors would influence response to treatment with miravirsen. Recently, a 5′-UTR C3U nucleotide change in HCV mRNA was demonstrated in patients who had a viral replication rebound (Ottosen et al., 2015). This mutant C3U is, in fact, insensitive to miravirsen. It would, therefore, make the virus independent of miR-122; the virus would then use an alternative mechanism to stabilize and replicate itself. The decrease in plasma levels of miR-122 in patients is still observed two months after the last injection of miravirsen, most likely due to the long half-life of miravirsen in tissues (about 30 days) (Matthes et al., 2016). Two pharmaceutical companies (*Roche* and *Regulus Therapeutics*) are currently involved in clinical trials to treat the HCV using antimiR-122.

In the case of CF, a team used TSBs which, by binding to the 3′-UTR of CFTR mRNA, prevents the binding of miR-101 and miR-145. The transfection of these molecules into the bronchial epithelial cells of patients with CF leads to an increase in CFTR expression and activity, suggesting that these TSBs could be used therapeutically (Viart et al., 2015). Other targets are being considered, like the protein ANO1 which, like CFTR, is involved in the secretion of chloride ions, pH regulation and the fluidity of airway surface liquid (Ruffin et al., 2013; Jun et al., 2016). It has shown that a TSB specifically preventing the binding of a miRNA (miR-9) on the 3′-UTR of the mRNA of the alternative chloride channel ANO1, made it possible, in *in vitro* and *in vivo* models, to increase its expression, and also its chloride activity, and cell migration, as well as mucociliary clearance independently of intracellular calcium concentration (Sonneville et al., 2017). The restoration of these parameters, deregulated at a physiological level, makes it possible to now propose the TSB ANO1 as a potential therapeutic target in the context of CF. Each patient would be able to benefit from this type of approach leading to the activation of a chloride secretion independent of CFTR. In this study, the TSB ANO1 was administered to animals intranasally, if this molecule were to be used in humans, its mode of administration would need to be determined. Inhalation seems to be the most appropriate method since these patients often have lung damage. Tissue-specific, it has less systemic exposure, reducing the risk of side effects. However, the patients' lungs have barriers, such as airways that are obstructed by mucus, that can make administration difficult. The passage of TSBs through the mucus therefore still needs to be studied.

CONCLUSION

Despite the improved care and recent progress in the identification of new therapies the predicted median survival of CF patients still remains in the low 40-years range in developed countries. New therapies and new strategies, alone or in combination with established therapies, are needed to prolong survival and improve the quality of life for all CF patients. The potential for modulating gene expression by the use of antisense oligonucleotides has become increasingly interesting in recent years, but safe delivery, long-term efficacy and side effects of prolonged treatment still need to be assessed. Advances in chemistry and molecular biology have provided the basis to develop antisense oligodeoxynucleotides and improve their selectivity, stability, and specificity of action. The antisense oligonucleotide drug platform is a really new approach for drug discovery, but basic science must be improved notably in understanding the molecular mechanisms that regulate CFTR and other chloride channels. Therapies targeting CFTR increase with ASO should be limited because channel increase will not necessarily induce increased activity especially for class III to VI mutations. The ease of delivery of modified antisense oligonucleotides seems to be linked with a lack of any major adverse side effects, making antisense oligonucleotides suitable candidates as a potential treatment for CF diseases.

AUTHOR CONTRIBUTIONS

PB, FS, and OT wrote the manuscript. PB, FS, HC, and OT participated in the design of this manuscript.

FUNDING

This research group was funded by grants from Inserm, Sorbonne University, sciences faculty (UPMC Paris 06) and the French non-profit organization "Vaincre la Mucoviscidose." PB received a Ph.D. fellowship from the "Ministère de l'Enseignement Supérieur et de la Recherche" in France. FS obtained a grant from the SATT Lutech and "Vaincre la Mucoviscidose."

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 08 February 2019
doi: 10.3389/fphar.2019.00074

Challenges Facing Airway Epithelial Cell-Based Therapy for Cystic Fibrosis

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Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene cause the life-limiting hereditary disease, cystic fibrosis (CF). Decreased or absent functional CFTR protein in airway epithelial cells leads to abnormally viscous mucus and impaired mucociliary transport, resulting in bacterial infections and inflammation causing progressive lung damage. There are more than 2000 known variants in the *CFTR* gene. A subset of CF individuals with specific *CFTR* mutations qualify for pharmacotherapies of variable efficacy. These drugs, termed CFTR modulators, address key defects in protein folding, trafficking, abundance, and function at the apical cell membrane resulting from specific *CFTR* mutations. However, some *CFTR* mutations result in little or no CFTR mRNA or protein expression for which a pharmaceutical strategy is more challenging and remote. One approach to rescue CFTR function in the airway epithelium is to replace cells that carry a mutant *CFTR* sequence with cells that express a normal copy of the gene. Cell-based therapy theoretically has the potential to serve as a one-time cure for CF lung disease regardless of the causative *CFTR* mutation. In this review, we explore major challenges and recent progress toward this ambitious goal. The ideal therapeutic cell would: (1) be autologous to avoid the complications of rejection and immune-suppression; (2) be safely modified to express functional CFTR; (3) be expandable *ex vivo* to generate sufficient cell quantities to restore CFTR function; and (4) have the capacity to engraft, proliferate and persist long-term in recipient airways without complications. Herein, we explore human bronchial epithelial cells (HBECs) and induced pluripotent stem cells (iPSCs) as candidate cell therapies for CF and explore the challenges facing their delivery to the human airway.

Keywords: cystic fibrosis, cell-based therapy, induced pluripotent stem cells, human bronchial epithelial cells, engraftment

OPEN ACCESS

Edited by:
Miquéias Lopes-Pacheco, Universidade de Lisbon, Portugal

Reviewed by:
Darcy Elizabeth Wagner, Lund University, Sweden
Daniel Weiss, University of Vermont, United States

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *26 September 2018*
Accepted: *21 January 2019*
Published: *08 February 2019*

Citation:
Berical A, Lee RE, Randell SH and Hawkins F (2019) Challenges Facing Airway Epithelial Cell-Based Therapy for Cystic Fibrosis. Front. Pharmacol. 10:74. doi: 10.3389/fphar.2019.00074

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive, multisystem, genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR)* gene resulting in deficient and/or defective CFTR protein (Cutting, 2014; Ratjen et al., 2015). CFTR is an anion channel present across a number of epithelia including the lungs, intestine, sinuses, pancreas, biliary tree, and vas deferens. The consequences of CFTR dysfunction are pronounced in the lungs where ineffective

chloride and bicarbonate ion transport results in an abnormally viscous and acidic apical surface layer (ASL). This abnormal environment is colonized by bacteria in early life and a cycle of infection and inflammation results in bronchiectasis and end-stage lung disease (Ratjen et al., 2015). Disease severity is determined to a large extent by the causative *CFTR* mutation(s). Over 2,000 variants in *CFTR* have been described, of which approximately 300 have been determined to be pathogenic (cftr2.org). These variants or combinations of variants have differing effects on the amount and function of CFTR protein. Some variants are associated with milder disease or particular organ involvement while others may be associated with very severe disease. For classification purposes, these mutations are grouped into six classes (I-VI) based on their effect on CFTR including: no protein synthesis (class I), protein misfolding (class II), dysfunctional channel gating (class III), reduced conductance (class IV), insufficient CFTR protein due to abnormal RNA splicing (class V), or increased protein turnover (class VI).

Mucus clearance techniques, antibiotics, and lung transplantation significantly improve the life expectancy of CF individuals. The recent discovery of CFTR modulators has ushered in a new era of precision medicine for CF patients with mutations that result in some residual druggable CFTR protein. For example, the major defect in patients with the class III mutation G551D is diminished channel activity at the apical surface. Ivacaftor is an FDA approved CFTR potentiator that increases CFTR activity and results in clinical improvement in patients with at least one copy of the G551D mutation (Ramsey et al., 2011). F508del is the most common CFTR mutation affecting approximately 90% of CF patients (Cutting, 2014). This mutation results in defective folding and trafficking of the CFTR protein. Corrector molecules such as lumacaftor and tezacaftor in conjunction with ivacaftor result in increased CFTR activity and some clinical improvement though not as robust as the response of gating and residual function mutations to ivacaftor therapy (Rowe et al., 2017; Taylor-Cousar et al., 2018). Recent progress with "triple combination" regimens including two correctors plus the potentiator ivacaftor indicates increased efficacy for those harboring the class II F508del mutation (Davies et al., 2018; Keating et al., 2018). Class I mutations are non-sense, frameshift or splice variants that result in premature termination of the CFTR transcript and no CFTR protein. These patients currently have no targeted therapies available and there are many barriers to a pharmacological approach to treatment.

The challenge is clear, how do we identify and develop effective therapies for all CF individuals? In theory, replacing the mutant *CFTR* sequence with the normal sequence could restore CFTR function regardless of mutation. Generally, this might be achieved by one of three approaches: (1) *in vivo* delivery of normal *CFTR* sequence, e.g., via viral vectors, (2) *in vivo* editing of the mutant *CFTR* sequence or, (3) delivery of cells carrying the normal *CFTR* sequence to replace cells carrying the mutant sequence. In this review, we focus on the approach of cell-based therapy for CF lung disease. Although there are compelling examples of effective cell-based therapies, such as hematopoietic stem cell transplantation, there are many challenges facing such an approach for lung disease. What cell type is best suited to restore CFTR function to the airways? How might cells be effectively but safely delivered to a CF patient's lungs? Here we will review the most promising cellular candidates to treat CF, human bronchial epithelial cells (HBECs) and induced pluripotent stem cells (iPSCs). Finally, we will discuss the major hurdles facing the field of CF cell-based therapeutics, including delivery and engraftment of cells into a diseased host.

OVERVIEW OF AIRWAY EPITHELIAL BIOLOGY IN CF

Airway epithelial cells, including club, goblet, multi-ciliated, basal and neuroendocrine cells occupy a vital environmental interface. Their normal physiological function is essential to maintain respiratory tract health, and the cells are intimately involved in the pathogenesis of multiple respiratory tract maladies, including the common diseases asthma and chronic obstructive pulmonary disease (Randell et al., 2009). In CF, a dehydrated and acidic ASL impairs critical innate defense mechanisms including mucociliary clearance (Randell et al., 2006; Button et al., 2012; Stoltz et al., 2015). There is current debate on the airway cell types that express *CFTR*, but it is detected in the submucosal glands (Jiang and Engelhardt, 1998) of the airway and in certain surface epithelial cells including multi-ciliated cells (low expression) and in the recently described pulmonary ionocyte (high expression) (Jiang and Engelhardt, 1998; Montoro et al., 2018; Plasschaert et al., 2018). There is debate whether cell autonomous loss of CFTR is hyper-inflammatory *per se*, but the combination of mucus stasis and chronic infection clearly results in highly inflamed CF airways (Roesch et al., 2018). Complex interactions during infection stemming from the epithelial defect ultimately cascade into pathologic changes including extensive mucus obstruction, ectasis of bronchi and bronchioles, and consequent loss of pulmonary function (**Figure 1**). It is likely that significant structural damage to the airways and surrounding parenchyma becomes irreversible, and a goal for airway epithelial cell therapy is to intervene early, likely in childhood.

HUMAN BRONCHIAL AND BRONCHIOLAR EPITHELIAL CELLS (HBECS)

Epithelial cell proliferation in the lung is relatively quiescent at steady state, but this organ retains the ability to regenerate rapidly after injury (Hegab et al., 2012). Much has been learned in recent years about lung stem cells and their roles in repair and regeneration, with particular emphasis on the alveolar parenchyma (Hogan et al., 2014; Kotton and Morrisey, 2014). Cell-based therapy for CF will logically be focused on the conducting airway epithelium, the primary site for mucus stasis, infection, and inflammation. The airways harbor distinct cell populations along the proximal to distal axis that exhibit self-renewal and differentiation capacity. Here, we propose that HBECs may serve as candidates for autologous cell therapy of CF. We review the stem cell properties of HBECs with

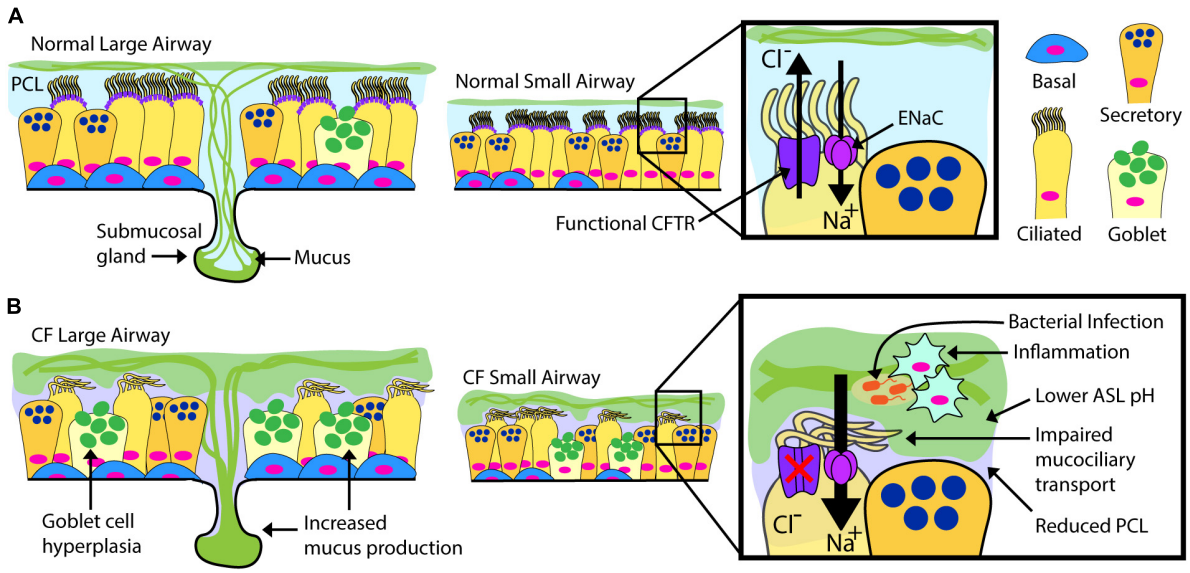

FIGURE 1 | Normal vs. CF Airway. Schematic drawings of normal and CF airways, illustrating selected components of epithelial apical ion transport. **(A)** Secreted mucins are produced by submucosal glands in the large airways and surface goblet cells, forming the overlying, mucin-rich component of the airway surface layer (ASL). This layer is normally transported by effective beating of cilia in the periciliary layer (PCL). ASL hydration is largely controlled by the balance of chloride secretion through CFTR and sodium reabsorption by the epithelial sodium channel (ENaC). **(B)** Mucin overproduction from hypertrophic submucosal glands and hyperplastic surface goblet cells characterize the CF large airways. Non-functional or absent CFTR leads to diminished chloride transport and increased sodium transport through ENaC. Airway dehydration reduces the PCL, impairing mucociliary transport. Accumulation of thick, sticky mucus increases overall ASL thickness and promotes a low pH environment favoring chronic bacterial infection and inflammation. The CF ASL environment is a likely barrier for cell therapy.

a focus on the unique advantages and challenges posed for therapeutic translation.

STEM CELLS IN THE CONDUCTING AIRWAYS

The human conducting airways are composed of a pseudostratified epithelium extending from the trachea to the proximal end of small bronchioles. The predominant evidence is that basal cells are the stem cells in this region (Rock et al., 2009). Basal cells are relatively undifferentiated and express a number of classic markers including *TP63* and *KRT5*. The regenerative capacity of basal cells has been extensively demonstrated through *in vitro* clonal growth assays, tracheal graft regeneration, and *in vivo* lineage tracing studies (Gray et al., 1991; Liu et al., 1994; Hong et al., 2004; Rock et al., 2009). Basal cells have the capacity to differentiate into the major cell types of the conducting airways including club (previously Clara) cells (Schoch et al., 2004; Rock et al., 2009), pulmonary neuroendocrine cells (Montoro et al., 2018), the recently described pulmonary ionocyte (Montoro et al., 2018; Plasschaert et al., 2018), and serous and mucus cells in the glands (Hegab et al., 2012, 2014). It is thought that ciliated and goblet cells are generated through a club cell intermediate (Tata et al., 2013; Widdicombe and Wine, 2015), but recent single cell mRNA sequencing studies also suggests direct differentiation of basal cells into ciliated cells (Plasschaert et al., 2018). Basal cell differentiation into pulmonary ionocytes and ciliated cells (either directly or indirectly) is of particular interest for CF cell therapy as these cell types express CFTR and are primarily responsible for epithelial ion transport (Rock et al., 2009; Montoro et al., 2018). The ability of lung basal cells to self-renew and replace luminal CFTR-expressing cell types is vital for cell therapy to be long-lasting.

The basal cell population is likely heterogeneous, containing subpopulations with different proliferation and differentiation capacities. At steady state, basal cell subsets express early differentiation markers for club or ciliated cells including the intracellular domain of Notch2 (N2ICD) or the proto-oncogene transcription factor MYB (C-Myb), respectively, suggesting subpopulations already primed for differentiation (Pardo-Saganta et al., 2015; Watson et al., 2015). Mechanisms regulating lung cell development, homeostatic maintenance, and repair have been recently reviewed (Lee and Rawlins, 2018). However, there is still debate about which cell lineage model is most applicable to the human pseudostratified airway epithelium. Are there rare populations of stem cells in specific niches that undergo asymmetric divisions, generating committed downstream progenitors, or is the stem cell compartment more widely distributed and stochastically regulated? While cell harvesting, gene correction, expansion, and delivery of intermediate progenitors may be efficacious, it is logical that starting with cells exhibiting extensive growth potential and differentiation capacity would be advantageous. Thus, identifying basal cell subsets and gene signatures corresponding to high clonal growth capacity remains an important goal that will theoretically serve as the basis for efficacious CF cell therapy.

Additional cell types within the airways should be considered as alternative candidates to restore CFTR function to the epithelium. Columnar epithelial cells from the pseudostratified regions demonstrate extensive plasticity in which they can dedifferentiate into basal cells (Tata and Rajagopal, 2017). Studies in the porcine model of CF suggest the importance of submucosal gland dysfunction in CF pathogenesis (Hoegger et al., 2014) and lineage tracing in mice illustrates a key role for gland myoepithelial cells in repair (Lynch et al., 2018; Tata et al., 2018). The ability to access, propagate and correct human gland myoepithelial cells, and their ultimate return to gland tubular and acinar niches will require more study.

OBTAINING, EXPANDING, AND GENE CORRECTING HBECS

Conceptually, HBECs can be obtained from the lungs of CF individuals using one of three minimally invasive techniques, induced sputum sample collection, bronchiolar lavage, and endobronchial brushing or biopsy. These methods yield approximately 2×10^3, 5.5×10^3, and 2×10^6 cells, respectively (Mou et al., 2012; Pollock et al., 2013; Butler et al., 2016). Considering how few cells effectively engraft by current approaches, the paucity of HBEC starting materials poses a considerable challenge to their use in cell therapy. As such, autologous cell-based therapy for CF with somatic epithelial cells will likely require *in vitro* culture for both cell expansion and gene correction. Fortunately, there is a long history of cell culture techniques to expand and subsequently differentiate a predominantly basal cell population from mouse and human large airways (see **Table 1** and Bove and Randell, 2015). Conventional expansion methods can be augmented to increase population doublings using dual SMAD inhibition or Rho kinase inhibition along with irradiated mouse 3T3-J2 fibroblasts co-culture (Suprynowicz et al., 2012; Mou et al., 2016). Key reagents such as irradiated 3T3-J2 feeder cells and irradiated serum developed for keratinocytes have been approved for clinical applications by some regulatory authorities and can be, similarly, employed in modern protocols to expand primary HBECs (Suprynowicz et al., 2012; Gentzsch et al., 2017).

While the larger airways are lined by a tall pseudostratified epithelium the more distal bronchi and bronchioles are lined by a progressively shorter epithelium, in which basal cells eventually extinguish. In the last generations of the small airways, the epithelium becomes simple columnar to cuboidal where stem cells are thought to be contained in the club cell compartment, with an independent lineage existing among pulmonary neuroendocrine cells (Hogan et al., 2014), although pulmonary neuroendocrine cell generation of club and ciliated cells has been observed (Song et al., 2012). This distal region is an important target since small airway obstruction and air trapping is thought to be a defining feature of early CF (Rosenow et al., 2017). Culture methods for human small airway epithelial cells are less well developed than for the large airway, and cells of the smallest bronchioles appear to be more dependent on mesenchymal signals (Lee et al., 2017; Zepp et al., 2017). Specific efforts to better understand human small airway stem cells, their propagation, and eventual engraftment are required.

TABLE 1 | Key studies in the development of culture methods for HBECs and directed differentiation protocols to derive airway epithelial cells from iPSCs.

Human bronchial epithelial cell culture methods	Reference
Clonal growth of human bronchial epithelial cells	Lechner et al., 1981
Detailed description of a media for proliferation of human bronchial epithelial cells on plastic	Lechner and LaVeck, 1985
Differentiation of serially passaged cells at an air-liquid interface (ALI)	Gray et al., 1996
Current detailed methods for ALI cultures using proprietary reagents	Neuberger et al., 2011
Current detailed methods for ALI cultures using non-proprietary reagents	Fulcher and Randell, 2013
Detailed methods for Conditionally Reprogrammed Cells (CRC, with feeder cells)	Gentzsch et al., 2017; Liu et al., 2017
Dual SMAD inhibition method for cell expansion	Mou et al., 2016
Method allowing clonal expansion of CRC primary HBECs	Peters-Hall et al., 2018
CRC method with proprietary media and no feeder cells	Zhang et al., 2018
Derivation of human airway epithelial cells from iPSCs	
TGFβ/BMP inhibition required to generate anterior foregut endoderm from iPSC	Green et al., 2011
Differentiation of human CF iPSCs into airway epithelial cells	Mou et al., 2012
Efficient differentiation of human iPSCs into lung epithelial cells comprised of both proximal and distal epithelial cells	Huang et al., 2013
Generation of multiciliated cells from human iPSCs	Firth et al., 2014
Human iPSC-derived lung organoids composed of epithelium and mesoderm	Dye et al., 2015
Surface marker sort method (Carboxypeptidase M) and culture conditions to generate neuroendocrine and functional multiciliated cells	Konishi et al., 2016
Fluorescent lineage reporters to purify early lung progenitors and generate epithelial-only organoid	Hawkins et al., 2017
Wnt withdrawal after early lung specification required for airway patterning	McCauley et al., 2017
Engraftment of iPSC-derived similar to embryonic lung-tips into immunocompromised mouse lungs	Miller et al., 2018

There have been tremendous advances in genome editing techniques in the last decade, including zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technique (Zhang et al., 2014). While of utmost importance for correcting a monogenic but complex lung disease such as CF, optimal methods for *CFTR* gene correction of HBECs with minimal off target, potentially carcinogenic risks, followed by cell expansion that maintains long-lived basal stem cells remain to be determined.

INDUCED PLURIPOTENT STEM CELLS (iPSCS)

iPSCs have several features relevant to their suitability as a source for cell-based therapies. Pluripotency refers to the

capacity to differentiate into all cell types in the body. iPSCs can now be routinely generated from any human while retaining an individual's unique genetic information, without the need for an invasive procedure. The capacity of these cells to proliferate while retaining pluripotency means sufficient numbers for cell-based therapy might someday be feasible. In the following section we review the essential studies that made the discovery of iPSCs possible, summarize the recent progress in deriving lung epithelial cells from iPSCs (**Table 1**), particularly cell types relevant to CF, and finally highlight the many challenges to be addressed before these cells can be transplanted into CF recipients.

HISTORY OF iPSCS

In 2006, Takahashi and Yamanaka made the remarkable discovery that the overexpression of four key factors in somatic cells could "re-program" cells into iPSCs (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). This breakthrough was made possible by decades of prior work in the field of embryonic stem cells (ESCs) that identified: (1) the transient presence of a population of pluripotent stem cells in the developing embryo, (2) the culture conditions sufficient to capture, *in vitro* expand, and maintain the pluripotency of cells isolated from early embryos, and (3) the gene-regulatory networks that control the ground-state of pluripotency (Solter et al., 1970; Stevens, 1970; Evans and Kaufman, 1981; Smith, 2001). Given the regenerative potential of ESCs there was much initial enthusiasm for their therapeutic promise but also controversy over the ethical and moral considerations regarding the use of fetal tissue. From a scientific perspective the main drawback for using ESCs as a cell-based therapy was that even if the cell type of interest could be generated efficiently and safely, the cells would be allogeneic to the recipient and the issues of rejection and immunosuppression that limit the effectiveness of solid organ transplants would persist. Dr. Jim Gurdon's pioneering nuclear transfer experiments in the 1960s first suggested the feasibility of reprogramming a somatic cell into an autologous pluripotent stem cell (Gurdon, 1962). When a frog egg nucleus was replaced with an intestinal cell nucleus a viable tadpole was produced confirming that the cellular identity could be "reset" to a pluripotent state. Takahashi and Yamanaka set about screening candidate factors that might have the same effect. They identified four factors, *Oct4*, *Sox2*, *Klf4,* and *c-Myc* that were sufficient to reprogram mouse somatic cells (initially skin fibroblasts) into pluripotent stem cells. These "induced" pluripotent stem cells were virtually indistinguishable from ESCs based on their similar transcriptional profiles and capacity to differentiate into all three germ layers when injected subcutaneously in a teratoma assay. Remarkably, iPSCs also contributed to embryogenesis when injected into a blastocyst. One year later, the same four factors were confirmed to reprogram human fibroblasts into iPSCs. Crucial to the emerging field of regenerative medicine, iPSCs have four key characteristics: (1) they are generated from the potential recipient, i.e., autologous, (2) they provide an unlimited supply of pluripotent stem cells without the need for embryonic tissues, (3) they are amenable to gene correction approaches, and (4) they are now routinely derived.

DIRECTED DIFFERENTIATION OF iPSCS INTO LUNG EPITHELIUM

Over the past decade, many groups contributed to major progress toward generating lung epithelial cells from iPSCs (**Table 1**). Directed differentiation refers to the approach of recapitulating, *in vitro*, the key embryonic developmental milestones that lead to the specification of the cell(s) of interest. This is typically achieved through the stepwise addition of signaling factors identified in mouse studies of organogenesis. The field of lung directed differentiation has evolved from generating relatively immature lung epithelial cells to recent reports of increasingly sophisticated protocols that produce relatively mature and functional lung epithelial cells that are currently being applied to lung disease modeling, drug screening and the first forays into *in vivo* transplantation.

In order to understand the directed differentiation process, a brief overview of lung development is first required. The lung develops from the foregut endoderm and is first identified by the expression of *Nkx2-1*, a key transcription factor. *Nkx2-1* is first detected at E9.0 in mice and day 28 in human embryos (Lazzaro et al., 1991; Morrisey and Hogan, 2010). During the first stages of lung organogenesis *Nkx2-1*$^+$ cells form two primary lung buds and the adjacent foregut separates into two tubes, a ventral trachea and dorsal esophagus. During the pseudoglandular stage, the lung buds undergo branching morphogenesis and proximal-distal patterning. This process, controlled by reciprocal signaling between the lung epithelium and surrounding mesenchyme, gives rise to a highly branched airway tree composed of basal, secretory, multi-ciliated and neuroendocrine cells and an alveolar compartment composed of types 1 and 2 pneumocytes. A number of signaling pathways are involved in the epithelial-mesenchymal interactions that control these stages of lung development including SHH, WNTs, FGFs, retinoic acid and BMPs (Hawkins et al., 2016).

To differentiate iPSCs into lung epithelial cells researchers seek to reproduce these key developmental events *in vitro*. The first major developmental stage in the road map of lung development is the formation of definitive endoderm. Mimicking the *in vivo* patterning of the primitive streak, the addition of the Nodal agonist Activin-A to iPSCs or ESCs produces a highly enriched population of endodermal cells that express classic endoderm markers (including *FOXA2* and *SOX17*) (Kubo et al., 2004; D'Amour et al., 2005; Christodoulou et al., 2011). The protocols to generate definitive endoderm are now well established and form the basis of all endodermal differentiation protocols including lung, liver, pancreas and intestine. The next important developmental step is directing definitive endodermal cells (*FOXA2*$^+$) toward an anterior-foregut fate (*FOXA2*$^+$/*SOX2*$^+$), analogous to the anterior-posterior axis patterning in the primitive gut tube. The field of

lung directed differentiation is relatively new and not surprisingly the published protocols from different groups vary significantly in terms of the timing and combination of growth factors and inhibitors applied at different stages. For example, differing approaches have achieved *in vitro* foregut patterning. In one method antagonism of BMP and TGFβ pathways after endoderm induction was identified as the most potent condition to maintain *FOXA2* expression, and to induce the anterior endoderm marker *SOX2* while suppressing a hindgut fate that expresses *CDX2* (Green et al., 2011). The inclusion of BMP and/or TGFβ inhibition has been adopted by others (Longmire et al., 2012; Mou et al., 2012; Firth et al., 2014; Dye et al., 2015; Hawkins et al., 2017). Alternative approaches include the combination of FGF2 and SHH signaling (Wong et al., 2012). Lung specification is the next major developmental milestone and is indicated by the expression of *NKX2-1* in foregut endoderm cells. Several groups have concluded that WNT, through recombinant WNT3a or glycogen synthase kinase 3 inhibitors, BMP4, and retinoic acid pathway activation are required for lung specification (Huang et al., 2013; Gotoh et al., 2014; Hawkins et al., 2017). Others have developed quite different approaches that led to specification of a lung program, for example using a combination of BMP and TGFβ inhibition, plus SHH and WNT activation (Dye et al., 2015). Early reports demonstrated lung competence of these cells via upregulation of differentiation markers over time. However, both proximal and distal lung markers were frequently present suggesting that the precise signals patterning lung progenitors toward proximal vs. distal fates and to specific differentiated cell types are incompletely known. Competence of these cells to differentiate into functional airway lineages was demonstrated by transitioning to air-liquid interface conditions used to differentiate primary HBECs, or by injecting the cells into the flank or kidney capsule of immune-deficient mice (Wong et al., 2012; Huang et al., 2013; Firth et al., 2014). More recently, there has been progress in generating lung organoids from iPSC-derived lung epithelium, reminiscent of the 3-D organization of lung tissue (Gotoh et al., 2014; Dye et al., 2015; Chen et al., 2017; Hawkins et al., 2017; Miller et al., 2018). In some cases, these protocols involve the co-development of mesodermal and endodermal compartments. To overcome the heterogeneity of cell types produced in these protocols, fluorescent lineage reporters or surface markers have been employed to prospectively isolate the lung epithelial cells of interest. Surface markers including Carboxypeptidase M or the combination of $CD47^{hi}/CD26^{lo}$ were identified to isolate immature *NKX2-1*$^{+}$ lung epithelial cells and to generate lung epithelial organoids depleted of other endodermal and mesodermal cell types (Gotoh et al., 2014; Hawkins et al., 2017). Although less relevant as a cell-based therapy for CF, it is worth highlighting some of the most compelling progress toward generating relatively mature, functional type 2 pneumocytes from iPSCs. Two groups, adopting the approach of targeting fluorescent reporters to the endogenous *SFTPC* locus, developed similar protocols to isolate and propagate long term a population of *SFTPC*$^{+}$ cells highly reminiscent of alveolar type 2 cells (Jacob et al., 2017; Yamamoto et al., 2017).

DIRECTED DIFFERENTIATION OF iPSCS INTO AIRWAY EPITHELIUM

To cure CF airway disease, a cell-based therapy would ideally replace the CFTR-expressing columnar luminal epithelial cells, including terminally differentiated multi-ciliated cells and perhaps ionocytes, but also the stem/progenitor cells that maintain the luminal cells. A number of groups have demonstrated the feasibility of generating CFTR expressing airway epithelium composed of multi-ciliated cells and cells reminiscent of basal and secretory cells (Wong et al., 2012; Firth et al., 2014; Konishi et al., 2016; McCauley et al., 2017). These airway epithelial cells broadly recapitulate the CFTR dysfunction seen in the primary cells of CF patients when interrogated using established CFTR functional assays including ion transport in Ussing chambers and forskolin-induced spheroid swelling. In an important proof-of-concept study, Crane et al., demonstrated the correction of the F508del mutation in iPSCs using zinc-finger nucleases and restoration of CFTR function in those cells after lung differentiation (Crane et al., 2015). Based on our current knowledge, an ideal iPSC-derived candidate cell for engraftment into CF airways is the airway basal cell. A number of groups have described the presence of a subset of basal-like cells after directed lung differentiation as evidenced by the expression of key basal cell markers (e.g., *TP63*, *KRT5*) (Dye et al., 2015; Konishi et al., 2016; Chen et al., 2017; Hawkins et al., 2017; McCauley et al., 2017). However, the efficient derivation of bonafide basal cells, as evidenced by transcriptional profiling and functional demonstration of differentiation into multi-ciliated and secretory cells comparable to those generated by primary basal cell controls, has not yet been convincingly achieved.

IN VIVO ENGRAFTMENT OF iPSC-DERIVED LUNG EPITHELIAL CELLS

Provided that it is possible to derive a candidate cell type *in vitro*, would that cell engraft and function once transplanted? This is a major question now facing this field and very little is known about the *in vivo* potential of iPSC-derived cells in the lung. Recently, the first report of human iPSC-derived lung epithelial cells engrafting in a murine lung injury model was described (Miller et al., 2018). Miller et al., identified conditions to capture and expand cells with a transcriptional profile similar to the multipotent cells of the developing lung bud tip. The iPSC-derived lung tip cells were delivered intra-tracheally into immunocompromised mice following airway injury with naphthalene. Engrafted cells persisted for up to 6 weeks after transplantation and these cells had adopted a predominantly airway and mostly secretory cell fate with rare multi-ciliated cells. This study is an important first step establishing the feasibility of using animal models to develop engraftment strategies, and suggests that the micro-environment

or niche may play an important role in the cell fate decisions of transplanted cells.

CHALLENGES FACING iPSCS AS A CELL-BASED THERAPY

Despite the progress made in the field of iPSC directed differentiation into lung cells, there are major challenges facing the application of cell-based therapy for the treatment of CF. To date, the derivation of a pure population of airway epithelial cells from iPSCs in sufficient numbers to engraft in human airways has not yet been achieved. How will adequate numbers of differentiated cells be generated for patients? How safe are iPSC-derived lung epithelial cells? The same factors and culture conditions that allow for the unimpeded self-renewal and pluripotency also predispose to tumorigenicity; in fact, a survey of the exomes of 117 human iPSC lines identified nine with mutations in the tumor suppressor gene, *TP53,* with evidence that iPSCs with the mutation had a survival advantage (Ben-David and Benvenisty, 2011; Merkle et al., 2017). Other groups have confirmed that gene editing with CRISPR/Cas9 selects against cells with a normal p53 pathway (Haapaniemi et al., 2018; Ihry et al., 2018). Initially iPSCs were generated using viral vectors that randomly integrated into the genome (Takahashi and Yamanaka, 2006). This approach raised obvious concerns about the malignant potential of iPSCs if an important tumor suppressor locus was disrupted. A non-integrating episomal-based method, such as Sendai virus, is a more logical approach to derive clinical-grade iPSCs (Fusaki et al., 2009). The delivery of safe cells will require a robust and reproducible process to generate iPSCs and differentiated cells, compliant with relevant current good manufacturing practice (cGMP) and regulatory agency approval. Confirmation that the resulting cells are free of integrations and somatic mutations will be needed (Baghbaderani et al., 2015). Transplanted cells will require *in vitro* gene-correction to express functional CFTR. With reprogramming and passaging, and additional time needed to gene-edit and expand cells, the probability of somatic mutations will increase. Furthermore, the process of gene-editing brings additional risk of off-target double-stranded breaks with the potential of disrupting other genetic loci (Zhang et al., 2015). As the goal of cell-based therapy is to replace diseased endogenous lung epithelium with engineered cells, it will be essential to generate pure populations of lung epithelial cells that are highly similar to their *in vivo* counterparts. The field is relatively new and most reports have not focused on the non-lung cells that might also be produced during the differentiation process. For example, an iPSC line expressing GFP from the NKX2-1 locus (*NKX2-1*^{GFP}) identified significant variability in the percentage of GFP^{+} cells, and analysis of the GFP^{-} population confirmed expression of liver and intestine markers (Hawkins et al., 2017; Serra et al., 2017). Even after a purification step to sort *NKX2-1*$^{GFP+}$ cells, McCauley et al. applied single-cell RNA sequencing to airway and alveolar iPSC-derived organoids and identified distinct populations of non-lung endoderm including hepatic-like and gastric-like cells (McCauley et al., 2018). The quest to generate pure populations of well-defined lung epithelial cells will continue and be aided by powerful new techniques such as single-cell RNA sequencing and expanding knowledge of the transcriptional profile and signaling pathways involved in human lung development (Treutlein et al., 2014; Nikolić et al., 2017; Miller et al., 2018). The appropriate number of donor cells required to repopulate a sufficient portion of the diseased airway epithelium is unknown. CFTR levels 15–20% of normal airway expression may be enough to effectively treat CF airway disease (McKone et al., 2003). Building on these aforementioned advances, the ultimate final hurdle to a cell-based therapeutic approach for CF is engraftment.

ENGRAFTMENT

The gold standard for cell-based therapy is hematopoietic cell transplantation. This now commonplace and life-saving procedure relies on delivering hematopoietic progenitor cells after inducing tissue receptivity through marrow-ablative doses of cytotoxic agents and radiation. The term engraftment is used to describe the reconstitution of host bone marrow by donor cells. For CF, successful airway engraftment is thought of in similar terms, recipient airway priming, donor cell survival and regeneration of the airway epithelium (**Figure 2**). The ideal strategy to deliver a cell-based therapy for CF, or any lung disease, has yet to be developed and the prospect of deliberately injuring the lungs of CF recipients to ablate endogenous cells is daunting. An ideal engraftment strategy would involve the minimal airway injury necessary, in particular to the small airways, to promote a microenvironment conducive to long-lived engraftment of cells in numbers sufficient to restore CFTR function. There are many hurdles the scientific community must overcome to achieve human airway engraftment such as determining: (1) the most suitable cell type(s), (2) the methods to safely scale-up cell production, (3) the optimal injury, (4) supportive measures for oxygenation/ventilation and (5) methods to control mucus and infection to allow engraftment of cells. Despite these challenges, there are promising reports of effective cell therapies for other organ systems that support the overall feasibility of this approach. Based on pioneering work from the laboratory of Howard Green, autologous epidermal keratinocytes have been extensively evaluated as treatment for burns (Green et al., 1979; Ter Horst et al., 2018). Recently, transgenic autologous keratinocytes with a correct copy of a gene mutated in the blistering skin disease junctional epidermolysis bullosa were used to replace nearly the entire epidermis of a critically ill child with 60% skin loss, miraculously allowing hospital discharge and return to school (Hirsch et al., 2017). This remarkable achievement not only alleviated patient suffering, but also provided vital insights about epidermal keratinocyte stem cell dynamics, indicating the importance of long-lived stem cells for epidermal restoration. Additional experience comes from corneal limbal epithelial stem cell transplantation in which damage to one eye can be treated with autologous stem cells from the contralateral eye (Rama et al., 2017; Sasamoto et al., 2018). In the lung field, a number of groups have recently reported evidence of lung engraftment in animal models (Rosen et al., 2015; Vaughan et al., 2015;

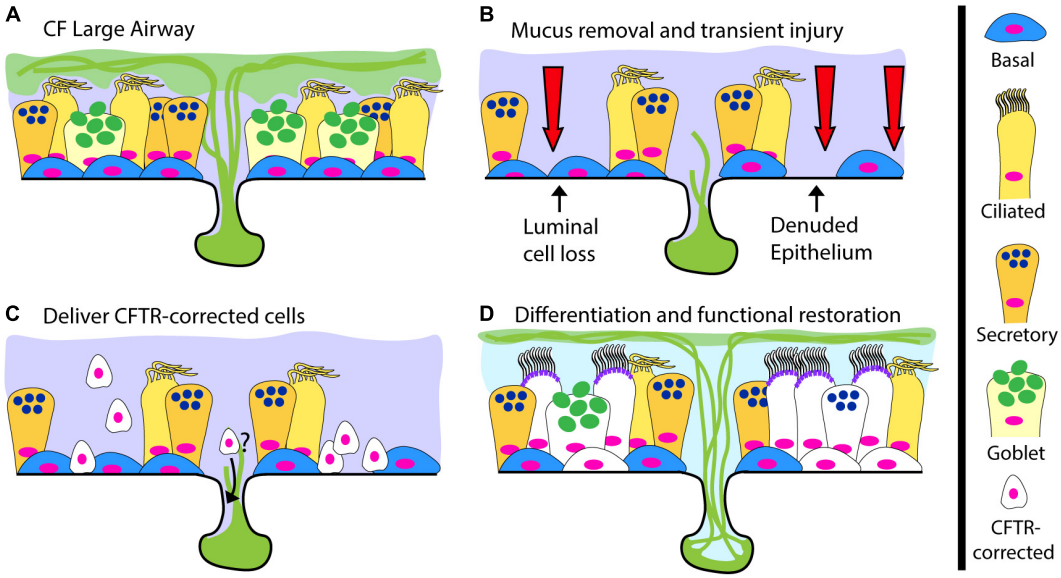

FIGURE 2 | A Cell-Based Therapy Model for CF. **(A)** The pre-treatment CF large airway. **(B–D)** A depiction of cell-based therapy in three steps: **(B)** Mucus removal and transient preconditioning of the airway epithelium, potentially requiring luminal epithelial surface cell loss and partial epithelial denudation; **(C)** Delivery of CFTR-corrected stem and progenitor cells to the surface epithelium and possibly the submucosal glands; **(D)** Differentiation of delivered CFTR-corrected cells into all relevant cell types, restoration of functional CFTR ion-transport and a return to normal airway physiology.

Ghosh et al., 2017; Nichane et al., 2017; Ma et al., 2018; Miller et al., 2018). Here we will briefly review this progress and highlight important next steps.

A number of injury models, including naphthalene, sulfur dioxide, radiation and detergents, have been developed to study the biology of the airway epithelium (Lamb and Reid, 1968; Plopper et al., 1992; Borthwick et al., 2001; Leblond et al., 2009; Rosen et al., 2015). It is unclear to what extent, if any, these models might be used in humans but they have provided a platform to study and develop engraftment strategies. Embryonic lung, both mouse and human, contain stem/progenitor populations with engraftment capacity (Rosen et al., 2015; Nichane et al., 2017). In one approach, intravenous administration of a single cell suspension of whole canalicular stage lung (mouse E15-16 or human week 20–22) into mice pre-conditioned with both naphthalene and total body irradiation, resulted in epithelial, mesenchymal, and endothelial patches of engrafted cells (Rosen et al., 2015). Higher rates of chimerism were detected in mouse-into-mouse compared to human-into-mouse experiments. It is surprising that intravenous delivery of a mixture of embryonic lung cell types resulted in homing of cells to their unique anatomic compartments. Further work is required to understand the safety of this approach including the consequence of cells being delivered to the wrong compartment within the lung or even to other organs. In terms of an approach for CF, the majority of epithelial engrafted cells had an alveolar phenotype although some airway-like cells were detected and some expressed CFTR. Interestingly, the co-administration of mesenchymal and endothelial cells was associated with improved epithelial cell engraftment. The multitude of practical, ethical and political issues surrounding the use of fetal tissue make this an unlikely future option for cell-based therapy. However, iPSCs may provide access to autologous cells at embryonic time points with capacity to engraft (Miller et al., 2018). Adult airway epithelial cells can also engraft in the lung (Vaughan et al., 2015; Ghosh et al., 2017; Nichane et al., 2017). The environment induced by H1N1 influenza infection has garnered much interest for its engraftment potential (Kumar et al., 2011; Vaughan et al., 2015). The viral infection causes widespread damage to the airway and alveolar epithelium and activates rare immature ($TP63^{+}/KRT5^{-}$) basal cells in the small airways (Yang et al., 2018). Orthotopic transplantation of a variety of airway epithelial cells into influenza-injured mice led to varying levels of engraftment (Vaughan et al., 2015). Perhaps most relevant to the CF field is a recent report of successful engraftment of primary airway basal cells into naphthalene injured mice (Ghosh et al., 2017). Transplanted mouse or human basal cells reconstituted patches of epithelium in the trachea and small airways with evidence of multi-lineage differentiation into secretory and multi-ciliated cells. Themes common to each of the above experiments include: (1) lung injury is required for engraftment of donor cells, (2) different injury models likely result in unique regenerative niches and (3) successful strategies to date suggest multipotent input donor cells are the most promising candidates for engraftment. Looking forward, a number of approaches might accelerate the field of cell-based therapy for lung disease. Engraftment after hematopoietic cell transplantation is monitored by sampling the peripheral blood. Assessing lung engraftment is far more challenging. Methods to interrogate whether donor cells are functionally integrated into the airway epithelium will be essential. Animal studies to optimize injury models and the efficiency, function and safety

of engrafted cells are necessary, but are rate-limiting due to low throughput, particularly in larger animals. Developing non-lethal experimental methods, including targeted airway injury and bioluminescent reporter cell lines to track engrafted cells in real-time without the need for necropsy would increase the efficiency of animal engraftment studies.

Methods to incite a targeted, clinically tolerable airway epithelial injury have been tested in humans albeit not in the context of cell-based therapy. Bronchial thermoplasty, an FDA-approved treatment for refractory severe persistent asthma, delivers brief thermal energy to the airways via flexible bronchoscopy and leads to initial epithelial sloughing and a prolonged decrease in smooth muscle with improvement in asthma severity (Miller et al., 2005). In a small pilot study, airway delivery of a metered liquid nitrogen cryospray via bronchoscopy resulted in airway epithelial damage followed by regeneration (Slebos et al., 2017). An alternative approach, with potential for clinical translation, is targeted airway de-epithelialization achieved by instilling mild detergents intratracheally (Dorrello et al., 2017). Despite the many limitations of these approaches in the context of CF, including the small size and large number of airways needed to treat CF, they provide preliminary support for the feasibility of a human injury platform. The situation is further complicated for targeting CF in more advanced disease where successful cell-based therapy must also overcome a highly inflamed, architecturally distorted organ characterized by thick mucus and superinfection. Similar to the blood product and growth factor support necessary after hematopoietic cell transplant, lung engraftment strategies will likely require lung supportive measures. The advances and more widespread use of extracorporeal membrane oxygenation (ECMO) provides a means of supporting oxygenation during respiratory failure (Squiers et al., 2016). Advances in the technology to maintain human lungs using *ex vivo* lung perfusion (EVLP) may provide insights into supporting the injured and regenerating lung (Makdisi et al., 2017). Future approaches for cell-based therapy incorporating these evolving technologies may be necessary. Alternatively, devising injury-independent strategies to promote lung engraftment of therapeutic cells may be an avenue for future research and development.

CONCLUSION

To date, CF remains the most common life-limiting hereditary lung disease. Decades of work resulted in important strides to understand molecular mechanisms in CF and treatment options for many patients. Despite this progress, a subset of patients lack effective treatment options and cell-based therapies could theoretically cure CF lung disease. The ideal cell type for this purpose would repopulate the airway epithelium with autologous, functional, and CFTR-expressing cells. Progress in our understanding of airway epithelial biology and endogenous stem/progenitor populations has revealed a number of candidate cell types for airway regeneration, in particular basal cells. The recent revolution in gene-editing techniques means genetic correction of the *CFTR* mutation in basal cells will likely be achieved in the near future and would provide a source of autologous airway stem cells with the normal *CFTR* sequence. In the iPSC field, directed differentiation protocols have evolved from initial studies demonstrating the presence of immature lung-like cells to more recent reports of increasingly efficient protocols to derive functional alveolar and airway epithelial cells. We have detailed some of the major challenges that must be overcome both in terms of generating the cells of interest and engrafting those cells in a safe and effective manner. While there is work yet to be done, there is cautious optimism that a curative cell-based therapy for CF will be achieved in the future.

AUTHOR CONTRIBUTIONS

All authors contributed to this manuscript. In collaboration, AB, FH, RL, and SR wrote all sections of this review. RL illustrated the figures. All authors read, edited, and approved of the final version of the manuscript.

FUNDING

The authors received funding support from NIH grants T32HL0007025 (AB) and R01HL139799 (FH), Cystic Fibrosis Foundation grants RANDEL15XX0 (SR), RANDEL17XX0 (SR), and HAWKIN15XX0 (FH), and Emily's Entourage (FH and SR).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 11 December 2018
doi: 10.3389/fphar.2018.01462

Esomeprazole Increases Airway Surface Liquid pH in Primary Cystic Fibrosis Epithelial Cells

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Respiratory failure, driven by airways mucus obstruction, chronic inflammation and bacterial infections, is the main cause of mortality and morbidity in people with cystic fibrosis (CF) due to defects in the Cl^- and HCO_3^- transport activity of the CF Transmembrane conductance Regulator (CFTR). Most recent pre-clinical and clinical studies have focused on restoring CFTR function by enhancing its trafficking or transport activity and show promising results. However, there are a significant number of patients that will not benefit from these CFTR-targeted therapies and it is therefore important to identify new non-CFTR targets that will restore lung function, by-passing CFTR dysfunction. The H^+/K^+-ATPase, ATP12A, has recently been identified as a potential novel target for CF therapies, since its acute inhibition by ouabain was shown to help restore mucus viscosity, mucociliary transport, and antimicrobial activity using *in vitro* CF airway models, and this effect was linked to an increase in the pH of the airway surface liquid (ASL). Here, we have evaluated the potential therapeutic use of ouabain by investigating the effect of chronically treating fully differentiated CF primary human airway epithelial cells (hAECs) with ouabain, under thin film conditions, resembling the *in vivo* situation. Our results show that although chronic treatment increased ASL pH, this correlated with a deleterious effect on epithelial integrity as assessed by LDH release, transepithelial electrical resistance, fluorescein flux, and ion transport. Since ATP12A shares approximately 65% identity with the gastric H^+/K^+-ATPase (ATP4A), we investigated the potential of using clinically approved ATP4A proton pump inhibitors (PPIs) for their ability to restore ASL pH in CF hAECs. We show that, despite not expressing ATP4A transcripts, acute exposure to the PPI esomeprezole, produced changes in intracellular pH that were consistent with the inhibition of H^+ secretion, but this response was independent of ATP12A. More importantly, chronic exposure of CF hAECs to esomeprazole alkalinized the ASL without disrupting the epithelial barrier integrity, but this increase in ASL pH was consistent with a decrease in mRNA expression of *ATP12A*. We conclude that PPIs may offer a new approach to restore ASL pH in CF airways, which is independent of CFTR.

Keywords: airway surface liquid pH, cystic fibrosis, ATP12A, ouabain, proton pump inhibitor, esomeprazole

OPEN ACCESS

Edited by:
Nicoletta Pedemonte, Istituto Giannina Gaslini (IRCCS), Italy

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *12 July 2018*
Accepted: *29 November 2018*
Published: *11 December 2018*

Citation:
Delpiano L, Thomas JJ, Yates AR, Rice SJ, Gray MA and Saint-Criq V (2018) Esomeprazole Increases Airway Surface Liquid pH in Primary Cystic Fibrosis Epithelial Cells. Front. Pharmacol. 9:1462. doi: 10.3389/fphar.2018.01462

INTRODUCTION

Cystic fibrosis (CF) is the most common autosomal recessive genetic disease in Caucasian populations and affects more than 70,000 people worldwide (Kelly, 2017). It is due to mutations in the Cystic Fibrosis Transmembrane conductance Regulator (*CFTR*) gene encoding an anion channel, transporting primarily Cl^- and HCO_3^- in epithelial tissues (Saint-Criq and Gray, 2017). CF is characterized by severe lung pathophysiology where thick, sticky, mucus provides a favorable environment for bacterial colonization, which, together with the initial CFTR defect, are the cause of a chronic inflammation that leads ultimately to organ failure. The CFTR channel is an essential regulator of the airway surface liquid (ASL) composition (Namkung et al., 2009; Van Goor et al., 2009; Luan et al., 2017). This thin fluid layer lines the airway epithelium, and contributes to the efficient physical and chemical barrier mechanism against inhaled particles and pathogens by regulating ciliary beating, mucociliary transport, and antimicrobial activity. Through its Cl^- and HCO_3^- transport activities, CFTR controls water movement across the epithelium and thus ASL hydration as well as its pH, respectively. However, the absolute value of the ASL pH in people with CF is still controversial as the measurement of this parameter in such a thin layer of fluid has proven very difficult. Although previous reports have shown an acidic ASL pH in human and animal models of CF airways (Coakley et al., 2003; Song et al., 2006; Pezzulo et al., 2012; Birket et al., 2018), the most recently published *in vivo* study reported no difference in ASL pH between children with or without CF (Schultz et al., 2017). Nevertheless, multiple studies have shown the importance of pH homeostasis in the ability of the airways to maintain ASL hydration (Garland et al., 2013), fight infections (Berkebile and McCray, 2014; Tang et al., 2016) and remove trapped microorganisms from the lungs (Quinton, 2008; Tang et al., 2016). Therefore increasing HCO_3^- or inhibiting H^+ secretion could be a suitable therapeutic strategy for lung disease in CF. To date, most pre-clinical research has focused on restoring CFTR function using CFTR-directed therapeutics. Gating mutants such as G551D (and others) respond very well to the CFTR potentiator, Ivacaftor, as well as a number of residual function mutations (De Boeck and Amaral, 2016) and next generation correctors appear able to restore some function to the most common CF-causing mutation (F508del) (Taylor-Cousar et al., 2017; Vertex, 2017). However, around 15% of people with CF lack the F508del mutation in both alleles and a certain percentage of these individuals who express F508del in at least one allele, experienced limited benefit from next generation CFTR modulators (NCT01225211; Boyle et al., 2014; Rowe et al., 2017). Therefore, there is an unmet need for alternative, mutation-independent, therapies that restore lung function in all people with CF. Accordingly, targeting non-CFTR H^+ or HCO_3^- channels or transporters, is a promising therapeutic strategy.

A recent study in mouse, pig and human airways has shown the essential role of the non-gastric H^+/K^+-ATPase, ATP12A, in ASL pH regulation in CF (Shah et al., 2016). Here the absence of expression of this ATPase in mice was linked to the mild pulmonary phenotype in the CF animals. On the other hand, acute (2 h) inhibition of this pump in pig and human airway cultures by a high concentration of apical ouabain, increased ASL pH and restored bacterial killing and mucus viscosity. This study showed for the first time the potential therapeutic use of ouabain in CF airways by targeting ASL pH homeostasis. However, to our knowledge no one has investigated the long-term effects of apical exposure to this widely used cardiac glycoside, that also inhibits the basolateral Na^+/K^+-ATPase, a key transporter essential for transepithelial ion and fluid transport, on airway epithelial cells. Moreover, as ATP12A belongs to the family of hydrogen-potassium ATPases (Crambert et al., 2002) and shares significant sequence and functional homology with the gastric form of the H^+/K^+-ATPase (ATP4A), for which there are a number of well-characterized proton pump inhibitors (PPIs) (Iwakiri et al., 2016) in use clinically, it was of interest to test the efficiency of these PPIs on airway cell function.

Therefore, in this study we tested the effects of long-term apical ouabain treatment on CF primary human airway epithelial cells (hAECs) ASL pH and epithelial integrity and compared this to the response to one of the commonly used PPIs, esomeprazole. We show for the first time that although apical treatment with ouabain increased ASL pH in a dose-dependent manner, this was positively correlated with an increase in cytotoxicity and disruption of epithelial barrier function. On the other hand, even though the gastric H^+/K^+-ATPase was not expressed in airway epithelial cells, exposure to esomeprazole acidified the cytosol and increased ASL pH of primary CF hAECs. We show that esomeprazole had a dual mechanism of action: acutely, it induced intracellular acidification in an ATP12A-independent manner but, chronic exposure, which importantly did not have any deleterious effect on epithelial integrity, was linked to decreased *ATP12A* mRNA levels. These results open up the possibility of repurposing PPIs as a new therapeutic approach for treating CF lung disease.

MATERIALS AND METHODS

Chemicals

Ouabain (O3125), esomeprazole (E7906), *N*-Acetyl-L-cysteine (A7250), amiloride (A7410), fluorescein (F6377), mitomycin C (M0503), and UTP (U6750) were purchased from Sigma-Aldrich. Forskolin (1099), Y-27632 (1254), and CFTRInh172 (3430) were purchased from Tocris (RnD). The fluorescent dyes, Alexa Fluor™ 488- Dextran (D22910); pHrodo™ Red Dextran (P10361) and BCECF, AM (2′,7′-*Bis*-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester) (B1150) were purchased from ThermoFisher Scientific.

Solutions

See **Table 1**.

Cell Culture

Primary non-CF (n = 3 donors) and CF (n = 3 donors, all F580del/F508del) hAECs were a kind gift from Dr. Scott H. Randell (Marsico Lung Institute, The University of North Carolina at Chapel Hill, United States). The cells were obtained under protocol #03-1396 approved by the University

TABLE 1 | Composition of the solutions used in this study. Concentrations are given in mM.

	HCO_3^- KRB	low Cl^-	NaCl HEPES	High K^+ HEPES/Nigericin	K^+-free solution (0K^+)	ASL pH standard curve solution	ASL pH standard curve solution	ASL pH standard curve solution
pH at 37°C	*7.4*	*7.4*	*7.4*	*6/6.9/7.5*	*7.4*	*5.5-6-6.5*	*7-7.5*	*8*
$NaHCO_3$	25	25			25			
NaCl	115		130	5	120	86	86	86
KCl	5	5	5	130		5	5	5
$CaCl_2$	1	1.2	1	1	1	1.2	1.2	1.2
$MgCl_2$	1	1.2	1	1	1	1.2	1.2	1.2
D-Glucose	5	5	5	5	5			
Na-gluconate		115						
K_2SO_4								
Ca-gluconate		2.8						
Mg-gluconate								
NaHEPES			10	10			100	
MES						100		
Tris								100

of North Carolina at Chapel Hill Biomedical Institutional Review Board. Primary cells from three different CF donors (all F580del/F508del) were obtained via the CFFT Biorepository. They were expanded using the conditionally reprogrammed cell (CRC) culture method as previously described (Suprynowicz et al., 2012). Briefly, cells were seeded on 3T3J2 fibroblasts inactivated with mitomycin C (4 μg/ml, 2 h, 37°C) and grown in medium containing the ROCK inhibitor Y-27632 (10 μM) until they reached 80% confluence. Cells then underwent double trypsinization to remove the fibroblasts first and then detach the hAECs from the P150 dish. At that stage, cells were counted and could be frozen down. Cryopreserved cells were seeded onto semi-permeable supports (6.5 or 12 mm) in bilateral differentiating medium (ALI medium) as previously described (Randell et al., 2011). The apical medium was removed after 3–4 days and cells then allowed to differentiate under air-liquid interface (ALI) conditions. Ciliogenesis started approximately 12–15 days after seeding and cells were used for experiments between days 25 and 35 after seeding.

Knock-Down of ATP12A Using CRISPR-Cas9

Guide RNA (gRNA) sequences targeting upstream (5′-GGCCGGAGGGAGTCGGACAG-3′) and downstream (5′-TCCCTCAGACTGAATGTCTG-3′) of *ATP12A* exon 2 were designed using the Optimized CRISPR Design Tool[1]. Single stranded DNA oligonucleotides (IDT) containing the gRNA sequence in addition to the *BbsI* restriction enzyme sequences were annealed (95 to 25°C, Δ-6°C/min). The double stranded oligonucleotides were then ligated into the CRISPR-Cas9 vector, PX462, following *BbsI* linearization, using T4 ligase (Invitrogen) overnight at 16°C. To validate the sequences, cells from the human chondrocyte cell line Tc28a2 were nucleofected [10^6 cells, 5 μg of plasmid DNA using the manufacturer recommended Cell Line 4D-Nucleofector X Kit in combination with the 4D-Nucleofector System (Lonza)] and selected with puromycin after 24 h. Following expansion, nucleic acids were extracted using the EZNA DNA/RNA Isolation kit (Omega Bio-Tek) according to the manufacturer's protocol. Deletion of the target region was confirmed using end-point PCR (**Supplementary Figure 6D**).

Following validation in the Tc28a2 cells, primary CF hAECs were transfected using an adapted version of a previously published protocol (Ramachandran et al., 2013). Briefly, DNA (3 μg) was incubated for 3 min in ALI medium without antibiotics, FuGENE HD Transfection Reagent was then added (3:1 ratio) and further incubated for 15 min at room temperature on the collagen-coated semi permeable supports. Freshly thawed cells were counted and seeded onto the semi-permeable support in antibiotics-free ALI medium. After 5 h incubation, medium was replaced and cells were grown as previously described in antibiotics containing ALI medium from day 3. After pH_i or ASL pH experiments, RNA was extracted as described below and *ATP12A* mRNA was quantified by RT-qPCR.

ASL pH Measurements

Cells grown on 6.5 mm transwells were washed apically with 120 μl glucose-free HCO_3^--containing Krebs solution (HCO_3^- KRB, **Table 1**) for 15 min at 37°C, 5% CO_2. The ASL was stained using 3 μl of a mixture of dextran-coupled pH-sensitive pHrodo Red (0.5 mg/ml, λex: 565 nm, λem: 585 nm) and Alexa Fluor® 488 (0.5 mg/ml, λex: 495 nm, λem: 519 nm) diluted in glucose-free HCO_3^- KRB, overnight at 37°C, 5% CO_2. Alexa Fluor® 488 was used as a loading control as pHrodo is not a ratiometric dye. The next day, fluorescence was recorded using a temperature and CO_2-controlled plate reader (TECAN SPARK 10M). After subtracting background values from pHrodo and Alexa Fluor® 488, ratios were generated for each time point and pH was calculated from a standard curve where pH was clamped using highly buffered solutions between 5.5 and 8 (see **Table 1** for composition). To prevent inter-experiment variability, the standard curve calibration was performed on each

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independent experiment. For ASL pH experiments involving apical exposure to chemicals, cells were treated overnight with the compounds, which were added with the fluorescent dyes at 0.1X final concentration. The final concentrations of the drugs added was calculated assuming a theoretical final ASL volume of 0.3 µl (10 µm × π × 3.25 mm^2), after absorption of the excess fluid by the epithelium. ASL pH measurements were performed in duplicate.

Transepithelial Electrical Resistance, Fluorescein Flux, and Short–Circuit Current Measurements in Ussing Chamber

Cells grown on 6.5 mm inserts were mounted into the EasyMount Ussing Chamber Systems (VCC MC8 Physiologic Instrument) and bathed in basolateral HCO_3^- KRB and apical low Cl^- (**Table 1**) continuously gassed and stirred with 5% (v/v) CO_2/95% (v/v) O_2 and maintained at 37°C. Monolayers were voltage-clamped to 0 mV and monitored for changes in short-circuit current (ΔIsc) using Ag/AgCl reference electrodes. The transepithelial short-circuit current (Isc) and the TransEpithelial Electrical Resistance (TEER) were recorded using Ag–AgCl electrodes in 3 M KCl agar bridges, as previously described (Saint-Criq et al., 2013), and the Acquire & Analyze software (Physiologic Instruments) used to perform the analysis.

Fluorescein Flux

A 100 µl sample (blank) was taken from the apical bath 5 min after mounting the monolayers and 100 µl of fluorescein (20 µg ml^{-1} final concentration) was then added to the basolateral bath. Apical samples (100 µl) were collected every 5 min for 45 min and replaced by the same volume of fresh warmed low Cl^- solution. Collected samples were loaded onto a 96 well plate and fluorescence was measured using a plate reader (λex: 460 nm; λem: 515 nm). Data is presented as the slope of fluorescein appearance in the apical bath and was obtained after plotting (sample-blank) vs. time and performing a linear regression. The addition of the fluorescein solution, as well as the collection and replacement of apical samples, did not have any effect on the TEER or Isc.

Short–Circuit Current Measurements

After the last fluorescein flux samples were collected, cells were left to equilibrate for a further 10 min and ion transport agonists and inhibitors were added following this sequence: amiloride (10 µM, apical), Forskolin (Fsk, 10 µM, bilateral), CFTRinh172 (172, 20 µM, apical) and UTP (100 µM, apical). Results were normalized to an area of 1 cm^2 and expressed as Isc (µAmp.cm^{-2}).

Intracellular pH Measurements

Primary airway epithelial cells were grown on 12 mm Transwell inserts and loaded with the pH-sensitive, fluorescent dye BCECF-AM (10 µM) for 1 h in a Na-HEPES buffered solution (see **Table 1**) at 37°C. Cells were mounted on to the stage of a Nikon fluor inverted microscope and perfused with a modified Krebs solution gassed with 5% (v/v) CO_2/95% (v/v) O_2. Solutions were perfused across the apical and basolateral membranes at 37°C at a speed of 3 and 6 ml min^{-1}, respectively. Intracellular pH (pH_i) was measured using a Life Sciences Microfluorimeter System in which cells were alternately excited at 490 and 440 nm wavelengths every 1.024 s with emitted light collected at 510 nm. The ratio of 490 to 440 nm emission was recorded using PhoCal 1.6 b software and calibrated to pH_i using the high K^+/nigericin technique (Turner et al., 2016) in which cells were exposed to high K^+ solutions containing 10 µM nigericin, set to a desired pH, ranging from 6 to 7.5. For analysis of pH_i measurements, ΔpH_i was determined by calculating the mean pH_i over 60 s resulting from treatment. The initial rate of pH_i change ($\Delta pH_i/\Delta t$) was determined by performing a linear regression over a period of at least 40 s.

RNA Extraction and Real-Time Quantitative PCR Analysis

RNA isolation from cells was performed using PureLink® RNA Mini Kit (12183018A, Ambion, Life Technologies), following the manufacturer's instructions. Briefly, lysates were mixed with 70% ethanol and loaded onto a silica-membrane column. Columns were washed with different buffers and total RNA was eluted in DNAse and RNAse-free water and stored at −80°C until use. DNase treatment was performed on 300 ng RNA prior to Reverse Transcription Polymerase Chain Reaction (RT-PCR) using RNAse-free DNAse I (04716728001, Roche) at 37°C for 10 min. Reaction was then stopped by increasing the temperature to 70°C for 10 min. Complementary DNA (cDNA) was synthesized from total RNA (300 ng) using M-MLV Reverse Transcriptase (Promega) as per supplier's protocol (1 h at 37°C followed by 10 min at 70°C).

Real-time quantitative PCR (qPCR) was performed in a total volume of 15 µl using 2× LightCycler® 480 SYBR Green I Master (Roche, 04707516001), 1.5 µl of cDNA, 2 µM forward primer and 2 µM reverse primer in a 96-well plate. Primer sequences are shown in **Table 2**. The expression of GAPDH was used as internal control. PCR was run with the standard program: 95°C 10 min, 40 times of cycling 95°C 15 s and 60°C 1 min in a 96-well plate. Results are shown as Threshold Cycle (Ct) or Relative quantity of mRNA copies to the determined control condition. A Ct value of 40 was applied for samples in which no messenger was detected.

TABLE 2 | qRT-PCR primers for mRNA quantification.

Target	Oligo sequence	PCR product length
ATP12A	5′-GGGGCACACTTGTTCATCTTCTGA-3′	128
	5′-GCAAAACATCAGTGAGCATCCTG-3′	
ATP4B	5′-GGCCTTCTACGTGGTGATGAC-3′	136
	5′-CCCGTAAACATCCGGCCTTA-3′	
ATP4A	5′-AAGATCTGCAGGACAGCTACGG-3′	200
	5′-CTGGAACACGATGGCGATCA-3′	
GAPDH	5′-TGC ACC ACC AAC TGC TTA GC-3′	87
	5′-GGC ATG GAC TGT GGT CAT GAG-3′	

Cell Cytotoxicity Measurement

Cell cytotoxicity was measured by quantification of secreted lactate dehydrogenase (LDH), after overnight exposure to vehicle or drugs. This was calculated after measurement of the apical + basolateral LDH release (LDH_{sec}) and expressed as a ratio to the cell lysates LDH (PBS/Triton X-100 0.8%; LDH_{lysate}). Samples were diluted in PBS/Triton X-100 0.8%, loaded onto a 96 well plate (Costar) and incubated with the assay reagent for 30 min at room temperature in the dark. After addition of the stop solution, absorbance was measured at 490 nm (kit CytoTox96® Non-Radioactive Cytotoxicity Assay kit, Promega) in a TECAN Infinite M200pro plate reader. Data were analyzed and cytotoxicity was calculated as follows:

$$\%\ \text{cytotoxicity} = \left(\frac{\text{OD LDH}_{\text{sec}}}{\text{OD LDH}_{\text{sec}} + \text{OD LDH}_{lysate}} \right) \times 100$$

Statistical Analysis

All analyses were performed using GraphPad Prism7. Where applicable, results are shown as mean ± SEM. Dose responses were analyzed by non-linear regression on a log scale (log[drug] vs. response) and IC_{50} were compared by the extra sum-of-squares *F*-test. For some data, correlations were tested and Pearson correlation coefficient given in the text. Parametric and non-parametric data distributions were assessed with the D'Agostino & Pearson normality test. Multiple group and two-group comparisons were performed using appropriate statistical tests for specific data sets (see details in individual figure legends).

RESULTS

The Activity of ATP12A Is Not Different Between CF and Non-CF hAECs

As ATP12A was suggested to be a key regulator of ASL pH (Shah et al., 2016), we investigated its expression and activity in fully differentiated primary cultures of CF and non-CF primary hAECs. Quantitative (q)PCR showed that the level of mRNA expression of *ATP12A* was not different between primary CF and non-CF hAECs (**Supplementary Figure 1**). Because ATP12A has previously been shown to be constitutively active in airway cells (Coakley et al., 2003; Shah et al., 2016; Lennox et al., 2018), inhibition of this pump, by acute exposure to ouabain, would be predicted to cause an intracellular acidification. Therefore, to functionally assess ATP12A activity, we measured the acute effect of increasing concentrations of apical ouabain on intracellular pH (pH_i) in primary CF and non-CF hAECs, loaded with the pH-sensitive dye BCECF-AM (see section "Materials and Methods"). **Figures 1A,B** shows that, as predicted, acute exposure to apical ouabain lead to a dose-dependent, reversible, acidification in both non-CF and CF cultures, respectively. Analysis of the dose-response curves showed that although the maximal change in pH_i

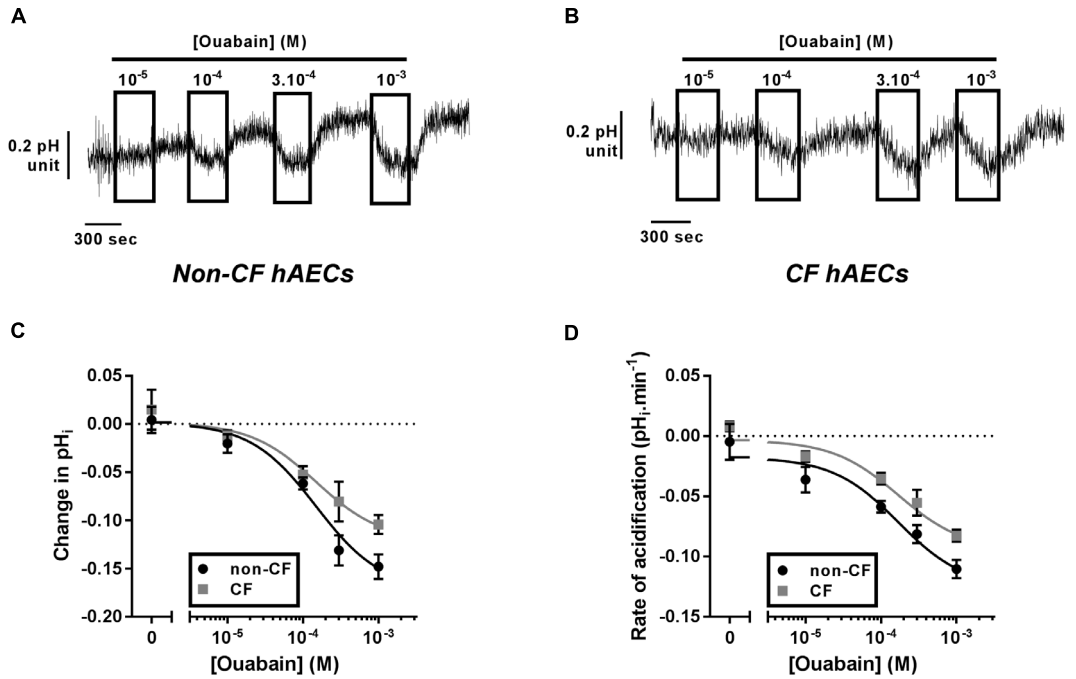

FIGURE 1 | Characterization of ATP12A activity in non-CF and CF primary hAECs. Non-CF and CF hAECS acidify in response to acute apical ouabain. Upper panels show representative traces of pH_i responses to acute increasing apical concentrations of ouabain in non-CF **(A)** and CF **(B)** hAECs. Change in pH_i **(C)** and rate of acidification **(D)** of non-CF (black circles, $n \geq 7$, three donors) and CF (gray squares, $n \geq 5$, five donors) hAECs in response to acute increasing concentrations of apical ouabain. Results show no significant difference in change in pH_i or rate of acidification between non-CF and CF hAECs (non-linear regression with comparison of fits; change in pH_i: $p = 0.51$; rate: $p = 0.64$).

induced by 1 mM ouabain was somewhat lower in CF versus non-CF cells, the IC_{50} calculated from the change in pH_i (**Figure 1C**) as well as from the rate of acidification (**Figure 1D**) induced by ouabain were not significantly different (IC_{50}: ΔpH_i non-CF = 165.2 μM, $n \geq 7$; CF = 102.3 μM, $n \geq 5$, $p = 0.506$; rates of acidification non-CF = 153.6 μM, $n \geq 7$; CF = 217.4 μM, $n \geq 5$, $p = 0.636$). Our results, obtained from 12 independent experiments ($n = 7$ non-CF/2 different donors and $n = 5$ CF/3 different donors), also showed that baseline pH_i was not significantly different between non-CF and CF hAECs. Due to the potential beneficial effect of inhibiting H^+ secretion in CF epithelia, the rest of the study then focused on primary CF hAECs.

Chronic Apical Ouabain Treatment Alkalinized the ASL

In view of the fact that acute treatment using 100 μM ouabain induced a significant intracellular acidification near to the IC_{50}, we then investigated the effect of treating CF cells overnight with 30 to 100 μM ouabain on ASL pH. After overnight incubation, the cells were transferred to a CO_2 and temperature-controlled plate-reader and ASL pH was recorded every 5 min for 2 h before addition of the cAMP agonist forskolin (Fsk) to the basolateral compartment, and ASL pH recorded for a further 4 h. In the vehicle (DMSO)-treated cells, ASL pH was stable over the entire course of the experiment (**Figure 2A**, black line) whereas, ouabain-treated cell cultures showed a slow but steady increase in ASL pH over time (**Figure 2A**, yellow, green, and blue lines). Although increasing concentrations of ouabain appeared to increase ASL pH in a dose-dependent manner it only reached significance with the highest dose tested, 100 μM (**Figures 2A,B**). Surprisingly, and contrary to what other groups have reported (Coakley et al., 2003; Shah et al., 2016) Fsk treatment did not induce ASL acidification in CF cells. As ATP12A is inhibited by ouabain or K^+-free ($0K^+$) solution, we confirmed our results using a different technique. Here, we measured changes in pH_i in response to ouabain and $0K^+$ in the absence or presence of

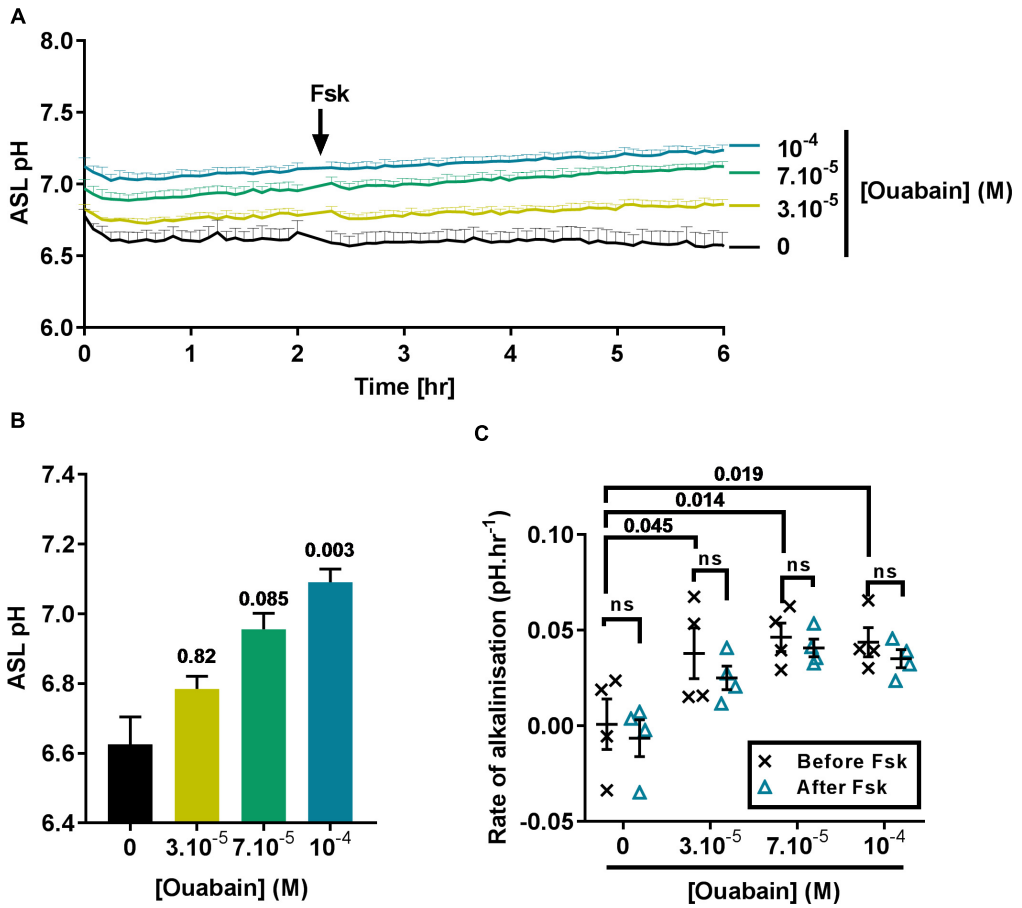

FIGURE 2 | Ouabain increases ASL pH in primary CF hAECs in a dose-dependent manner. CF hAECs were treated overnight with increasing concentrations of apical ouabain and ASL pH was then measured in real time **(A)** under resting conditions and after stimulation with forskolin (Fsk, basolateral, 10 μM) ($n = 4$ in duplicate, three donors; data plotted as mean ± SEM). **(B)** Chronic ouabain increased resting ASL pH. Resting ASL pH was calculated as an average of five points before addition of Fsk (Friedman's test comparing ouabain-treated vs. vehicle-treated). **(C)** CF hAECs treated with ouabain show a slow and continuous increase in ASL pH but do not respond to Fsk. The rate of increase in pH was calculated before and after addition of basolateral Fsk and compared using a two-way repeated measures (RM)-ANOVA with Tukey's test.

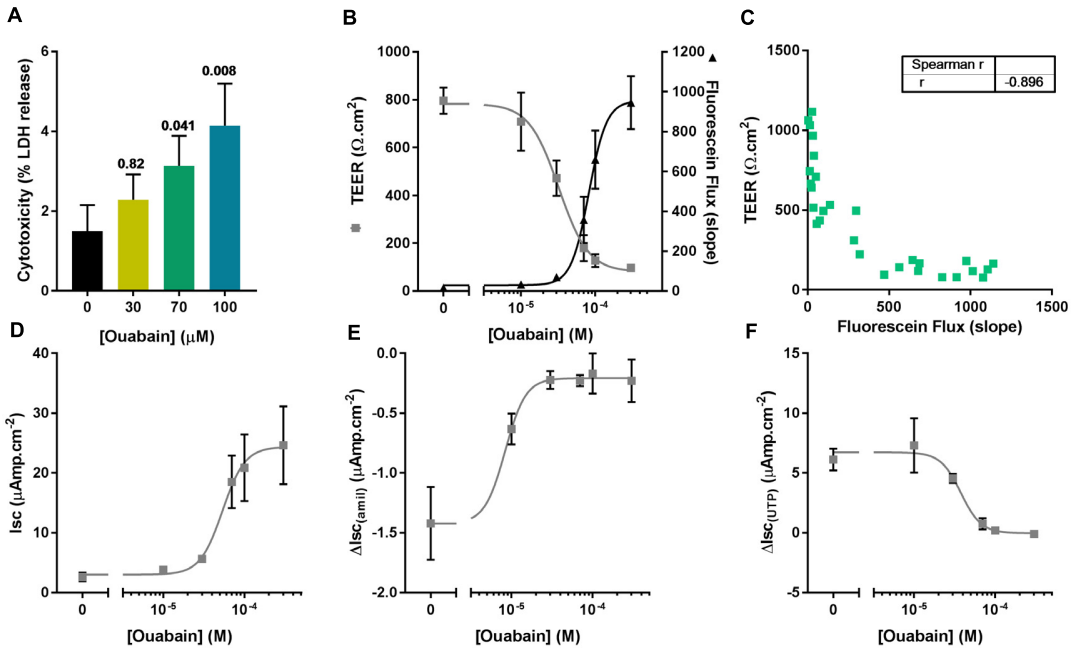

FIGURE 3 | Apical ouabain disrupts epithelial integrity of primary CF hAECs. **(A)** LDH release in response to increasing concentrations of apical ouabain in primary CF hAECs (n = 4, three donors; Friedman's test comparing ouabain-treated vs. vehicle-treated). **(B)** Effect of increasing concentrations of apical ouabain on TransEpithelial Electrical Resistance (TEER) and Fluorescein Flux assessed in Ussing chambers in the presence of a basolateral to apical Cl^- gradient (n = 4). **(C)** Correlation between TEER and Fluorescein Flux (Spearman correlation test: $p < 0.001$). Effect of increasing concentrations of apical ouabain on resting short-circuit current (Isc, **D**, n = 4), amiloride-sensitive Isc ($\Delta Isc_{(amil)}$, **E**, n = 4) and UTP-induced Isc ($\Delta Isc_{(UTP)}$, **F**, n = 4).

10 μM Fsk. Results showed that Fsk itself, (1) did not alter pH_i (**Supplementary Figure 2A**) and (2) did not change the extent or rate of ouabain and $0K^+$-induced acidification (**Supplementary Figures 2A–C**).

As ASL pH slowly increased after addition of Fsk, we determined if ouabain induced changes in CF hAECs that would have sensitized them to Fsk (thereby inducing an increase in ASL pH via CFTR), by measuring the rates of alkalinization before and after addition of Fsk. As shown in **Figure 2C**, the initial rates of alkalinization increased significantly in ouabain treated samples when compared to vehicle-treated cells, but these rates were unchanged after Fsk treatment, suggesting that chronic apical ouabain treatment induced changes that allowed for a slow and gradual increase in ASL pH. We hypothesized that chronic apical ouabain treatment was having a deleterious effect on CF hAECs and therefore assessed the effect of these treatments on epithelial integrity.

Chronic Apical Ouabain Treatment Disrupts Epithelial Integrity by Inhibiting the Basolateral Na^+/K^+-ATPase

We first assessed the potential cytotoxic effect of the same concentrations of ouabain used in the ASL pH experiment by measuring LDH release after overnight exposure to the drug. To do this, the apical surface of CF hAECs was washed with 50 μl of HCO_3^- KRB for 30 min (37°C, 5% CO_2) and LDH was measured in the apical washes as well as the basolateral media. **Figure 3A** shows that 70 and 100 μM ouabain induced a significant increase in LDH release. Even though the percentage released remained under 10%, this increase in LDH release was positively correlated with the increase in ASL pH (Pearson r = 0.5278; p = 0.036). To confirm the deleterious effect of chronic apical ouabain treatment on epithelial integrity, we measured TEER and fluorescein flux across the epithelia as well as the activity of the main ion channels expressed in primary CF hAECs. Increasing concentrations of the ATP12A inhibitor significantly decreased TEER and increased fluorescein flux across the epithelia (**Figure 3B**). These two parameters were inversely correlated as shown in **Figure 3C** (Pearson $R = -0.809$, $p < 0.001$). Resting short circuit current increased with increasing concentrations of ouabain (**Figure 3D**), whereas amiloride-sensitive and UTP-induced currents were inhibited in a dose dependent manner (**Figures 3E,F**, respectively). Fsk-induced and CFTRinh172-sensitive Isc were minimal and remained unchanged after treatment with increasing concentrations of apical ouabain (data not shown).

To investigate if the deleterious effects of apical ouabain treatment were due to an inhibition of the basolateral Na^+/K^+-ATPase, cells were treated overnight with either 70 μM apical ouabain or the equivalent basolateral concentration (30 nM), assuming complete equilibration of the inhibitor from the apical to the basolateral compartment (70 μM × 0.3 μl/700 μl). As shown in **Figure 4**, treatment of CF hAECs with 30 nM basolateral ouabain induced a decrease in TEER (**Figure 4A**), an increase in fluorescein flux (**Figure 4B**) and resting Isc

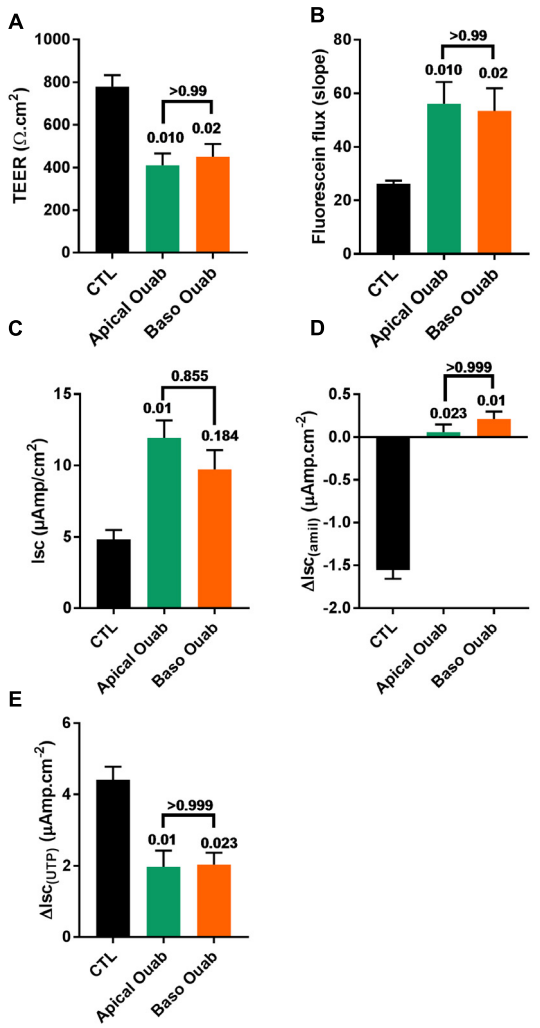

FIGURE 4 | Ouabain (Ouab)-induced epithelial disruption is mimicked by inhibition of the basolateral Na^+/K^+-ATPase in primary CF hAECs. The effect of 50 μM apical ouabain was compared to 30 nM basolateral ouabain on TEER **(A)**, fluorescein flux **(B)**, resting Isc **(C)**, $\Delta Isc_{(amil)}$ **(D)**, and $\Delta Isc_{(UTP)}$ **(E)** (all, $n = 7$, five donors, Friedman's test comparing all conditions).

(**Figure 4C**) as well as an inhibition of amiloride-sensitive (**Figure 4D**) and UTP-induced (**Figure 4E**) changes in Isc, that were not significantly different from the changes induced by the overnight apical treatment with 70 μM ouabain. Taken together, these results show that the potential use of ouabain to target ASL pH in CF is very likely to be deleterious and cytotoxic to airway epithelial cells. To further investigate this, CF hAECs were treated basolaterally with conditioned medium derived from the basolateral compartment from cells treated apically with 30 μM ouabain (for 24 h). This revealed that conditioned medium did not fully reproduce the effects seen with apical exposure alone, which suggests that either ouabain is metabolized during the 48 h incubation or that the cytotoxic effect of ouabain cannot be solely explained by the inhibition of the serosal Na^+/K^+-ATPase (**Supplementary Figures 3A–E**). It was thus of interest to identify molecules that could specifically target apical H^+ secretion, without affecting the Na^+/K^+-ATPase, as a non-cytotoxic strategy to increase ASL pH in CF airways.

Esomeprazole Affects Intracellular pH and Raises ASL pH in CF hAECs

Using Blastn, we found that the mRNA of *ATP12A*, the non-gastric H^+/K^+-ATPase, shares 70% identity with the gastric H^+/K^+-ATPase, ATP4A mRNA, which translates to around 65% identity in the protein products (Blastp). This H^+ pump is targeted in gastric ulcers and gastroesophageal reflux diseases (GERDs) by antacids, and especially PPIs. We therefore investigated the effect of the PPI, esomeprazole (Eso) on pH homeostasis in CF hAECs. It has been previously published that airway epithelial cells do not express the gastric H^+ pump and this was confirmed in our cells in which we found an average Ct value of 37.50 ± 1.53 in CF hAECs and 40 in non-CF hAECs (compared to averaged values of 21.61 ± 0.21 in CF cells and 22.03 ± 0.66 in non-CF cells for *ATP12A*, **Supplementary Figure 4**). We then assessed the effect of increasing concentrations of apical Eso on pH_i and showed that CF hAECs responded in a dose dependent manner (**Figures 5A,B**). To elucidate whether Eso targeted H^+ or HCO_3^- transport, pH_i experiments were performed in the absence (Hepes) or presence of HCO_3^- (HCO_3^- KRB). **Figures 5C,D** show that Eso, as well as ouabain, inhibited H^+ secretion and that the effect of both was enhanced in the absence of HCO_3^-.

As ouabain caused detrimental effects on epithelial integrity, we then evaluated the effect of chronic exposure of CF hAECs to increasing concentrations of Eso on TEER, fluorescein flux and ion channel activity. Concentrations of Eso, ranging from 10 to 300 μM, showed no effect on any of these parameters (**Figures 6A–C**) supporting a non-toxic effect of Eso on CF hAECs.

Considering its effect on pH_i (**Figure 5**) we then investigated the effect of Eso on ASL pH. Primary CF hAECs were treated overnight with 50, 100, or 300 μM Eso and the next day ASL pH was monitored for 2 h before FSK was added basolaterally. Although increasing concentrations of Eso appeared to increase ASL pH in a dose-dependent manner (**Figure 7A**) it only reached significance with the highest dose tested, 300 μM, which raised resting ASL pH by 0.10 ± 0.02 ($p = 0.001$, $n = 8$, paired t-test, **Figures 7A–C**) but did not change the response to Fsk by CF hAECs (**Figure 7D**). This result, taken together with the Ussing chamber experiments in which Eso did not change either Fsk-or UTP induced Isc (**Figure 6C**), suggests that Eso does not increase ASL pH in CF hAECs via an increase in either mutant CFTR, or calcium-mediated anion secretion, but rather, it works via inhibiting proton secretion.

Chronic Esomeprazole-Induced Alkalization of the ASL but Not Acute Intracellular Acidification Is ATP12A Dependent

We then investigated the molecular target of acute and chronic exposure to Eso. Intracellular pH was monitored during exposure

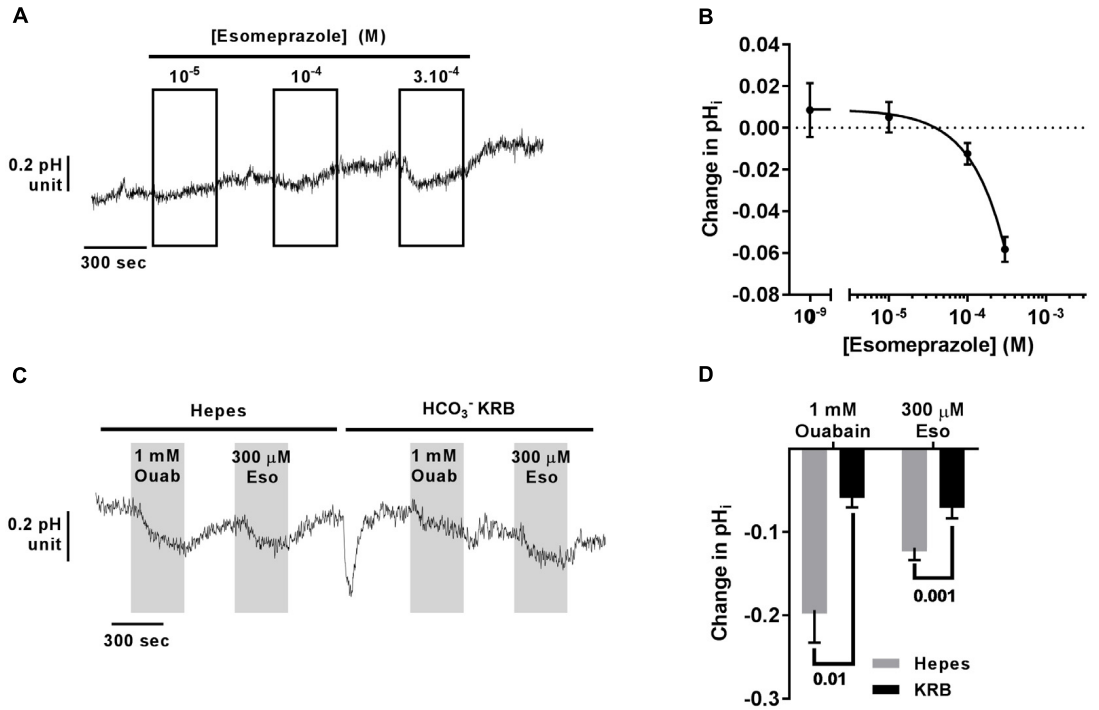

FIGURE 5 | Acute esomeprazole decreases intracellular pH in primary CF hAECs by inhibiting H^+ secretion. **(A)** Representative trace of the effect of increasing concentrations of acute esomeprazole on pH_i. **(B)** Summary data of the esomeprazole dose response on pH_i ($n \geq 7$, three donors). **(C)** Representative trace of the effect of 1 mM apical ouabain (Ouab) or 300 μM apical esomeprazole (Eso) in the absence (Hepes) or presence of HCO_3^- (HCO_3^- KRB). **(D)** Summary data of the effect of 1 mM apical ouabain (Ouab) or 300 μM apical esomeprazole (Eso) in the absence (Hepes) or presence of HCO_3^- (HCO_3^- KRB) presented as mean ± SEM ($n = 3$, two donors, two-way ANOVA with Sidak's post-test).

to acute Eso, $0K^+$, or Eso + $0K^+$. Eso and $0K^+$ induced intracellular acidification in a similar manner (**Figure 8**, $p = 0.28$, $n = 11$, RM ANOVA). However, Eso and $0K^+$ induced a further decrease in pH_i when compared to either $0K^+$ alone (**Figure 8B**, $n = 13$, $p < 0.001$, RM ANOVA) or to Eso alone (**Figure 8B**, $n = 13$, $p < 0.001$, RM ANOVA) suggesting that the acute effect of Eso on pH_i is independent of ATP12A and targets another H^+ transporter. Similar results were found using ouabain (1 mM) instead of $0K^+$ in the same type of experiment (**Supplementary Figure 5**, ouabain vs. Eso+ouab $p = 0.03$; Eso vs. Eso+ouab $p < 0.001$; $n = 10$, RM ANOVA). Furthermore, using CF hAECs in which ATP12A expression was reduced by CRISPR-Cas9 (**Supplementary Figure S6C**), we found that $0K^+$ and ouabain-induced acidification were reduced by 44 and 52%, respectively (**Supplementary Figures 6A,B**) whereas Eso-induced acidification was only decreased by 9% (**Supplementary Figures 6A,B**).

In contrast, we identified ATP12A as a potential target of chronic Eso treatment in CF hAECs. Indeed, RT-qPCR showed that 24 h treatment with 300 μM Eso decreased significantly mRNA levels of *ATP12A* (**Figure 9A**, $p = 0.049$, $n = 7$, paired *t*-test). We confirmed this result using the CRISPR-Cas9 method to knock down *ATP12A* expression. Results showed that in cells where *ATP12A* levels were reduced (**Figure 9B**, −17.1 ± 4.4%, $p = 0.02$, $n = 6$, paired *t*-test), ASL pH increased (**Figure 9C**, $p = 0.003$, $n = 6$, two-way ANOVA) and chronic Eso was not able to induce a further increase in ASL pH (**Figure 9C**, $p = 0.347$, $n = 6$, two-way ANOVA).

DISCUSSION

In the past few years, there has been increasing evidence that extracellular ASL pH plays a major role in airway homeostasis and defense against inhaled pathogens. Thus an acidic pH was shown to reduce bacterial killing (Pezzulo et al., 2012), increase ENaC activity and therefore ASL dehydration (Garland et al., 2013; Tan et al., 2014) and increase mucus viscosity (Tang et al., 2016). An acidic ASL in CF airways can be explained by a defect in HCO_3^- secretion (due to defective CFTR) and/or an unregulated H^+ secretion. Recently, it was shown that ATP12A, was responsible for this unchecked H^+ secretion in human and pig CF airways and accounted for the increased mucus viscosity and decreased bacterial killing ability (Shah et al., 2016) promoting this pump as a valid target for CF airway disease.

We have shown that inhibition of the non-gastric H^+/K^+-ATPase using ouabain concentrations close to the IC_{50} significantly increased ASL pH in CF hAECs. However, this increase in ASL pH was not stable after overnight incubation with the drug and a slow, but constant, alkalinization was observed throughout the ASL pH measurement. This led us to suggest

that upon chronic treatment with apical ouabain, the epithelia were becoming leaky, allowing for the equilibration of the pH between the basolateral (pH 7.4) and the apical compartments. In kidney epithelia, it has been shown that chronic treatment (3 days) with a low dose (1 μM) of ouabain induced the disassembly of tight-junction proteins as well as cell adhesion associated protein, leading to cell to cell and cell to surface detachment (Larre et al., 2010; Rincon-Heredia et al., 2014). Using a shorter treatment time frame, we showed that ouabain disrupted CF airway epithelial integrity in a dose-dependent manner, leading to an increase in LDH release, fluorescein flux as well as a profound drop in transepithelial resistance. It was also linked to a decrease in ENaC-mediated and calcium-activated transepithelial transport, but not to an increase in CFTR conductance (data not shown). The lack of effect of ouabain on CFTR in CF cells is in contrast to the study from Zhang et al. (2012) which showed that 24 h treatment with low concentrations of cardiac glycosides, including ouabain, induced trafficking of the F508del-CFTR to the cell surface in a cell line over-expressing the mutated CFTR, mimicking the effect of low temperature incubation (Zhang et al., 2012). This may be explained either by the difference in concentrations used (0.1 vs. 10–300 μM) or by a difference in the cell models. Indeed, the cell line used in Zhang's study overexpressed F508del-CFTR and it is therefore more likely to detect small changes in CFTR trafficking and/or transport activities and report effects that do not happen in a non-overexpressing systems. Our experiments were performed on primary CF hAECs from three different donors (all F508del homozygous). Inter-donor variability was compensated for by doing the statistical analysis using paired/repeated measure settings and therefore we are confident about the lack of effect of ouabain on mutant CFTR activity.

Surprisingly and contrary to what other groups have published (Shah et al., 2016), forskolin did not appear to stimulate ATP12A and acidify the ASL (**Figures 2A**, **7A,D**). We confirmed our results, by measuring the activity of ATP12A (using apical $0K^+$ or ouabain) in the presence or absence of forskolin. The results showed that (i) forskolin did not induce any change in pH_i (**Supplementary Figure 2**) and (ii) the ouabain or $0K^+$-induced changes in pH_i were not significantly different whether forskolin was present or not, indicating that ATP12A activity was not altered by a rise in cAMP. The study by Shah et al. (2016) showed that Fsk and IBMX induced CF ASL acidification, but did not explicitly demonstrate the role of ATP12A in this process in *human* cells. Moreover, in cultured porcine airway epithelial cells, ouabain only partially inhibited the cAMP-induced acidification. Although we cannot ascertain the reason behind this discrepancy, differences in the methodology in assessing ASL pH or culture conditions could potentially explain it.

Several groups have studied the *in vivo* effect of cardiac glycosides, including ouabain, in inhaled form, on bronchial reactivity in asthma (Agrawal et al., 1986; Knox et al., 1988; Hulks and Patel, 1989). Indeed in the 1980s, a study conducted in England and Wales showed a correlation between asthma mortality and dietary salt intake (Burney, 1987) and it was thought that inhibiting Na^+/K^+-ATPase would increase the intracellular concentration of Na^+, inhibiting Na^+-Ca^{2+} exchange, and thereby increasing contractility of smooth muscle cells. In one study, Agrawal et al. (1986) showed that a low dose of ouabain-induced bronchodilatation in 6 out of 10 asthmatic subjects, whereas higher doses induced bronchoconstriction. However, two more studies reported no effect of inhaled ouabain on FEV_1 or bronchial reactivity to histamine in asthmatic patients (Knox et al., 1988; Hulks and Patel, 1989). Only one study has reported the effect of topical ouabain in the airways of CF patients (originally to study the role of the Na^+/K^+-ATPase on Nasal Potential Difference (NPD) in CF nasal epithelium)

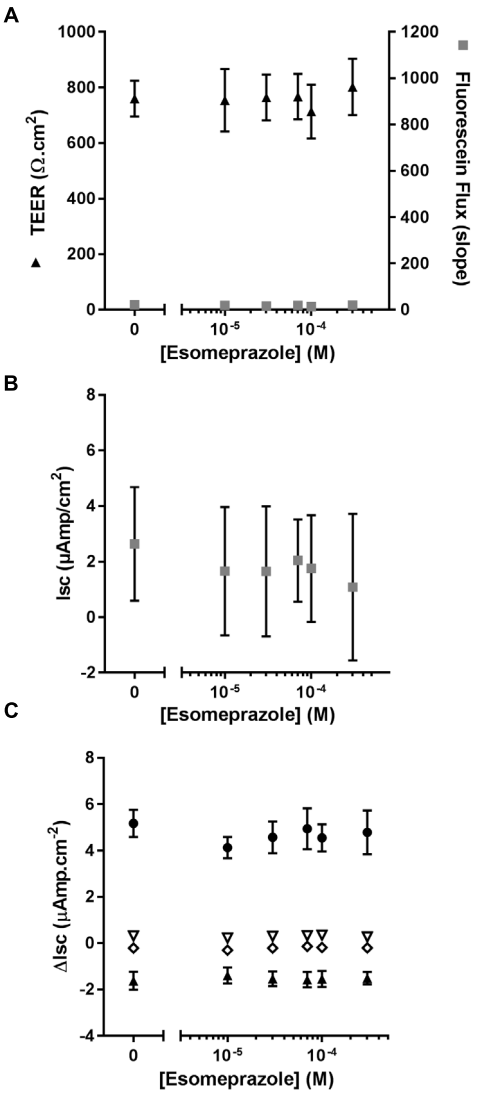

FIGURE 6 | Chronic esomeprazole does not disrupt epithelial integrity of CF hAECs. **(A)** Effect of increasing concentrations of apical esomeprazole on TransEpithelial Electrical Resistance (TEER) and Fluorescein Flux measured in Ussing chambers in the presence of a basolateral to apical Cl^- gradient (n = 5, five donors). **(B,C)** Effect of increasing concentrations of apical eso on resting short-circuit current (Isc, **B**, n = 5), and **(C)** amiloride-sensitive Isc ($\Delta Isc_{(amil)}$, black triangle, n = 5), Fsk-induced Isc ($\Delta Isc_{(Fsk)}$, open triangle, n = 5), CFTRinh172-sensitive Isc ($\Delta Isc_{(172)}$, open diamond, n = 5) and UTP-induced Isc ($\Delta Isc_{(UTP)}$, black circles, n = 5).

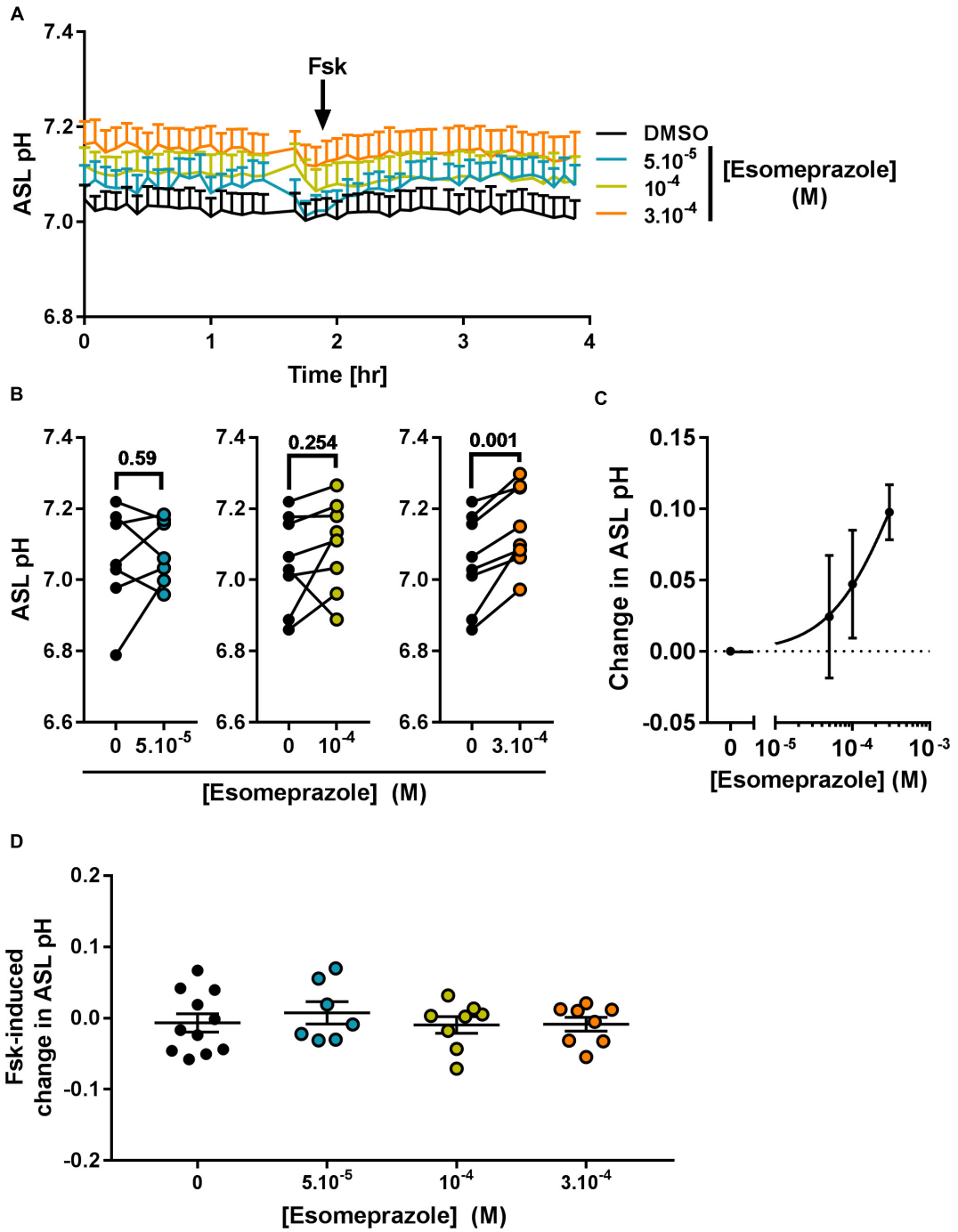

FIGURE 7 | Chronic esomeprazole increases ASL pH in primary CF hAECs. CF hAECs were treated overnight with 50, 100, or 300 μM apical esomeprazole and ASL pH was measured under resting condition (**A–C**, $n \geq 7$, four to six donors, paired *t*-test) and after stimulation with forskolin (**A,D**, Fsk, basolateral, 10 μM, $n \geq 7$, four to six donors, ANOVA with Dunnett's post-test). **(A)** Mean kinetic data of ASL pH of CF hAECs treated with increasing concentrations of esomeprazole. **(B)** Paired data of ASL pH for each independent experiment after treatment with 50 μM (left panel), 100 μM (middle panel), or 300 μM (right panel) of Eso. **(C)** Dose response of ASL pH in response to Eso. **(D)** Forskolin-induced changes in ASL pH in cells treated with 50, 100, 300 μM esomeprazole.

and showed no effect on NPD in people with CF and control subjects (Peckham et al., 1995). However, this was done over a short period of time: NPD was measured every 15 min for an hour after application of ouabain. This time-frame would be too short to observe any effect on pH. Taken together with our results, these studies suggest that ouabain is not a good candidate to target pH homeostasis as a means to improve CF airway disease.

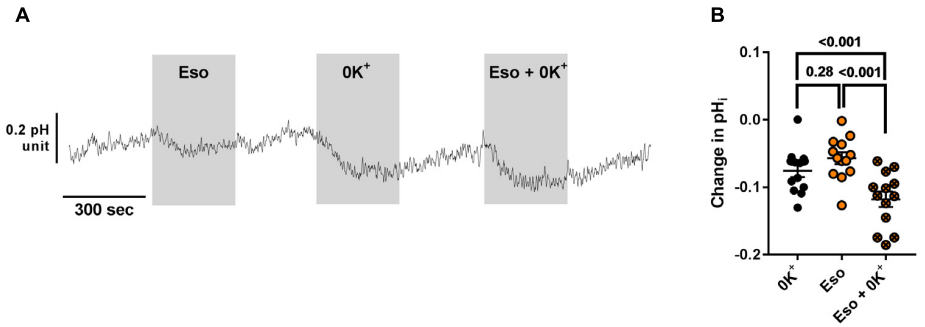

FIGURE 8 | Additive effect of acute esomeprazole and K^+-free solution on pH_i. **(A)** CF hAECs were exposed acutely to apical esomeprazole (Eso, 300 μM), K^+-free solution ($0K^+$) or both. **(B)** Summary data of the effect of Eso and K^+ free solution on pH_i (n = 11, four donors, Repeated Measures (RM)-ANOVA with Tukey post-test).

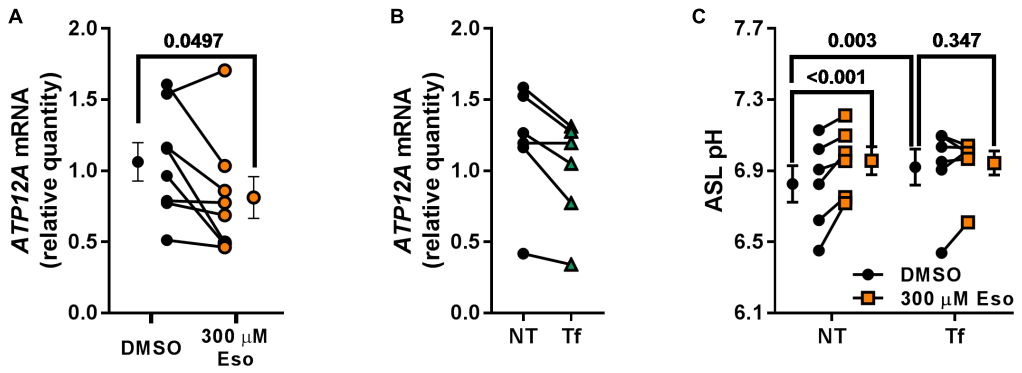

FIGURE 9 | Chronic esomeprazole treatment targets ATP12A. **(A)** CF hAECs were treated for 24 h with 300 μM apical esomeprazole (Eso) and *ATP12A* mRNA levels measured by RT-qPCR (n = 8, four donors, paired t-test). **(B)** CF hAECs were transfected with the CRISPR-Cas9 vector, PX462, containing gRNA sequences targeting upstream and downstream of *ATP12A* exon 2. ATP12A knock-down was confirmed by RT-qPCR which showed a decrease of 17.1 ± 4.4% (NT: non-transfected, Tf: Transfected; n = 6, four donors, paired t-test). **(C)** CF hAECs transfected or not were treated overnight with 300 μM Eso and ASL pH was monitored the following day (n = 6, four donors, two-way ANOVA with Tukey post-test).

Because of the significant sequence homology between different members of the proton/potassium ATPase family, and the fact that PPIs inhibit the gastric ATPase by binding to cysteine residues, we hypothesized that these inhibitors could also target the non-gastric H^+/K^+-ATPase. To test this we investigated the effect of the PPI esomeprazole. It belongs to the second generation of PPIs, which are more stable than the 1st generation, and remain bound to the gastric H^+/K^+-ATPase, thus inhibiting H^+ secretion until new proteins are synthesized (Andersson et al., 2001). Importantly, our results showed that esomeprazole increased the ASL pH in CF hAECs, and furthermore, that this was not accompanied by any deleterious effects as observed with ouabain treatment. Although the intracellular acidification induced by acute esomeprazole exposure appeared to be independent of the non-gastric H^+/K^+-ATPase, its chronic effect on ASL pH was linked to a decrease in *ATP12A* mRNA expression (**Figure 9A**) and CRISPR-Cas9 experiments suggested that esomeprazole targeted the non-gastric H^+/K^+-ATPase in order to increase ASL pH (**Figure 9B,C**). Interestingly, the inhibitory effect of PPIs on ATP12A has been reported as a potential therapeutic approach in treating the inflammatory process in chronic rhinosinusitis (Min et al., 2017). PPIs are mainly prescribed for GERD and the association between GERD and lung diseases has been established although the mechanism of action and causality have not. Accordingly, there has been an interest in the effect of PPI on chronic lung diseases and although results are controversial (Laheij et al., 2004; Filion et al., 2014; Benson et al., 2015; Lee et al., 2015; Othman et al., 2016), PPIs appear to have anti-inflammatory properties in COPD and IPF (Ghebremariam et al., 2015; Xiong et al., 2016). One of the hallmarks of the lung pathophysiology in CF is the chronic inflammation. It is characterized by an excessive neutrophilic infiltration of the airways, abundant protease (neutrophil elastase (Le Gars et al., 2013) and bacterial elastase (Saint-Criq et al., 2018)) and elevated levels of IL-1 and IL-8 in the CF airways lumen (Konstan et al., 1994; Balough et al., 1995; Armstrong et al., 1997; Montgomery et al., 2018). We can therefore hypothesize that the use of esomeprazole in CF could also dampen the chronic inflammation observed in CF airways. In addition to anti-inflammatory properties, PPIs have shown antioxidant features in various cell types, another hallmark of the CF airway pathophysiology mainly due to the large neutrophilic infiltration.

In vitro, the first generation PPI, omeprazole, was shown to scavenge hypochlorous acid (Lapenna et al., 1996) and *in vivo*, this PPI prevented stress-induced ulcer formation by blocking the generation of *OH (Biswas et al., 2003), whereas esomeprazole prevented the depletion of the antioxidant glutathione induced by indomethacin in rat gastric mucosa (Pastoris et al., 2008). PPIs have also shown antimicrobial properties. *In vitro*, esomeprazole decreased *Pseudomonas aeruginosa* and *Staphylococcus aureus* biomass and increased killing by conventional antibiotics. It also largely inhibited biofilm formation by these two bacterial species (Singh et al., 2012). Sasaki also reported an antiviral effect of lansoprazole in tracheal cells by reducing their susceptibility to rhinovirus infection (through a decrease in ICAM-1 that serves as the receptor for rhinovirus) (Sasaki et al., 2005). Although we have not determined the effects of chronic esomeprazole on ASL hydration, mucus viscosity or bacterial killing in primary CF hAECs, other groups have reported that small changes in ASL pH (<0.2 pH) were linked to an increased bactericidal activity and a decreased mucus viscosity in human non-CF airway cells (Shah et al., 2016). This suggests that the changes in ASL pH we have observed could have beneficial effects in CF airway disease.

In CF, 80 to 90% of patients have silent or symptomatic gastroesophageal reflux (Ledson et al., 1998; Button et al., 2005; Blondeau et al., 2008) and therefore a large proportion of these patients use PPIs. PPIs serve to reduce acid reflux but also compensate for the lack of gastrointestinal secretion (due to defective CFTR), and thereby enhance the benefit of pancreatic enzyme replacement therapy. Indeed, several studies have shown the beneficial effect of PPI use in children with CF when looking at steatorrhea and nutritional status (Tran et al., 1998; Proesmans and De Boeck, 2003), and it is well acknowledged that in these cases, PPIs such as omeprazole and lansoprazole, show improvement in fat absorption. However, the effect of PPI on pulmonary function is more controversial. PPIs decrease acid content of gastric juice and Palm et al. (2012) showed that there was a negative correlation between non-acid reflux burden and FEV_1. Another study, looking at children with CF between 2009 and 2014, reported that PPI use was associated with a decline in percent predicted (pp)FEV_1 as well as an increase in future pulmonary exacerbation rate (van Horck et al., 2018). This was also reported in adults with CF in which PPI use was associated with an increase in the number of hospitalizations due to pulmonary exacerbations (Ayoub et al., 2017). However, by differentiating those on gastric acid inhibitors because of GERD, from those taking PPIs because of fat malabsorption, van der Doef et al. (2009) showed that only the children with CF taking gastric acid inhibitors because of GERD presented a reduced pulmonary function. This challenges the role of PPIs in pulmonary exacerbations in CF and suggests that GERD rather than PPI use is involved in pulmonary decline.

Since our current study showed a beneficial effect of esomeprazole on CF airway ASL pH homeostasis, and considering the other potential benefits of PPIs against bacterial and viral infections, oxidative stress and inflammatory processes, we propose that inhaled use of PPIs in CF airways might alleviate CF lung pathophysiology. Interestingly, novel H^+/K^+-ATPase inhibitors, termed P-CABs (Potassium-Competitive Acid Blockers), such as Vonoprazan, are more potent than PPIs for eradication of clarithromycin-resistant *Helicobacter pylori* (Li et al., 2018). Thus it will be interesting to test the effects of these new H^+ pump inhibitors on inflammation, infection, and pH homeostasis in CF airways.

ETHICS STATEMENT

The cells were obtained under protocol #03-1396 approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board.

AUTHOR CONTRIBUTIONS

LD, JT, AY, and SR performed experiments and analyzed data. MG designed and performed experiments, provided reagents, analyzed data and edited the manuscript. VS-C designed and performed experiments, analyzed data and wrote the manuscript.

FUNDING

This work was supported by two CF Trust Strategic Research Centre grants (SRC003 and SRC013) and a Medical Research Council (MRC) Confidence in Concept grant (MC_PC_15030). SR was supported by Versus Arthritis (grant 20771), and by the MRC and Arthritis Research UK as part of the Centre for Integrated Research into Musculoskeletal Ageing (CIMA, grant references JXR 10641, MR/P020941/1 and MR/R502182/1). Cells from Dr. Randell were supported by Cystic Fibrosis Foundation grant (BOUCHE15R0) and NIH grant (P30DK065988).

ACKNOWLEDGMENTS

We wish to thank Dr. Robert Tarran for the advices on setting up the ASL pH measurements. We would like to acknowledge Drs. Scott H. Randell and Leslie Fulcher (Marsico Lung Institute, The University of North Carolina at Chapel Hill, United States) for providing CF primary airway epithelial cells from the CFFT collection and CF and non-CF primary airway epithelial cells from the UNC CF Center Tissue Procurement and Cell Culture Core.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01462/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

MINI REVIEW
published: 07 November 2018
doi: 10.3389/fphar.2018.01270

Elucidating the Interaction of CF Airway Epithelial Cells and Rhinovirus: Using the Host-Pathogen Relationship to Identify Future Therapeutic Strategies

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OPEN ACCESS

Edited by:
Frederic Becq, University of Poitiers, France

Reviewed by:
Richard B. Moss, Stanford University, United States
Raymond Pickles, University of North Carolina at Chapel Hill, United States

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *31 July 2018*
Accepted: *17 October 2018*
Published: *07 November 2018*

Citation:
Ling K-M, Garratt LW, Lassmann T, Stick SM, Kicic A, WAERP, AusREC and Australian Respiratory Early Surveillance Team for Cystic Fibrosis (2018) Elucidating the Interaction of CF Airway Epithelial Cells and Rhinovirus: Using the Host-Pathogen Relationship to Identify Future Therapeutic Strategies. Front. Pharmacol. 9:1270. doi: 10.3389/fphar.2018.01270

Chronic lung disease remains the primary cause of mortality in cystic fibrosis (CF). Growing evidence suggests respiratory viral infections are often more severe in CF compared to healthy peers and contributes to pulmonary exacerbations (PEx) and deterioration of lung function. Rhinovirus is the most prevalent respiratory virus detected, particularly during exacerbations in children with CF <5 years old. However, even though rhinoviral infections are likely to be one of the factors initiating the onset of CF lung disease, there is no effective targeted treatment. A better understanding of the innate immune responses by CF airway epithelial cells, the primary site of infection for viruses, is needed to identify why viral infections are more severe in CF. The aim of this review is to present the clinical impact of virus infection in both young children and adults with CF, focusing on rhinovirus infection. Previous *in vitro* and *in vivo* investigations looking at the mechanisms behind virus infection will also be summarized. The review will finish on the potential of transcriptomics to elucidate the host-pathogen responses by CF airway cells to viral infection and identify novel therapeutic targets.

Keywords: cystic fibrosis, airway epithelium, rhinovirus, innate immune response, therapy, transcriptomic

RESPIRATORY INFECTIONS IN THE CYSTIC FIBROSIS LUNG

Chronic obstructive lung disease remains the primary cause of mortality and morbidity in CF (Cutting, 2015). The defective function of the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene initiates a lifelong cycle of neutrophilic inflammation, progressive bronchiectasis, mucus obstruction and recurrent microbial infection of the CF airway. These processes typically begin in the first years of life and lead to eventual lung failure during early adulthood. The CF airway environment is vulnerable to colonization by particular bacterial

and fungi species including *Haemophilus influenzae*, *Staphylococcus aureus, Aspergillus fumigatus,* and *Pseudomonas aeruginosa* (Gangell et al., 2011). Infection by these common pathogens typically trigger neutrophilic responses, however, these fail to eradicate the infection and lead to a sustained release of oxidants and proteases, particularly neutrophil elastase (NE) (Hartl et al., 2007; Painter et al., 2008). This neutrophil-based inflammation has been associated with the progression of structural abnormalities specifically bronchiectasis and air trapping, from as early as 3 months of age (Mott et al., 2012; Sly et al., 2013).

In addition to colonization by bacteria and fungi, the CF airway will be infected with respiratory viruses and viral infections are a major cause of PEx in the pediatric CF population (Goffard et al., 2014; Dijkema et al., 2016). The significance of viral infections in CF has been identified by the advancements in molecular diagnostic technologies to detect virus (Wat, 2015). The prevalence of respiratory viruses during CF PEx can vary from 5% up to 60% (Billard et al., 2017) and include; rhinovirus (RV), influenza A and B, respiratory syncytial virus (RSV), parainfluenza (PIV; Type 1–4), metapneumovirus, coronavirus and adenovirus (Waters and Ratjen, 2015; Flight and Jones, 2017). Earlier work suggested influenza viruses (A & B) (Pribble et al., 1990; Hiatt et al., 1999) and RSV (Abman et al., 1991; Armstrong et al., 1998) were the major cause of PEx in CF. However, studies utilizing more sensitive virological methods in the last 5 years have comprehensively established RV as the most common respiratory virus detected in CF airway (Burns et al., 2012; Wark et al., 2012; Kieninger et al., 2013; Etherington et al., 2014; Dijkema et al., 2016; Stelzer-Braid et al., 2017). Despite numerous studies into the virology of CF airways, the mechanistic link between virus infection, airway inflammation and structural lung disease remains largely unknown. Further investigation into the interaction of these disease components is warranted.

IMPACT OF RV INFECTION IN CF LUNG

A member of the *Picornaviridae* family within the Enterovirus genus, RV features a positive sense single stranded RNA genome ∼7.2 kb in length. The airway epithelium is the primary site of RV infection and replication (Vareille et al., 2011). As reviewed by Palmenberg and Gern (2015), 11 viral proteins form the non-enveloped icosahedral structure. The external capsid proteins comprise of VP1, VP2, VP3, while VP4 is located between the interface of capsid protein and RNA genome. These capsid proteins feature a high degree of heterogeneity and consequently the significant antigenic diversity among RV has precluded vaccine development (Glanville and Johnston, 2015; Lewis-Rogers et al., 2017). Currently, there are more than 150 serotypes of RV, which have been classified into 3 species; RV-A, RV-B, and RV-C. Within RV-A and RV-B, strains are clustered into major and minor RV groups based upon their specificity for the intracellular adhesion molecule (ICAM-1) receptor or low-density lipoprotein receptor (LDLR), respectively (Palmenberg, 2017). Recently, the cell receptor for RV-C species has been putatively identified as cadherin related family member 3 (CDHR3), whose expression is largely confined to ciliated cells (Bochkov et al., 2015; Griggs et al., 2017; Palmenberg, 2017).

Rhinovirus infections occur all year round and children experience on average six to eight episodes per year (Worrall, 2011). Although the "common cold" is largely self-limiting, it still poses a burden on the activity and productivity of the general population (Stein, 2017). Additionally, RV infection has a more pronounced effect on vulnerable individuals such as children with CF, as summarized in **Table 1**. These include increased PEx (Asner et al., 2012), more severe respiratory symptoms (Burns et al., 2012; Wark et al., 2012), greater inflammation (Kieninger et al., 2013), reduced quality of life and hospitalization and prolonged antibiotic treatment (Smyth et al., 1995). Prevalence and symptoms of RV infection in patients with CF can vary between cohorts (reviewed by Billard et al., 2017), with some reporting similar rates of RV detection in both children with and without CF (de Almeida et al., 2010; Esposito et al., 2014), while others have reported significant correlations with disease progression in those with CF (Hiatt et al., 1999; van Ewijk et al., 2005). Other features such as age preference, RV serotype, viral load, impact on lung function were assessed in several studies. Susceptibility to particular RV serotype in children with CF requires further investigation due to inconsistent observations (de Almeida et al., 2010; Shah et al., 2015). RV load has been observed to be significantly higher in children with CF (>100 times) when compared to healthy controls and children with asthma (>10 times; Kieninger et al., 2013). This study also illustrated that viral load was negatively correlated to pulmonary function (Kieninger et al., 2013). Cousin et al. (2016) observed that RV-induced PEx in children with CF resulted in failure of pulmonary function recovery for up to 6 weeks. An age preference for RV-associated CF exacerbations has also been reported for young children <5 years old (Stelzer-Braid et al., 2017). However, several other studies have detected a higher frequency of RV in upper and lower airway of adults with CF via screening of sputum and throat swabs (Etherington et al., 2014; Goffard et al., 2014). Adults with CF who have viral associated PEx have been shown to have worse lung function and require more days of intravenous antibiotic treatment (Flight et al., 2014; Goffard et al., 2014). Others have also reported that adult patients who are less responsive to treatment are re-admitted for a subsequent exacerbation within a shorter time frame (Etherington et al., 2014). Finally, Flight et al. (2014) found that RV infection in adults is accompanied by an increased risk of PEx, prolonged antibiotic prescription, higher respiratory symptom scores and heightened level of C-reactive protein. As RV has a large clinical impact on those with CF, it is critical to elucidate how this virus alters host antiviral and inflammatory responses.

AIRWAY EPITHELIUM AND RV INFECTION IN CF

A pseudostratified epithelium lines the surface of the lung (trachea, primary bronchi, secondary bronchi, tertiary bronchi, and bronchioles) and is composed of several cell types including ciliated cells, basal cells, secretory cells and goblet cells. These

TABLE 1 | Summary of clinical studies and outcomes related to Human Rhinovirus infection in CF.

References	Cohorts	Sample Type	Virus Detection								Clinical Data		
			Positive Samples	Detection Method	RV (%)	Influenza (%)	RSV (%)	Parainfluenza (%)	Adenovirus (%)	Others	FEV1	Antibiotic	Hospitalization
Smyth et al., 1995	CF patients (mean age 7.9 years)	Nasopharyngeal Aspirate, Serum Specimen	44/157	Virus Immunofluorescene, Culture, Serology and PCR	58	12	9	12	9	NR	Higher than other virus induced exacerbation	Prolonged treatment	No Difference
Collinson et al., 1996	CF patients (median age 7.3 years)	Nasopharyngeal Specimens	51/119	Virus Culture, PCR	41	NR	NR	NR	NR	NR	Significantly lower	More oral and intravenous antibiotic treatments for those with more infection annually	No Difference
Armstrong et al., 1998	80 infants diagnosed with CF before 12 months of age (31 infatns were hospitalized for persistent respiratory symptoms	BALs/ Nasopharyngeal Samples	14/26	Virus Immunofluorescene, Culture	14.3	14.3	43	28.5	NR	NR	NR	Not for virus infection	Higher
Hiatt et al., 1999	22 infants <2 years of age with CF (30 patient-seasons) and 27 age-matched controls (28 patient-seasons) participated	Nasopharyngeal samples	26/150	Serology, Culture Inoculation	NR	30	23	17	17	27% Picornavirus	NR	NR	RSV infection CF infants has higher rate of hospitalization
Olesen et al., 2006	75 children (media age 8)	Sputum, Laryngeal aspirations	96/606	PCR	87	3	2	6	2	NR	Significantly lower (when excluding HRV infection)	8 patients received antibiotic treatments	NR
Wat et al., 2008	71 CF patients (median age 9)	Nasal swabs and sputum samples	63/138	NASBA	15.9	15.2	2.9	10.9	NR	1% Coronavirus, 36.2% Any	NR	NR	NR
de Almeida et al., 2010	103 CF patients (median age 8.9)	Nasopharyngeal aspirates and nasal mucus specimens, sputum and oropharyngeal samples	203/408	PCR	34.1	1.2	3.7	0.6	0.2	5.9% Enterovirus, 5.6% Human Bocavirus, 4.7 Human Coronavirus, 0.7% Human metapneumovirus	NR	NR	NR

(Continued)

TABLE 1 | Continued

References	Cohorts	Sample Type	Virus Detection								Clinical Data		
			Positive Samples	Detection Method	RV (%)	Influenza (%)	RSV (%)	Parainfluenza (%)	Adenovirus (%)	Others	FEV1	Antibiotic	Hospitalization
Asner et al., 2012	112 CF patients	Mid-turbinate swabs, sputum, throat swab	26/43	Immunofluroescene, multiplex PCR	23	7.6	35	15.4	11.5	34.6% Coxsackie/echovirus, 15.4% Coronavirus, 7.7% Human Metapneumovirus	No Difference	No Difference	No Difference
Stelzer-Braid et al., 2012	37 Participants (median age of 11.4) with CF	Nasal swabs and sputum samples	17/37	Multiplrc PCR	35	2.7	2.7	10.8	NR	2.7% Metapneumovirus; 46% has more than one viral or bacteria pathogen	NR	NR	NR
Kieninger et al., 2013	299 Children (median age 8.2). 195 children with CF (88 stable, 107 exacerbation), 40 children with Non CF Bronchiectasis, 29 children with Asthma and 35 Control Subjects	BALs	73/299	PCR	24.4	NR	NR	NR	NR	NR	Inversely associated with RV load	Increase use of antibiotic when increase respiratory symptoms were recorded	NR
Goffard et al., 2014	46 patients (median age of 29)	Sputum	16/64	PCR	24	3	3	3	NR	8% Coronovirus	No Difference	No Difference	NR
Esposito et al., 2014	47 CF patients with acute pulmonary exacerbation (median age of 16.7) and 31 CF patients in stable clinical condition (median age of 17.3)	Nasopharyngeal Swabs	23/78	PCR	61	17.4	4.3	NR	NR	8.6% Bocavirus, 4.3% Metapeumovirus, 4.3% Enterovirus	No Difference	NR	NR

(Continued)

TABLE 1 | Continued

References	Cohorts	Sample Type	Virus Detection								Clinical Data		
			Positive Samples	Detection Method	RV (%)	Influenza (%)	RSV (%)	Parainfluenza (%)	Adenovirus (%)	Others	FEV1	Antibiotic	Hospitalization
Etherington et al., 2014	180 patients participated ion treatment with intravenous antibiotics for an acute pulmonary exacerbation. 42 patients (media age 26.5) with positive viral detection	Viral Throat Swabs	42/432	PCR	69	19	2.4	4.8	2.4	2.4% Metapneumovirus	Significantly Lower	Intravenous antibiotic for longer period	NR
Flight et al., 2014	100 adults with CF (median age of 28)	Sputum, Nose Swabs and Throat Swabs	191/626	PCR	72.5	6.1	2	2.5	4.1	13.2% Human Metapneumovirus	Lower acute fall in FEV1	Increase number of prescription	NR
Dijkema et al., 2016	20 Children with CF (0–7 years) and age matched healthy control	Nasopharyngeal Swabs	161/352 (only HRV was tested)	Nested PCR, Southern Blotting and Sequencing	45.7	NR	NR	NR	NR	NR	NR	Increase use of antibiotic prophylaxis	NR
Stelzer-Braid et al., 2017	110 children with CF	upper (nasal swab, oropharyngeal suction, and sputum) and lower (bronchoalveolar washings) respiratory tract	59/263 (< 5 years old); 23/202 (older children) only HRV was tested	PCR, Nested PCR	43% (< 5 years old); 12% older children	NR	NR	NR	NR	NR	NR	NR	NR

airway epithelial cells form the first point of contact with inhaled environmental insults, including respiratory viruses. To provide a physical barrier against particulates/pathogens from entering the lung tissue, numerous cell-cell connections are formed including tight junctions, adherent junctions, gap junctions, and desmosomes (Whitsett and Alenghat, 2015). To clear inhaled particles/pathogens, intraepithelial goblet cells, and submucosal glands mucous cells secret mucins. These large glycoproteins bind matter including microbes and allows effective cough clearance by the mucociliary escalator (Foster, 2015). Mucins are transported from the bronchioles to the trachea via beating cilia, expressed by airway epithelial cells of the luminal airway surface (Ma et al., 2018).

Perhaps more significantly for viral pathogens, the airway epithelium plays a crucial role in innate immunity. It has been suggested that the inflammatory responses induced by airway epithelial cells give rise to associated clinical symptoms (Jacobs et al., 2013). RVs can disrupt epithelial tight junctions including zona occludens 1 (ZO-1) protein by stimulating the production of reactive oxygen species (ROS) during viral replication (Unger et al., 2014). Work utilizing airway epithelial cells *in vitro* have shown reduced expression of other tight junction proteins, loss of epithelial integrity, disruption of extracellular matrix and subepithelial fibrosis and induction of proangiogenic molecules which enhance angiogenesis and airway remodeling (Bossios et al., 2005; Leigh et al., 2008; Bochkov et al., 2010; Tacon et al., 2010; Yeo and Jang, 2010; Looi et al., 2018).

The uptake of RV via clathrin-dependent or -independent endocytosis or through micropinocytosis occurs when RV binds to its specific receptors. Upon binding and in a low-pH environment, uncoating of RVs occurs and the virus undergoes conformational changes. The loss of the protein capsid protein VP4, and the externalization of the hydrophobic N-terminal of VP1, facilitates RVs to cross the host cell membrane (Jacobs et al., 2013; Blaas and Fuchs, 2016). Following viral uncoating and membrane rupture, RV "pathogen-associated molecular patterns" (PAMP) are recognized by the host cell via interaction with pattern recognition receptors (PRRs) including; Toll like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain-like receptors (NLRs). The signaling pathways induced by TLRs and RLRs are typically host defense antiviral pathways as well as the production of antiviral substances, namely IFNs, B-defensins (Proud et al., 2004), and nitric oxide (Sanders et al., 1998). The airway epithelium also responds to RV infection by activating pro-inflammatory signaling pathways which trigger the release of chemokines and cytokines including IL-8, RANTES/CCL5, and granulocyte-macropahge colony-stimulating factor (GM-CSF), that in turn recruit neutrophils, esoinophils, natural killer cells (NK cells), and macrophages to the infected tissue. IL-6 has an important role in innate immune responses induced by RV infection and IL-6 production has been shown to be inversely correlated to cold symptoms scores and disease severity (Doyle et al., 2010). IL-15 exerts important antiviral and cytotoxic effects and is involved in the activation, differentiation, survival and recruitment of NK cells and CD8+ T cells (Jayaraman et al., 2014). IL-8 has been associated with RV infection as well as cold symptom scores (Gern et al., 2002). Furthermore, it has also been associated with neutrophilic infiltration in sputum (Gern et al., 2000). Taken together, it is evident that innate immune signaling induced by the airway epithelium is essential for effective antiviral responses.

However, in many chronic airway diseases including CF, antiviral responses are defective. Due to the pre-existing genetic defect, normal functions of the CF airway epithelium are often disrupted. As the primary site for virus entry and replication during viral infection, understanding the consequence that lack of CFTR function has on pathophysiology during virus infection is critical for effective disease management. Relevant *in vitro* experimental studies investigating RV infection in CF epithelium have been summarized in **Table 2**. Most studies performed to date assessed cells obtained from adult CF cohorts who had significant disease and structural lung damage. These studies report similar levels of interferon production post infection despite higher viral load being detected (Chattoraj et al., 2011; Dauletbaev et al., 2015). Studying cells from pediatric CF cohorts may generate more relevant data and potentially reveal new insights into early life RV infections that could be exploited therapeutically. Also important is the level of pro-inflammatory cytokines produced by CF epithelium following RV infection. Many studies have reported similar level of IL-8, IL-6, type I, and III IFN production, while others reported higher level of production dependent on virus strain and infectious titer (**Table 2**). These contradictory observations may be due in part to the age of patients involved, disease severity, RV strain, dose, and length of infection. Most studies to date including ours have focused on specific host response targets at the gene or protein level which might not reflect the global innate immune changes during RV infection. The translation of such a targeted approach would be the identification of a single molecule to address a single pathway and ultimately target one downstream effect such as the production of a single cytokine. However, knowing that the interaction of RV and the airway is multifaceted, an alternative approach that addresses this complexity is needed.

PAST AND CURRENT THERAPIES

To date, there have been no studies performed that have focused on potential treatments for RV infection in CF individuals. As RV continues to be the most prevalently detected virus in the all individuals including CF airway, additional evidence is needed to specify its connection with the existing factors such as lack of CFTR and airway inflammation through molecular intermediates and cellular signaling pathways. Common anti-inflammatories including oral corticosteroids and high-dose ibuprofen are unsuitable for treatment in infants and preschool children due to their long-term side effects (Lai et al., 2000; Fennell et al., 2007). Azithromycin may have some interesting antiviral properties, specifically in reducing RV replication via amplification of the IFN pathway-mediated antiviral responses (Schögler et al., 2014). Nevertheless, clinical studies are necessary

TABLE 2 | Summary of *in vitro* studies of Human Rhinovirus infection in CF Airway Epithelium.

Subject	Sampling Sources	RV Serotype	Inoculation Dose	Viral Load	Other Pathogens	Antiviral Cytokines	Inflammatory Cytokines	Apoptosis	References
Adult CF (16–33 years)	BEC (ALI)	RV39	3×10^6 $TCID_{50}$	10^4 TCID50/mL Similar to normal cells	*Pseudomonas aeruginosa* (PA)	RV: IFNβ, λ1, 2 mRNA and protein ↑ Similar to normal cells RV + PA: IFNβ, λ1, 2 mRNA and protein ↓ Only in CF cells	RV: IL8 mRNA ↑ RV + PA: IL-8 mRNA ↑↑ Similar to normal cells	Not measured	Chattoraj et al., 2011
Adult CF (19–41 years)	BEC from explant lung	RV16	MOI 0.1	$>10^4$ copy number Higher in CF	*Pseudomonas aeruginosa* (PA)	RV: IFNβ mRNA↑ Similar to normal cells; OAS1 mRNA↑ Similar to normal cells	RV: IL8 mRNA ↑ Similar to normal cells	Not measured	Dauletbaev et al., 2015
Young children with CF (1–7 years)	AEC	RV1b	MOI 3, 25	∼1500 copy/ng RNA	No	Not measured	IL-6 and IL-8 protein ↑ in CF cells only for infection with RV1b of MOI 25 at 48 hours	Reduced compared to normal cells	Sutanto et al., 2011
Children and adults with CF (4.5–48.9 years)	NEC, BEC and cell lines	RV16, RV1b	MOI 2	Not measured	No	Not measured	IL-6, IL-8, IP-10, MCP-1, RANTES↑ Similar to normal cells	Similar apoptosis, ↑necrosis compared to normal cells	Kieninger et al., 2013
Children with CF (3–11 years)	BEC	RV16, RV1b	MOI 4	Not measured	No	RV16: ↓ IFNs (IFN-λ1, IFN-λ2/3 and IFN-β), PRRs (RIG-1 and MDA-5) and ISGs (PKR, OAS1, viperin and MxA). RV1b: ↑ PRRs (TLR3 and RIG-1) and IFN pathway (IFN-λ1 and IFN-λ2/3 compared to normal cells	↑ CXCL8/IL-8, IL-6 and CXCL10/IP-10	Not measured	Schögler et al., 2014

AEC, Airway Epithelial Cells; BEC, Bronchial Epithelial Cells; NEC, Nasal Epithelial Cells.

to elucidate the clinical impact of azithromycin against RV-induced PEx in patients with CF. Vaccination is an important part of CF clinical care, however vaccine development for RV has been rather challenging due to the wide range of antigenic diversity of more than 150 serotypes of RV across three different strains. Technical difficulties in producing sufficient amounts of antigen against multiple RV serotypes using animal models (as reviewed in Del Vecchio et al., 2015) also remains a challenge and as result, the development of a long-lasting RV vaccine has not been successful. This has been further compounded by the lack of a suitable model (other than human) that is fully permissive to RV infection as well as insufficient clinical data to identify and prioritize dominant RV serotypes. In addition, no suitable animal models for CF exhibit complete spectrum of CF phenotype besides CF pig and ferret which are strictly limited for research use (as reviewed in Rosen et al., 2018).

Since vaccination is unavailable, other approaches have been explored in healthy and disease cohorts other than CF. An early approach by Turner et al. (1999) aimed to prevent HRV binding to its receptors via administration of inhaled recombinant soluble ICAM-1 (Tremacamra). Although the reduction in symptom severity and viral shedding were promising, the high costs and dosing regimen recommended (6 times daily) made translation of this therapy into the clinical setting prohibitive (Turner et al., 1999). Targeting viral replication has perhaps been the highest priority in past therapeutic development, where capsid-binding drugs bind to the hydrophobic "pocket" of the viral capsid (reviewed in McKinlay et al., 1992). Pleconaril and Pirodavir were discontinued due to unforeseen side effects and drug efficacy. Reformulated Pleconaril and Vapendavir have completed clinical trials although results have yet to be published [ClinicalTrials.gov (NCT00394914 & NCT01175226)]. A recently discovered compound IMP-1088 offers more promise (Mousnier et al., 2018). This molecule was shown to inhibit human N-myristoyltransferases NMT1 and NMT2, prevent virus assembly and suppress RV replication and infection across various RV strains without inducing cytotoxicity. However, most of this work was demonstrated using cell lines or adult primary cells and further assessment of IMP-1088 on primary cells from young children with CF is necessary. The outcome of such studies would be informative as to whether this compound exerts similar efficacy across all cohorts, as more than 30 polymorphic DNA loci associated with host variation in gene expression called responsereQTLs to rhinovirus infection has been previously reported (Çalişkan et al., 2015).

Alternatively, the roles of type I IFN administration in enhancing the primary antiviral signaling pathway of innate immunity have also been assessed. Early studies involving the prophylactic administration of IFN-α2 or IFN-β were found to demonstrate a reduction in number of RV-induced episodes but no difference in symptom severity or duration (Farr et al., 1984; Hayden et al., 1986; Monto et al., 1986; Sperber et al., 1988). Multiple side effects from high dose administration of IFNs, including nasal bleeding, transient leukopenia and sore throat have also been reported (Sperber et al., 1988). A more recent study assessed low dose IFN-β administration and although antiviral activity was enhanced, it did not aid in reducing cold symptoms of viral induced exacerbations asthma cohorts (Djukanović et al., 2014). Ruuskanen et al. (2014) also suggested that short-term subcutaneous pegylated IFN-α in combination with oral ribavirin treatment rapidly decreased RV RNA in recurrent or chronic rhinovirus infection in immunocompromised patients.

Alternative therapies have not been thoroughly assessed in CF cohorts. While ongoing clinical trials are comprehensive in evaluating the efficacy of CFTR potentiators and correctors for application on mutation specific patients, improvements in infection and inflammation therapies would be highly desirable for all individuals with CF. Indeed, Ivacaftor has been found to reduce sputum *P. aeruginosa* density (>60-fold) and airway inflammation significantly (Hisert et al., 2017). Whether improvements in CFTR folding or function will enhance antiviral responses in children with CF warrant further investigation. Multi-target drug design also holds potential and could be employed to exert both antiviral and anti-inflammation effects. Understanding how host anti-viral and inflammatory responses differ in CF airways, particularly young children, is critical in facilitating the development of new therapeutic treatments that can limit CF disease progression.

NEW THERAPEUTIC FOR RV INFECTIONS

Current therapies directed at RV are mainly focusing on specific viral proteins or inhibition of viral cycle. However, some of these drugs are not effective on drug-resistant viral strains. The current review proposes an alternative approach that focuses on host cellular pathways and factors. To expedite novel therapeutic strategies, investigation on how cellular signaling pathways can be altered by RV infection and how these alterations can be manipulated by new compounds or drugs are crucial for new therapeutic development. The current field of system biology and adoption of high-throughput technologies through transcriptomics not only facilitates characterization of the host-pathogen interaction in a more comprehensive manner, but also aids in understanding how developed and repurposed compounds exert their antiviral properties on RV infection in CF patients.

Knowing viruses can manipulate the host signaling processes and thus altering the host-pathogens interactions (Christiaansen et al., 2015), evaluating the global changes in gene expression during infection via employing gene/transcriptomics could elucidate crucial messages for therapeutic target identification. Transcriptomics is used to study the total RNA output of a cell. Early transcriptomic analyses were performed using microarrays which have customized probes, while current transcriptomic analyses rely on high-throughput RNA sequencing which capture global transcriptome (Mortazavi et al., 2008). These techniques allow analysis for "all molecules" regulated at the gene level. By illustrating their interaction within the cells and the complexities of host-pathogen interactions, enhancing or diminishing specific molecules as well as precise characterization of specific targets can be a more promising therapeutic approach (as reviewed in Cesur and Durmuş, 2018). Generally, computational approaches

are used to organize or manage these data sets and interpret the biological inference, including network analysis (such as InnateDB, NetworkAnlayst, and Cytoscape) and pathway analysis (such as Reactome, Kegg Pathway, and Pantherdb).

In the context of host-viral interaction, transcriptomic analysis has been successfully applied to identify the uncharacterized isoforms from wild-type dengue infected host RNA from human hepatoma cells. The authors demonstrated that infection with wild-type dengue virus elicited a different host response compared to infection with a vaccine sensitive strain, highlighting the potential of strain-specific responses (Sessions et al., 2013). Transcriptional profiling of blood specimens from

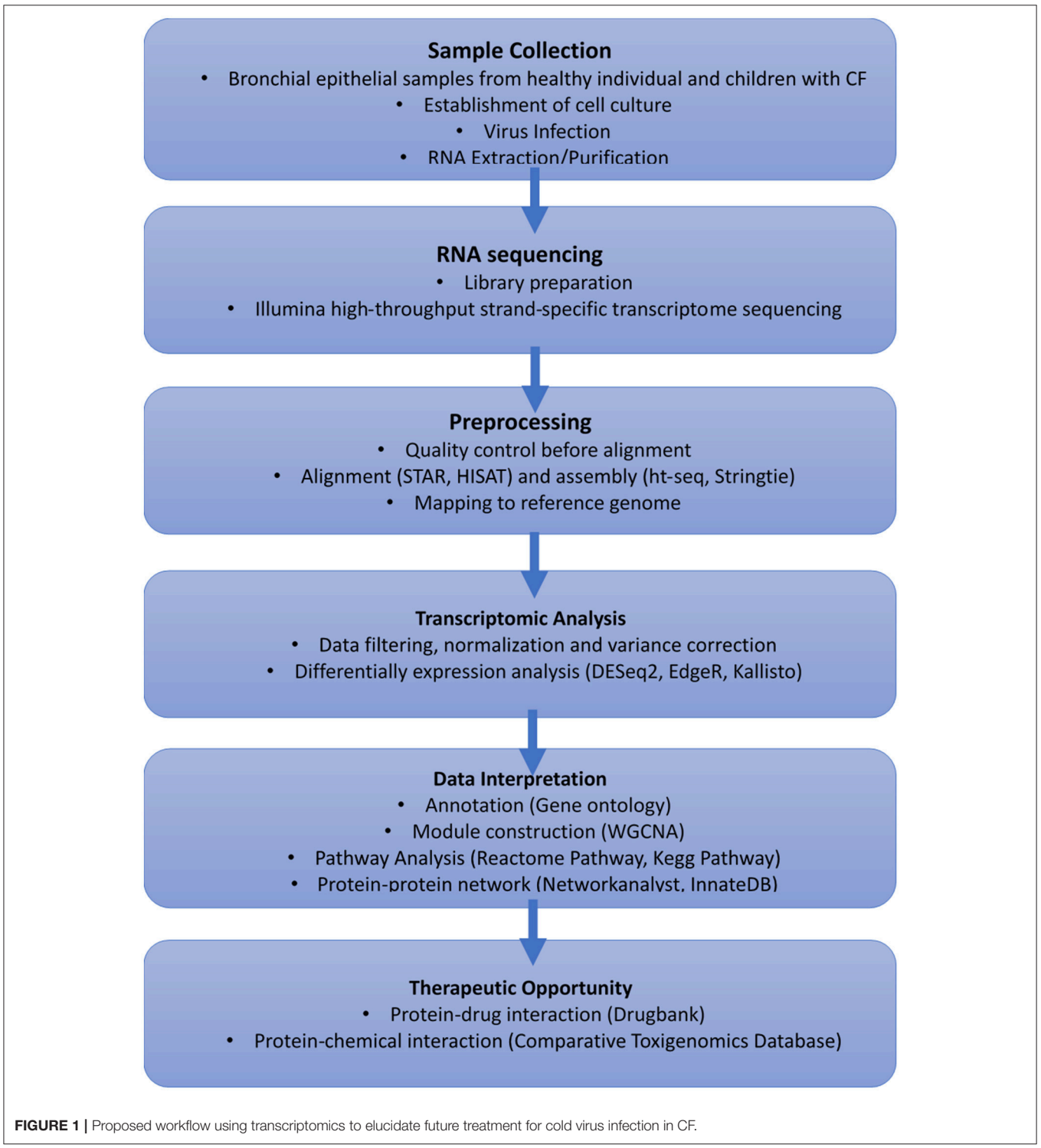

FIGURE 1 | Proposed workflow using transcriptomics to elucidate future treatment for cold virus infection in CF.

symptomatic and asymptomatic patients with RV infection have revealed that individuals with active infection demonstrate a robust transcriptional signature of immune-related genes (Heinonen et al., 2016). In other disease settings including asthma and allergic rhinitis, transcriptomic responses of human respiratory cells to surrogate RV infection [Poly(I:C) stimulation] have potentially identified disease-specific signatures (Wagener et al., 2014). Therefore, it is imperative to assess the global transcript expression and investigate the host-viral interaction, given that CF is a defined genetic disease condition and live RV infection can truly represent an active infection which might involve modification of the host response. This approach is not only applied to protein-coding RNA but also provide insights to critical non-coding RNA such as short non-coding RNAs (miRNAs) and long non-coding RNAs (lncRNAs) which are key regulators for modulation of gene expression (Delpu et al., 2016).

Mapping genes on a complete network allows identification of key hub genes and central genes with high connectivity which exert large effects on signal transduction. Molecular network analysis also allows enrichment of functional modules to identify which area group of genes are cooperatively working together to perform specific biological function and could be associated with disease setting (reviewed by Csermely et al., 2013). Some examples include, the identification of 16 strongly connected hub genes as potential antifungal drug targets against *Candida albicans* (Altwasser et al., 2012), the identification of new key genes for type 1 diabetes (Safari-Alighiarloo et al., 2016) as well as certain cancers (Zaman et al., 2013; Jin et al., 2015). Moreover, omics data has discovered disease modules and revealed substantial inter-patient heterogeneity, highlighting the potential importance of customize treatments to conditions. Numerous algorithms have been introduced to identify disease modules, including ModuleDiscoverer that identified a rodent model of non-alcoholic steatohepatitis (NASH), as well as a severe form of non-alcoholic fatty liver disease (NAFLD) (Vlaic et al., 2018). To maximize the efficacy and treatment outcome, patient individual characteristics, including their genetic profile needs to be considered. Although using biological network analysis can expedite the drug discovery process, the timeline from target identification to clinic application can still be lengthy.

An alternative strategy is to explore drug repurposing. Integrated analysis of disease-gene profiles, pathway analysis, and mining of FDA approved drug databases can be carried out to identify correlations of common pathways with certain compounds or molecules at the network level. Successful examples of drug repurposing based on transcriptomic analyses include the identification of topiramate for the treatment of inflammatory bowel disease (IBD) and cimetidine for the treatment of lung adenocarcinoma (Dudley et al., 2011; Sirota et al., 2011). Using a large-scale expression signature, Lee et al. (2016) have also identified that ivermectin, trifluridine, astemizole, amlodipine, maprotiline, apomorphine, mometasone, and nortriptyline show significant anti-proliferative activity against glioblastoma. With the recent establishment of ImmPort, a data repository that promotes research dataset repurposing (Bhattacharya et al., 2018), the identification of novel targets and repurposed drugs that target these has been accelerated further. Currently, there is paucity of data in CF-related RV-therapy given its impact on CF lung disease and thus new interventions are urgently required. The strategy to repurpose already approved drugs could advance antiviral therapies by reducing cost and improving and quality of life for affected individuals.

CONCLUSION

RV infection remains a significant cause of pulmonary exacerbation in CF. There has been little investigation into antiviral therapies in CF especially in young children who are more susceptible to these types of infection. However, modern virological procedures and omic technologies now facilitate more in-depth studies of the genes and molecular pathways involved in aberrant CF antiviral responses to RV. We propose transcriptomics could be leveraged to elucidate future therapeutic intervention for treatment of rhinovirus infection in CF. For example, a global gene expression profile of bronchial epithelial cells from patients with CF, under baseline conditions and after RV infection will be profiled following next-generation RNA sequencing (**Figure 1**). Sequences can be aligned and mapped to already available reference genomes to identify differentially expressed genes pre- and post-infection. The identified genes could then be annotated using online repositories or libraries to investigate their enriched functional biological pathways. Moreover, networks or subnetworks can then be constructed by mapping identified genes to explore their relationship using curated protein-protein interaction databases. Therapeutic opportunities can also identify by exploring protein-protein interaction and protein-transcription factor, protein-drug interaction as well as chemical interaction databases. Finally, monolayer cell cultures which have previously been found to be more susceptible for RV infection (Bochkov et al., 2010) represent an oversimplified model for the multicellular interactions of epithelial (ciliated cells, goblet cells) and immune cells (dendritic cells, neutrophils). Indeed, functional validation utilizing human *in vitro* 3D airway models (Boda et al., 2018) will be needed to further elucidate to host-pathogen interactions. The emergence of single cell transcriptomics could be used to compliment 3D airway models and accelerate progress in this new era of scientific research. Overall, the advancement of these promising tools should aid in expediting new therapeutic intervention in this sphere.

AUTHOR CONTRIBUTIONS

K-ML, LG, and AK conceptualized the contents of the manuscript. K-ML wrote the first draft of the manuscript. K-ML, LG, TL, SS, and AK contributed to the drafting and editing of the manuscript. WAERP, AusREC, and AREST CF approved the final submission of the manuscript.

ACKNOWLEDGMENTS

The members of Western Australian Epithelial Research Program (WAERP) are: AK, SS, Darryl A. Knight, Elizabeth Kicic-Starcevich, LG, Marc Padros-Goosen, Ee-Lyn Tan, Erika N. Sutanto. Kevin Looi, Jessica Hillas, Thomas Iosifidis, Nicole C. Shaw, Samuel T. Montgomery, K-ML, Kelly M. Martinovich, Francis J. Lannigan, Ricardo Bergesio, Bernard Lee, Shyan Vijaya-Sekeran, Paul Swan, Mairead Heaney, Ian Forsyth, Tobias Schoep, Alexander Larcombe, Monica Hunter, Kate McGee, Nyssa Millington. The full membership of the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) is available at www.arestcf.org. The Australian Respiratory Epithelium Consortium (AusREC) acknowledges the following members; AK, SS, Elizabeth Kicic-Starcevich, LG, Erika N. Sutanto, Kevin Looi, Jessica Hillas, Thomas Iosifidis, Nicole C. Shaw, Samuel T. Montgomery, K-ML, Kelly M. Martinovich, Matthew W.-P. Poh, Daniel R. Laucirica, Craig Schofield, Samantha McLean, Katherine Landwehr, Emma de Jong, Nigel Farrow, Eugene Roscioli, David Parsons, Darryl A. Knight, Christopher Grainge, Andrew T. Reid, Su-Kim Loo, and Punnam C. Veerati. This work was supported by grants from the National Health and Medical Research Council of Australia (NHMRC; APP1069101 and APP1142505) and United States Cystic Fibrosis Foundation (#STICK15A0). SS is a NHMRC Practitioner Fellow. LG is a NHMRC Peter Doherty Fellow.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 28 November 2018
doi: 10.3389/fphar.2018.01330

Use of a Primary Epithelial Cell Screening Tool to Investigate Phage Therapy in Cystic Fibrosis

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OPEN ACCESS

Edited by:
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Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
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† *The members of these groups are listed in the Acknowledgments section.*

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *29 July 2018*
Accepted: *29 October 2018*
Published: *28 November 2018*

Citation:
Trend S, Chang BJ, O'Dea M, Stick SM, Kicic A, WAERP, AusREC and AREST CF (2018) Use of a Primary Epithelial Cell Screening Tool to Investigate Phage Therapy in Cystic Fibrosis. Front. Pharmacol. 9:1330. doi: 10.3389/fphar.2018.01330

Antimicrobial-resistant microbes are an increasing threat to human health. In cystic fibrosis (CF), airway infections with *Pseudomonas aeruginosa* remain a key driver of lung damage. With few new antibiotics on the development horizon, alternative therapeutic approaches are needed against antimicrobial-resistant pathogens. Phage therapy, or the use of viruses that infect bacteria, is one proposed novel therapy to treat bacterial infections. However, the airways are complex microenvironments with unique characteristics that may affect the success of novel therapies. Here, three phages of *P. aeruginosa* (E79, F116, and one novel clinically derived isolate, designated P5) were screened for activity against 21 *P. aeruginosa* strains isolated from children with CF. Of these, phage E79 showed broad antibacterial activity (91% of tested strains sensitive) and was selected for further assessment. E79 genomic DNA was extracted, sequenced, and confirmed to contain no bacterial pathogenicity genes. High titre phage preparations were then purified using ion-exchange column chromatography and depleted of bacterial endotoxin. Primary airway epithelial cells derived from children with CF (n = 8, age range 0.2–5.5 years, 5 males) or healthy non-CF controls (n = 8, age range 2.5–4.0 years, 4 males) were then exposed to purified phage for 48 h. Levels of inflammatory IL-1β, IL-6, and IL-8 cytokine production were measured in culture supernatant by immunoassays and the extent of cellular apoptosis was measured using a ssDNA kit. Cytokine and apoptosis levels were compared between E79-stimulated and unstimulated controls, and, encouragingly, purified preparations of E79 did not stimulate any significant inflammatory cytokine responses or induce apoptosis in primary epithelial cells derived from children with or without CF. Collectively, this study demonstrates the feasibility of utilizing pre-clinical *in vitro* culture models to screen therapeutic candidates, and the potential of E79 as a therapeutic phage candidate in CF.

Keywords: cystic fibrosis, phage therapy, preclinical models, airway epithelial cells, *Pseudomonas aeruginosa*, infection

INTRODUCTION

Cystic fibrosis (CF) is the most common autosomal recessive lethal genetic disease of Caucasian populations (Tian et al., 2016). In the lung, loss of CFTR function leads to production of viscous mucus, which is difficult to clear by standard mucociliary action (Koehler et al., 2004). This in turn contributes to the development of chronic infections with opportunistic bacterial pathogens such as *Pseudomonas aeruginosa* (Friman et al., 2013). The severity of lung bronchiectasis, excessive lung inflammation in children, and rate of decline in lung function in infants with CF is directly correlated with airway infections with *P. aeruginosa* (Koehler et al., 2004; Farrell et al., 2009; Pillarisetti et al., 2011).

Current therapies for CF lung infections include intravenous or inhaled antibiotics (National Guideline Alliance, 2017). However, antibiotic-resistant bacterial strains have emerged as major causes of mortality in hospitals worldwide, and in Western Australia, approximately 30% of *P. aeruginosa* isolates from CF patients are carbapenem-resistant (Tai et al., 2015). The World Health Organization has recognized antibiotic resistance as a significant threat to human health requiring urgent action (World Health Organization, 2014). Bacteriophages (phages) are viruses that infect bacteria, and present a novel treatment option in CF, however, little has been investigated regarding their potential in this setting. Concomitant with this need for new antibacterial treatments is the need for relevant *in vitro* models to screen potentially therapeutic phages and facilitate understanding of how these preparations act in the context of the human airway (Trend et al., 2017). Phage therapy holds enormous potential benefit for people with CF, and may complement existing antimicrobial strategies, since phages can replicate at the site of infection inside the target bacterial cells and subvert existing antimicrobial resistance in bacterial pathogens (Alemayehu et al., 2012; Sahota et al., 2015).

While the investigation and development of phage-derived products as therapeutic agents requires employment of *in vitro* antimicrobial assays, the effectiveness of any antimicrobial therapy *in vivo* may not always correspond to expected outcomes, due to a range of human factors not considered in the models (Henry et al., 2013). For example, host immune mediators directly interact with phage particles to inactivate them *in vivo* (Majewska et al., 2015). Furthermore, the patient's innate immune system may induce an inflammatory response upon exposure to the phage, particularly if the phage preparations derived from culture in bacterial hosts are inadequately purified. Relevant *in vitro* models are thus essential in order to elucidate and understand the responses of the airway to this new potential therapy.

To develop phages for clinical trials, researchers should characterize and screen candidates for antimicrobial activity and carriage of bacterial pathogenicity genes. Given the inflammatory nature of many bacterial-derived products, stringent purification steps must be applied to phage preparations after propagation in bacterial hosts (Merabishvili et al., 2009), the success of which can then be confirmed on human cells using relevant exposure models. Previous investigations of this type have utilized immortal cell lines or animal models. Although the ability to screen the suitability of phage preparations using human CF airways cells would lead to more relevant findings, investigations involving primary airway epithelial cells have traditionally been limited due to their restricted expansion potential (Lane et al., 2005). However, our laboratory has recently described the development of an *in vitro* culture expansion methodology of primary airway epithelial cells in a realistic model of the airway (Martinovich et al., 2017). Using primary airway cells derived utilizing this technique, we performed screening analyses of a number of phages. Due to its broad range of activity against CF clinical isolates, phage E79 was purified using ion-exchange chromatography and endotoxin depleted to clinically acceptable levels. We showed that purified E79 does not induce apoptosis or inflammatory cytokine production in primary airway cells derived from children with CF or healthy non-CF individuals exposed to the phage preparations for 48 h. Moreover, the phage was virulent, stable, and genome sequencing did not reveal any known bacterial virulence genes. Collectively, these findings suggest that E79 may be a good candidate for phage therapy.

MATERIALS AND METHODS

Bacteriophage and Bacterial Sources and Culture

E79 and F116 bacteriophages (Slayter et al., 1964) were obtained from the University of Western Australia Department of Microbiology Culture Collection (MCC), and utilized as examples of virulent and temperate phages to compare to novel phage isolates. Novel *P. aeruginosa* phage P5 was isolated from pediatric CF patient sputum. A panel of 21 isolates of *P. aeruginosa* (designated PMH-) were derived from sputum of patients with CF-related infections, supplied by PathWest Laboratory Medicine, Perth, Western Australia. An independent collection of 14 isolates of *P. aeruginosa* isolated from sputa of children with CF were derived from the AREST CF culture collection (designated ARESTCF-). Reference strain PAO1 (Dunn and Holloway, 1971) was obtained from MCC. *P. aeruginosa* strains were routinely propagated in heart infusion agar (HIA) or broth (HIB) aerobically at 37°C.

Antimicrobial Susceptibility of Clinical *P. aeruginosa* Isolates

Sensitivity of 14 clinical isolates (AREST CF collection) of *P. aeruginosa* to gentamicin, ceftazidime, meropenem, amikacin, piperacillin, cefepime, tobramycin, ciprofloxacin, norfloxacin, timentin, piperacillin/tazobactam, and aztreonam was determined by the Royal Children's Hospital Melbourne pathology laboratory using disk diffusion assays. Susceptibility was determined according to Clinical and Laboratory Standards Institute (CLSI) breakpoints (CLSI, 2016).

Isolation of Clinically Derived Phages

Sputa from 20 CF patients were screened for the presence of phages that were able to infect any member of the panel of 21 clinical *P. aeruginosa* isolates (PMH collection). Sputa

were mixed, and large materials sedimented by centrifugation at 1,750 × *g* for 20 min at room temperature (RT). Following filter-sterilization of the supernatant using a 0.22 μm pore sterile membrane, an aliquot (10 μL) was pipetted onto a lawn of each bacterial strain as described for phage propagation. Plaques appearing on bacterial lawns, either derived from sputa or from lysogenic phage spontaneously induced from the bacterial genome, were transferred to 150 μL of phage buffer (Tris–HCl 10 mM, $MgCl_2$ 10 mM [pH 8]) (Matsumoto et al., 1986). The suspension was purified as described for phage propagation and tested for phage titre. Phages were plaque-purified twice, prepared at high titre and stored at 4°C.

Phage Imaging and Classification

Electron microscopy was performed on phage suspensions that were purified using a modified methodology (Bruttin and Brussow, 2005). Briefly, following centrifugation at 4,000 × *g* for 15 min at RT the resulting supernatant was separated by centrifugation at 35,000 × *g* for 25 min at RT. The phage-containing pellet was resuspended and applied to carbon-coated formvar grids for 5 min, which were charged prior to phage co-incubation using 1% (w/v) Alcian blue. Grids were negatively stained for 10 min using a 2% sodium silicotungstate solution, dried at RT, then analyzed using Transmission Electron Microscopy (TEM) using a Philips 401 TE microscope. Phage head, tail, and sheath sizes were measured, and data presented as a mean and standard deviation of three particle measurements.

For classification, phage genetic material was treated with the class II restriction endonucleases, either *Bam*HI or *Hind*III, for 2 h at 37°C. A 2 μL sample was then combined with 3 μL of tracking dye containing bromophenol blue (2.5 mg/mL) and sucrose (400 mg/mL) and electrophoresed on a 0.8% (w/v) agarose gel containing ethidium bromide (10 μg/mL) in Tris-Acetate Buffer with Ethylenediaminetetraacetic acid (0.5 M) for 60 min at 90 V. The gel was then photographed using UV illumination to detect if multiple bands were present, indicating digested DNA. The genome size was estimated by comparison to a *Hind*III ladder, while the genome sequencing results provided a more precise genome size.

Phage Propagation and Host Range

Phages were propagated to high titre according to a modified version of the double agar layer method (Adams, 1959). Molten overlay agar containing 1% Bacto agar and HIB, supplemented with calcium and magnesium (0.01 M) was mixed with an aliquot of overnight broth culture of host bacteria and phage at approximately 10^5 PFU/mL. The mixture was overlaid onto a HIA plate, allowed to set at RT and then incubated overnight at 37°C in 5% CO_2. The following day, the overlay agar with semi-confluent phage plaques was scraped off the HIA plate and mixed with 3.5 mL of isotonic saline. All phage preparations were preliminarily purified by mixing followed by centrifugation at 1,750 × *g* at RT. The supernatant was filtered through a 0.22 μm pore-size membrane. Phage titre was then determined using the drop-on plate method. Briefly, 10 μL of 10-fold dilutions of phage preparations were dropped in triplicate onto a HIA plate overlaid with overlay agar containing an overnight culture of PAO1, and the plaques in the bacterial lawn counted after incubation overnight at 37°C in 5% CO_2.

Host range experiments were carried out on 21 clinical *P. aeruginosa* strains. Sensitivity to a phage was determined by a zone of clearing or plaques on a bacterial lawn where 10 μL of high titre phage preparation (10^8–10^{12} PFU/mL) was spotted onto the lawn in triplicate, followed by incubation at 37°C overnight.

Growth Inhibition by E79

The inhibitory capacity of E79 for clinical *P. aeruginosa* isolates (AREST CF collection) was determined using the following method. Briefly, bacteria were grown to approximately 0.5 MacPharland standard (∼1 × 10^8 CFU/mL,) determined by spectroscopy and 100 μL of bacterial suspension was then added to wells of a 96-well flat-bottom polypropylene plate. E79 was diluted to 10^9 PFU/mL in sterile isotonic saline, then serially diluted in 10-fold dilutions down to 10^4 PFU/mL. Dilutions of phage or a control of saline only were added in equal volumes at a multiplicity of infection (MOI) of 10:1 and 1:1, and plates incubated at 37°C in 5% CO_2 overnight. Optical density of wells was then measured at 595 nm, and the percentage of growth in treated wells quantified by dividing this value by the absorbance in the control well. A bacterial strain was considered sensitive to phage at that MOI if the optical density in the test well was ≥10% lower than that of the untreated well.

Phage DNA Extraction and Genome Sequencing

High titre E79 was pre-treated with DNase I to reduce contaminating bacterial DNA. A 200 μL aliquot containing ∼10^{11} PFU of phage was incubated for 30 min at 37°C with 1 U of DNase I from the Invitrogen PureLink RNA Mini Kit. DNA was subsequently extracted using a QIAamp DNA mini spin column kit (QIAGEN) per manufacturer's instructions and sequenced (LotteryWest Sequencing Facility, University of Western Australia, Perth, WA, Australia). The concentration of DNA was determined using a Qubit fluorometer according to manufacturer's instructions. The sample (10 ng) was sheared using a S2 ultrasonicator (Covaris) and sequencing libraries prepared using a NEBnext Ultra library kit (New England Biosciences). Sequencing was performed on a 318 chip using an Ion Torrent PGM semiconductor sequencer for 820 flows. Data were collected using Torrent Suite 5.0. Reads were imported into CLC Genomics Workbench V 10.1.2, and *de novo* assembled, before the phage genome was annotated using RASTtk (Brettin et al., 2015). The annotated phage genome was then imported into Geneious V10.2.4 for visualization and manual curation.

Purification of High Titre Phage Preparation for Cellular Assays

Filter-sterilized high titre phage preparations were purified using a CIMmultus quaternary amine (QA) advanced composite monolithic column (BIA separations) in combination with a Bio-Rad Econopump. Phage was diluted 1:10 in 20 mM Tris–HCl (pH 7.5) loading buffer, and 10 mL was passed through the column.

TABLE 1 | Clinical details of CF-related characteristics in cell donors with CF at time of cell collection.

Sample	CFTR genotype	CFTR mutation class	Neutrophil elastase (nM)	Bronchiectasis (0–12 score)	Respiratory pathogens
CF1	p.Gly85Glu/Unknown	–/–	100	0	Mixed oral flora
CF2	p.Phe508del/p.Phe508del	II/II	1260	n/a	None
CF3	p.Phe508del/p.Phe508del	II/II	270	n/a	*Moraxella catarrhalis*, *Haemophilus influenzae,* MRSA, Mixed oral flora
CF4	p.Phe508del/p.Asn1303Lys	II/II	270	n/a	*Aspergillus fumigatus*, *Stenotrophomonas maltophilia*
CF5	p.Phe508del/p.Thr966ArgfsX2	#N/A	170	n/a	None
CF6	p.Phe508del/Ala455Glu	#N/A	70	n/a	*H. influenzae*
CF7	p.Phe508del/p.Phe508del	II/II	140	n/a	*Streptococcus pneumoniae*
CF8	p.Phe508del/p.Phe508del	II/II	60	n/a	None

CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; MRSA, methicillin-resistant Staphylococcus aureus.

The column was washed with 20 volumes of loading buffer, and then 20 mM Tris–HCl supplemented with successive increases of saline (250 mM–1.2 M NaCl) in 2 mL volumes, which were collected as separate fractions. The titre of phage in each fraction was tested as previously described and the fraction with the highest titre of phage selected for further purification through endotoxin-depletion.

Endotoxin was removed from the purified phage preparation using the EndoTrap HD 5/1 kit (Hyglos) per the manufacturer's protocol. Here, phage preparation was diluted 1:5 in 20 mM Tris–HCl containing 450 mM NaCl and 0.125 mM $CaCl_2$, passing the phage solution three times through the column. Concentrations of endotoxin in the phage preparations before and after purification were determined using the Endpoint Chromogenic Limulus Amebocyte Lysate (LAL) assay (Lonza), using endotoxin-free reagents and equipment. A high titre phage preparation (10^{11} PFU/mL), generated as previously described, was tested diluted in endotoxin-free water diluted 10^{-8} (10^3 PFU/mL) to reach the detectable range of the kit. Purified phage preparations were tested in duplicate at 1:5 dilution in endotoxin-free water and the concentration interpolated from a standard curve using a 3PL curve fit in GraphPad Prism software.

Study Participants and Epithelial Cell Collection

This study was carried out in accordance with the recommendations of the National Health and Medical Research Council of Australia's National Statement on Ethical Conduct in Human Research with written informed consent was obtained from the parent or guardian of all participants. All participants gave written informed consent in accordance with the Declaration of Helsinki. The study was approved by St. John of God Health Care Human Research Ethics Committee Ref#901 sub-study 901.1050 and by the Princess Margaret Hospital for Children Ethics Committee Ref#1762EPP. Sixteen individual cell cultures derived from children with CF (n = 8) and from healthy non-CF children (n = 8), aged from 0–6 years were collected. Median ages of participants with or without CF were not significantly different (3.0 vs. 3.2 years old, respectively; p = 0.88), nor proportions of females in each of these groups (37.5% vs. 50%, respectively; p = 0.60). Parental consent was obtained prior to bronchial brushing and cells collected as described previously (Kicic et al., 2006; Sutanto et al., 2011). Epithelial cells were purified by removal of CD68+ cells and the epithelial phenotype confirmed (Lane et al., 2005; Kicic et al., 2006; McNamara et al., 2008). Demographics of the study cohort are shown in **Table 1**.

Cell Cultures

Children with CF had cell samples collected during bronchoscopy as part of their annual clinical surveillance program (Sutanto et al., 2011). In addition, cells from non-CF controls were collected from children during elective non-respiratory related surgery where intubation was required.

Primary airway epithelial cells derived from bronchial brushings were conditionally reprogrammed, a method which we previously reported resulted in cells that retain their phenotype and functionality in culture (Martinovich et al., 2017). Briefly, airway epithelial cells were co-cultured with irradiated fibroblasts and grown on tissue culture-grade flasks that were pre-coated with fibronectin and type I collagen (Kicic et al., 2006). Cells were grown to 90% confluence in pre-coated flasks in culture media for approximately 5 days, and then seeded into 96-well plates at ∼20,000–40,000 cells/well in growth media containing bronchial epithelial basal medium (BEBM®; Lonza, Basel, Switzerland) supplemented with SingleQuot additives. All cell cultures were at passage 3 at time of experiments and were maintained at 37°C in an atmosphere of 5% CO_2 under aseptic conditions. Experiments were conducted on multi-layered primary airway epithelial submerged cultures which exhibit typical cobblestone morphology (**Figure 1**).

Stimulation of Cell Cultures

Prior to exposure to phage, media in 96-well plates was changed to starvation media (growth medium minus bovine pituitary extract, epidermal growth factor, and antibiotics) for 24 h. Cells were then exposed to saline control or phage treatments in infection media (media minus bovine pituitary extract and antibiotics). The following treatments were applied to cells as 10% of the medium in 90% v/v infection media: (1) isotonic saline, (2) E79 phage (ratio 1:1 per epithelial cell), and (3) E79 (ratio of 10:1

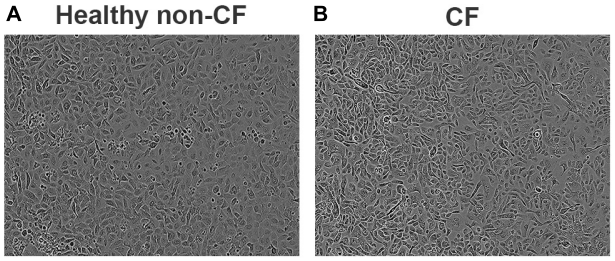

FIGURE 1 | Phase contrast images of confluent primary airways cell cultures obtained using conditionally reprogrammed method. Epithelial cobblestone morphology is typically maintained and observed in both healthy non-CF **(A)** and CF **(B)** primary cell cultures. Representative image of eight subjects per phenotype. Magnification 100×.

per epithelial cell). Cells were then incubated for 48 h at 37°C in 5% CO_2. After exposure, cell supernatant was collected from replicate wells, pooled and frozen at −80°C until batch cytokine analyses were performed. Following removal of supernatant from 96-well plates, cell viability was assessed.

Cytokine Detection in Cell Culture Supernatants

The concentration of IL-8 in culture supernatants was determined using enzyme-linked immunosorbent assays (ELISA; R&D Systems) previously used in our laboratory (Kicic et al., 2016). Values were interpolated from a standard curve with a range of 3.125–200 pg/mL of IL-8. IL-6 was measured in culture supernatants using a time-resolved fluorescence immunoassay as previously described (Taylor et al., 2007; Sutanto et al., 2011) with a minimum detectable value of 3 pg/mL. The concentration of IL-1β in cell culture supernatants was tested using commercially available ELISA kits, according to the manufacturer instructions (eBioscience), with a limit of detection of 2 pg/mL. Fluorescence and absorbance measurements for immunoassays were detected using the ClarioStar plate reader (software version 5.20 R5 from BMG Labtech). Concentrations of cytokines were interpolated from measurements using a standard curve used in the same assay. Data were transformed using the calculation x = log(x) and a 4-parameter logistic curve fit in GraphPad Prism software. Any values falling below the limit of detection were assigned a value of half the lowest detectable concentration multiplied by the dilution factor. Following interpolation of concentrations, they were back-transformed using $x = 10^x$.

Cell Viability and Apoptosis Measurements

Airway epithelial cell viability was determined using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega). Following incubation as instructed, absorbance of each well was measured at 492 nm using a plate reader. A control well without cells was used to determine background absorbance. The percentage viability in each treatment well was then compared to the calculated mean control value. Mean values from at least three technical replicate wells were used in final calculations.

Apoptosis was measured in cells exposed to phage for 48 h using a ssDNA apoptosis ELISA Kit (Merck Millipore) as previously described (Sutanto et al., 2011; Kicic et al., 2016). Briefly, following exposure, culture supernatant was removed, and cells were fixed using 200 μL per well of a solution containing 80% methanol and 20% PBS. Plates were then air dried for 30 min at RT and stored at 4°C until batch analyses of apoptosis were performed. The percentage of apoptotic cells compared to untreated controls was then determined where data were normalized to the control apoptosis value for that plate.

Statistical Analyses

Continuous variables were tested for normality using a Shapiro–Wilk test. Since most variables did not follow a normal distribution, non-parametric tests were used. Categorical variables in the CF and healthy non-CF cohorts were compared using a Chi-squared test, and continuous clinical variables were compared using a Mann–Whitney test. Cytokine measurements were presented as both absolute concentrations measured in supernatants adjusted for viable cell numbers, and for the purposes of comparing between the CF and healthy non-CF groups, cytokine levels in phage treated wells were normalized to the saline well as a ratio of 1. Paired data including results from cell culture experiments and bacterial minimum inhibitory concentration assays were compared using a Friedman test or Wilcoxon signed-rank test, comparing phage treatments to the control (saline), with Dunn's post-test applied for multiple comparisons where appropriate. Unpaired data comparing cytokine fold-change in phage-treated wells between CF and HNA were compared using Mann–Whitney tests. All statistical analyses were performed in SPSS v24 (IBM). In all tests, a p-value <0.05 was considered statistically significant.

RESULTS

Isolation of Clinically Derived Phage

One *P. aeruginosa* phage (P5) was successfully isolated from patient sputum by direct plating and propagated on patient strain PMH5. Six other phages were observed as plaques on lawns of bacterial isolates derived from CF patients, but could not be successfully propagated to high titre or were unstable at 4°C in storage, and thus were not investigated further. In total, 6/20 (30%) CF-derived *P. aeruginosa* were demonstrated to carry spontaneously inducible lysogenic phages.

Phage Host Ranges

Plaque formation by E79 was observed on lawns of 20/22 (91%) of *P. aeruginosa* strains tested (**Table 2**), making it a strong candidate for further testing as a potential therapeutic phage. F116 and P5 had more limited host ranges (36 and 64% strains susceptible, respectively), and were known (F116) or suspected (P5) temperate phages, and were therefore eliminated from further screening, since these were not considered to be ideal for phage therapy.

TABLE 2 | Host range of phages within the panel of clinical *P. aeruginosa* isolates and PAO1 strain.

P. aeruginosa strain	Colony phenotype	Phage susceptibility		
		E79	F116	P5
PAO1	Smooth	++	++	++
PMH1	Mucoid	++	–	++
PMH2	Smooth	++	–	–
PMH3	Mucoid	++	–	–
PMH4	Mucoid	++	–	+
PMH5	Smooth	++	+	++
PMH6	Rough	++	–	–
PMH7	Mucoid	++	–	+
PMH9	Smooth	–	–	++
PMH10	Mucoid	–	–	++
PMH11	Rough	++	+	++
PMH12	Mucoid	++	–	–
PMH13	Smooth	++	+	++
PMH14	Smooth	++	–	–
PMH15	Mucoid	++	–	–
PMH16	Rough	++	–	–
PMH17	Smooth	++	–	+
PMH18	Rough	++	–	–
PMH19	Mucoid	++	+	+
PMH20	Mucoid	++	++	++
PMH22	Mucoid	++	+	+
PMH23	Rough	++	+	+

++Indicates complete clearing, +indicates partial clearing, –indicates no visible clearing of the bacterial lawn.

E79 Genetic and Morphological Characterization

Both E79 and P5 were confirmed to be double-stranded DNA phages, demonstrated by digestion by *Hind*III and *Bam*HI, respectively (not shown). TEM of E79 identified the phage to have a head diameter of 63.6 ± 3.8 nm, head length of 60.5 ± 5.7 nm, tail width of 8.4 ± 0.8 nm, tail length of 128.1 ± 3.7 nm, and a sheath width of 16.6 ± 2.4 nm. Genome sequencing of E79 generated 2.2 M reads with an average length of 252 bp. The sequencing analysis returned a complete genome of 66,061 bp, with BLASTN analysis revealing 98% query cover and 97% identity to a range of widespread PB1-like *Pseudomonas* phages in the *Myoviridae* family (Ceyssens et al., 2009). The E79 genome contained 96 open reading frames, 75 of which were for hypothetical phage proteins. No virulence factors or antibiotic resistance genes were detected, nor any genes from the host PAO1 bacterial genome. The full sequence of E79 is deposited in GenBank under accession #MH536736.

Phage Purification

Phage fractions were collected from the ion-exchange chromatography column and examined for remaining viable phage. Approximately 80% of the added phage was recovered in the saline fractions collected. Maximal phage recovery occurred from the elution at approximately 450 mM NaCl (**Figure 2B**). The concentration of endotoxin in the filter-sterilized phage (unpurified) and the purified, endotoxin-depleted phage preparations was approximately 10^8 and 1.9 EU/mL, respectively. E79 phage preparations were stored at 4°C and retained the high infective titre for >20 weeks of testing (**Figure 2A**).

Bacterial Growth Inhibition

Growth of clinical *P. aeruginosa* isolates was significantly decreased (median 33% decrease) in the MOI 1:1 (low phage ratio) and MOI of 10:1 (high phage ratio) (median 23% decrease). Of 14 clinical isolates tested, a variety of growth inhibition and clinical antibiotic resistance phenotypes were observed (**Table 3**) with 11 (79%) showing some growth inhibition by E79. Of these, eight (57%) exhibited single or multiple antibiotic-resistance.

Response of Airway Epithelial Cells to Stimulation

Viability of airway epithelial cells from children with CF or healthy non-CF controls was not affected when treated with

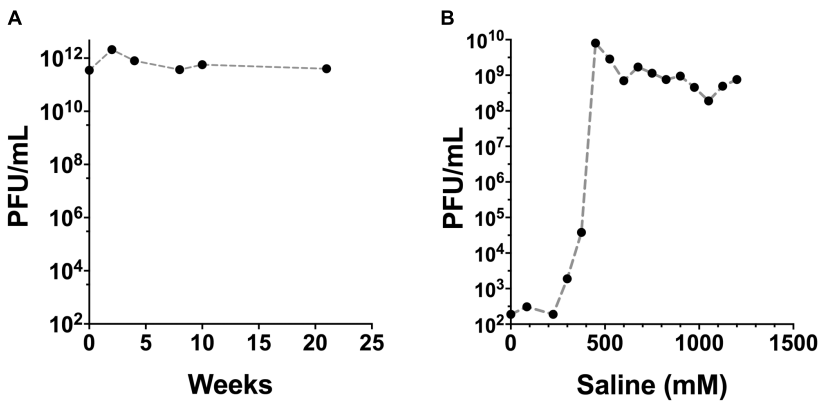

FIGURE 2 | Stability and recovery of E79 phage after purification steps. **(A)** Titre of E79 phage was retained over long term storage at 4°C, determined by the drop-on-plate method using PAO1 as the propagating host strain. **(B)** Recovery of E79 in fractions from ion-exchange chromatography column. Data show average values obtained from three replicate measurements of a single solution.

TABLE 3 | Comparative sensitivity of clinical *P. aeruginosa* isolates to E79 phage at high MOI (10:1 ratio to bacterial cells) or low MOI (1:1 ratio to bacterial cells) and to antibiotics.

Sample ID	Gentamicin	Ceftazidime	Meropenem	Amikacin	Piperacillin	Cefepime	Tobramycin	Ciprofloxacin	Norfloxacin	Timentin	Piperacillin/ Tazobactam	Aztreonam	E79 at low MOI	E79 at high MOI
ARESTCF-1	S	S	S	S	S	S	S	S	S	**I**	S	S	S	S
ARESTCF-2	S	S	S	S	S	S	S	S	S	**I**	S	**I**	**R**	S
ARESTCF-3	S	S	S	S	S	S	S	S	S	S	S	**I**	S	S
ARESTCF-4	S	S	S	S	S	S	S	S	S	S	S	S	S	S
ARESTCF-5	S	S	S	S	S	S	S	S	S	**I**	S	S	S	S
ARESTCF-6	S	S	S	S	S	S	S	S	S	**I**	S	S	S	S
ARESTCF-7	S	S	S	S	S	S	S	S	S	**R**	S	**R**	**R**	**R**
ARESTCF-8	S	S	S	S	S	S	S	S	S	S	S	S	S	S
ARESTCF-9	S	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**
ARESTCF-10	S	S	S	S	S	S	S	S	S	S	S	S	S	S
ARESTCF-11	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S
ARESTCF-12	S	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**
ARESTCF-13	**I**	S	S	S	S	S	S	S	S	S	S	S	**R**	S
ARESTCF-14	S	S	S	S	S	S	S	S	S	S	S	S	S	S

MOI, multiplicity of infection; S, sensitive; I, intermediate; R, resistant. Results with intermediate resistance or resistance are indicated with bolded font and gray shading.

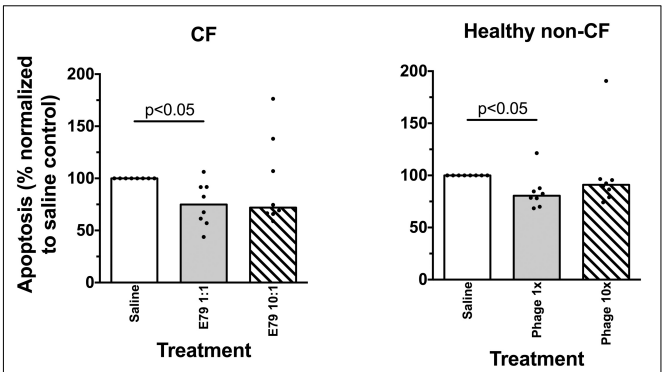

FIGURE 3 | Survival of primary airways cells exposed to phage. Apoptosis in cell cultures exposed to phage at high or low concentration relative to saline controls. Average individual values are shown from three replicate wells, and bars indicate the group median value. Statistically significant differences in Friedman post-tests ($p < 0.05$) are indicated by p-values.

phage at either ratio (data not shown). Similarly, E79 phage did not induce apoptosis in either phenotype at either the low or high phage ratio (**Figure 3**); rather exposure to phage was associated with lower apoptosis levels than observed in control wells.

Comparing 8 non-CF and 8 CF cultures treated with phage suspensions at low or high ratio, there was no induced inflammatory cytokine production (**Figure 4**). Specifically, no significant increase in IL-1β, IL-6, and Il-8 was observed when CF or healthy non-CF cells were treated with low or high phage titres ($p > 0.05$; **Figure 4**).

DISCUSSION

New antibacterial therapies are urgently required to counteract the rise of multidrug-resistant microbes. In this work, we successfully propagated and screened three bacteriophages and identified that the *P. aeruginosa*-infecting phage E79 had the broadest host range of these. Moreover, the protocol utilized to purify phage was successful in removing bacterial contaminants to the extent that no significant apoptosis, or inflammatory cytokine production was detected in either airway epithelial cells from children with CF or healthy non-CF controls after 48 h of co-culture with purified E79 phage. Collectively, this work demonstrates a proof of concept that a screening approach utilizing bacteriophage and primary epithelial cells derived from the CF population could be applied for the development of new therapies for people with CF-associated antibiotic-resistant bacterial lung infections.

Given the association of *P. aeruginosa* airway infections and pulmonary decline in people with CF, early intervention when children are first infected could be critical in preventing lung damage and increase life expectancy (Ranganathan et al., 2017). Inhaled phage therapy is one option that could be utilized in combination with current therapies to improve outcomes. The optimal dosing formulations and regimes would

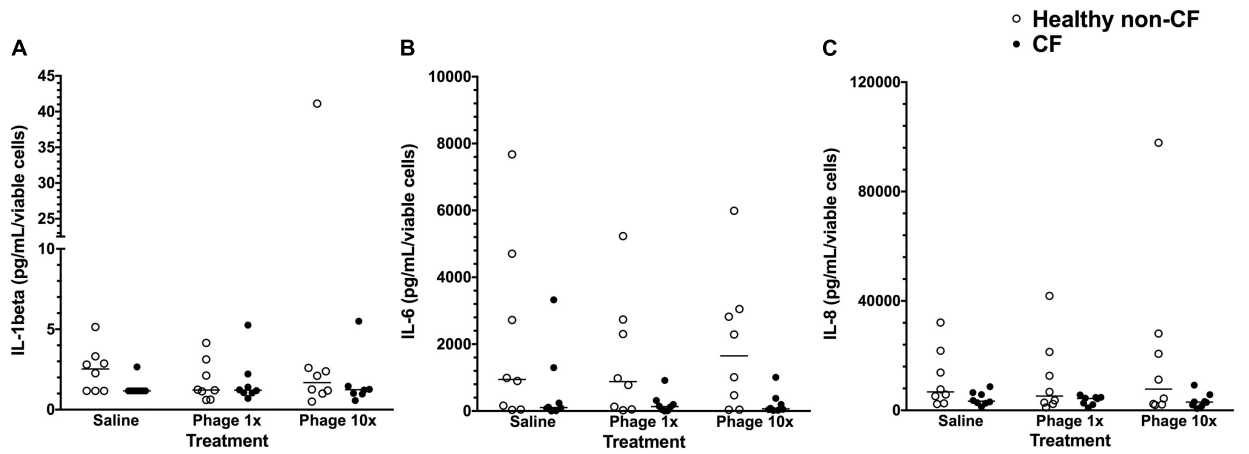

FIGURE 4 | Cytokine production in primary airways cells exposed to phage. Levels of **(A)** IL-1β, **(B)** IL-6, and **(C)** IL-8 cytokines in culture supernatants of cells exposed to treatments for 48 h compared with untreated controls cell. Cytokine concentrations were adjusted for the ratio of viable cells in the phage treatment wells compared with the control well. Data show values for healthy non-CF children (open circles; $n = 8$) or children with CF (closed circles; $n = 8$) representing the mean calculated from three replicate treatment wells per individual. Horizontal lines represent the group (healthy non-CF or CF) median.

need to be determined in clinical trials following identification of ideal phage candidates, using pre-clinical models such as the one described here. A limited number of case studies are described in the literature on treatment of airways infections in children with CF with phage therapy, as well as a number of reports on use in uncontrolled studies in CF and non-CF adults (Sulakvelidze et al., 2001; Kutateladze and Adamia, 2008; Kvachadze et al., 2011; Abedon, 2015; Hoyle et al., 2018). Encouragingly, these early reports suggest that phage therapy may be feasible in the pediatric CF population.

Our pre-clinical screening method demonstrated that almost half of *P. aeruginosa* isolates carried one or more spontaneously released phages. It is likely that these phages were temperate, and therefore may not be ideal for therapeutic use (Hraiech et al., 2015), although we did not confirm this with genome sequencing. Isolation and propagation to high titre proved difficult for most of these phages. The prevalence of non-propagatable isolates in this study is not unique (Serwer et al., 2004), and isolation techniques could be optimized in future, such as inclusion of antibiotics in culture media (Santos et al., 2009). Despite the potential for lysogenic phages to confer resistance to related phages (Labrie et al., 2010; James et al., 2012; van Houte et al., 2016), each bacterial isolate utilized for screening of host ranges here was susceptible to at least one phage tested for activity, indicating that the children from whom the bacteria were isolated could potentially be treated with phage therapy.

We found that the virulent phage, E79, had many ideal characteristics for further development as a therapeutic phage, including stability in high titre preparation, broad infective capacity of clinical *P. aeruginosa* isolates, and lack of known bacterial pathogenicity genes. Indeed, PB1-like phages such as E79 are considered by some to be among the most promising for application in phage therapy (Krylov et al., 2015) due in part to the low frequency of mutation to phage-resistance (Ceyssens et al., 2009). On the other hand, an earlier study suggested that E79 had restricted access to the deeper layers of a biofilm of PAO1 generated on glass (Doolittle et al., 1996), although the authors stated that fluorescently labeling the phage significantly affected infective capacity and that they could not determine whether the decrease in signal deeper in the biofilm was an experimental artifact. In another study, biofilm challenge with E79 resulted in a transient increase at 24 h in biofilm formation, although following further incubation, biofilm decreased (Hosseinidoust et al., 2013). Despite the limitations of extrapolating artificial biofilm methods to human CF lungs, which have very different environmental conditions that might affect bacterial growth, these are important issues to address for phage therapy. Certainly, further characterization would be required for clinical development in a more disease-appropriate model system. Here, to determine bacterial infective capacity, we used a qualitative drop-on-plate assay and an indirect optical density assay of bacterial viability. Whilst these assays may give some indication of a phage's broad infective capacity of various bacterial strains, more accurate prediction of clinical outcomes in patients would ideally involve quantitative measurement of phage activity in a biofilm model, ideally including live/dead cell imaging.

Phage-host dynamics are complex with pharmacokinetic interactions that may not be correctly predicted without including as many components as possible of the phage-bacterial-host cell interaction in a model. *In vivo*, it is predicted that the human immune system would work in a complementary manner to phage to control bacterial growth. Roach et al. (2017) reported that neutrophils are important for immunophage synergy and immunodeficient mice did not respond to phage treatment of *P. aeruginosa* pneumonia in the same manner as immunocompetent mice. Therefore, simple antimicrobial assays may not accurately predict clinical consequences of

novel treatments. Given the differences between healthy and CF neutrophil phenotypes (Gray et al., 2018), it is critical to examine immunophage synergy for people with CF in a relevant model for these individuals, and as such it may be possible to include neutrophils or other immune cells in an airways co-culture model. Although we did not include any non-epithelial cells in this model, one major focus was adequate purification of phage preparation. Given that human airways epithelial cells express a range of Toll-like receptors (Ritter et al., 2005), and our work assessed upregulation of downstream cytokines from NF-κB activation, our model was sufficient for this aim. The response of primary cells derived from different children was personalized; though overall, phage preparations did not induce cytokine production, in some individuals, production of inflammatory cytokines in response to phage exposure was elevated when compared to untreated cells. Despite this, as a group (CF or non-CF children) the differences between phage-exposed and control cell cultures were not statistically significant. Therefore, our data suggest that personalized screening approaches may be best to determine the appropriate treatment for people with CF, where grouped results may not be representative of outcomes for all individuals within that group. However, the method used here in this pilot study could be significantly improved in future by one or all of the following: (a) use of air-liquid interface (ALI) in primary respiratory cell cultures, (b) examination of biofilm, (c) addition of professional immune cells in co-culture with respiratory cells, and (d) delivery of aerosolised phage preparations.

The phage purification methods used here were comparable to those used successfully by others, and resulted in depletion of endotoxin and other bacterial signatures that might have activated epithelial cell inflammatory responses. However, it is likely that without extra preparatory steps, host DNA contamination may be an issue for phage preparations (Kleiner et al., 2015). In addition, it is possible that the decreased levels of apoptosis in phage suspension-treated cells compared with saline-exposed cells is a host cell pro-survival response to damage caused by other PAO1 contaminants remaining in the phage preparation (Wu et al., 2011). Given that we did not find any PAO1-associated genes in the E79 genome, our methods to remove free host DNA were successful, and therefore, it may be appropriate to utilize a similar DNase treatment step to avoid potential transfer of host bacterial genes to bacteria in the human airways, as previously suggested (Pirnay et al., 2015). Previous work with other phages has shown different methods of pharmacological preparation affect viability and particle sizing, which are relevant for inhalation of phages (Leung et al., 2016). In the CF lung environment, it will be important to determine the pharmacological formulations that best allow phages to survive the preparation steps and to enter the lungs with optimal aerodynamic properties in an inhaled formulation (Chang et al., 2017). The activity of optimized phage formulations in the lung environment can be investigated using models of the CF airway, although more purification steps should be taken with phage preparations in future.

In this study, of eight clinical isolates of *P. aeruginosa* that displayed some level of resistance to single or multiple antibiotics, seven exhibited E79 phage-susceptibility at the highest MOI tested, while two out of six antibiotic-susceptible isolates were E79 phage-resistant. This preliminary result suggests that the combined use of traditional antibiotics and phages might be a good approach in at least some cases of antibiotic-resistant infections. Rossitto et al. (2018) have noted the limited number of investigations of combined antibiotic-phage treatment, but consider this a promising approach for the future of anti-*P. aeruginosa* therapy in CF. The rate of progress from bench to bedside that might be predicted by the ease with which novel phages are isolated is in fact limited by the uncertainty surrounding their safety. This situation arises, in part, from inadequate characterization of these phages in clinically relevant CF models. Therefore, in future, the process of selecting phages for therapeutic development could be performed as a high-throughput methodology for people with CF, identifying phages that are suitable for their particular infecting bacterial strain and immunological idiosyncrasies, and determining appropriate dosage using appropriate personalized *in vitro* models.

AUTHOR CONTRIBUTIONS

BC, ST, and AK developed the research concept and designed the experiments. ST and AK carried out the experimental work. ST and MO conducted the data analysis. ST wrote the first draft of the manuscript. All authors contributed to the drafting and editing of the manuscript.

FUNDING

This work was funded by the Telethon Kids Institute Blue Sky Research Grant.

ACKNOWLEDGMENTS

We would like to thank Ms. Jessica Hillas for assistance with cell culture and Dr. Richard Allcock for assistance with genome sequencing. We also thank Dr. Rosemary Carzino for technical assistance in providing AREST CF bacterial isolates. We sincerely thank the participants and their families on the Western Australian Epithelial Research Program (WAERP) for their contribution to this study, and the Telethon Kids Institute for supporting part of this work through the Blue Sky Research Grant.

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Anthony Kicic, Stephen M. Stick, Darryl A. Knight, Elizabeth Kicic-Starcevich, Luke W. Garratt, Marc Padros-Goosen, Ee-Lyn Tan, Erika N. Sutanto, Kevin Looi, Jessica Hillas, Thomas Iosifidis, Nicole C. Shaw, Samuel T. Montgomery, Kak-Ming Ling, Kelly M. Martinovich, Francis J. Lannigan, Ricardo

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Conflict of Interest Statement: AK, SS, and ST have registered a provisional patent related to the use of primary airway cells as a screening tool (P104811.AU).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 04 January 2019
doi: 10.3389/fphar.2018.01475

Animal Models in the Pathophysiology of Cystic Fibrosis

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Our understanding of the multiorgan pathology of cystic fibrosis (CF) has improved impressively during the last decades, but we still lack a full comprehension of the disease progression. Animal models have greatly contributed to the elucidation of specific mechanisms involved in CF pathophysiology and the development of new therapies. Soon after the cloning of the CF transmembrane conductance regulator (*CFTR*) gene in 1989, the first mouse model was generated and this model has dominated *in vivo* CF research ever since. Nonetheless, the failure of murine models to mirror human disease severity in the pancreas and lung has led to the generation of larger animal models such as pigs and ferrets. The following review presents and discusses data from the current animal models used in CF research.

Keywords: cystic fibrosis, CF mice, animals in CF, CF ferrets, CF pigs, CF rats

OPEN ACCESS

Edited by:
Miquéias Lopes-Pacheco, Universidade de Lisboa, Portugal

Reviewed by:
Susan E. Birket, University of Alabama at Birmingham, United States
Rainer Schreiber, University of Regensburg, Germany

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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *27 September 2018*
Accepted: *03 December 2018*
Published: *04 January 2019*

Citation:
Semaniakou A, Croll RP and Chappe V (2019) Animal Models in the Pathophysiology of Cystic Fibrosis. Front. Pharmacol. 9:1475. doi: 10.3389/fphar.2018.01475

INTRODUCTION

Cystic fibrosis, is the most common lethal autosomal recessive disorder in the Caucasian population, affecting one in 2,500 to 3,000 newborns and 70,000 people worldwide (C. G. App, Epidemiology). In 1938, CF was differentiated from celiac disease and named "cystic fibrosis of the pancreas" by Andersen (1938; Davis, 2006). Paul di Sant' Agnese, discovered the sweat electrolyte defect, and the standardization of the sweat test in 1959 made the diagnosis of CF accurate (Gibson and Cooke, 1959; Wine, 2010). Thirty years later, Riordan et al. (1989) discovered the gene responsible for CF, (*CFTR*), located on human chromosome 7. In 1989, they established the basic defect to be in a cAMP-regulated chloride channel (Welsh and Smith, 1993). Since then, an explosion of basic and clinical research has led to therapeutic progress, and the median age of survival today ranges from 40 to 53 years old, with the highest in Canada, which is a remarkable improvement from only 6 months in 1938 (Riordan et al., 1989; Rommens et al., 1989).

In addition, CF is caused by the dysfunction or absence of the CFTR channel from the exocrine tissues due to mutations in the gene (Riordan et al., 1989). While more than 2,000 mutations have been reported to date, the most common disease-causing mutation, occurring in 70% of patients, is F508del, a deletion of three nucleotides that results in the loss of phenylalanine (F) at the 508th position on the protein.

Abbreviations: rAAV, adeno-associated virus; AA, amino acid; ATP, adenosine triphosphate; ASL, airway surface liquid; BAL, bacterial alveolar lavage; *Bcc, Burkholderia cepacia* complex; CACC, Ca^{2+}-activated Cl^- channel; CFTR, cystic fibrosis transmembrane conductance regulator protein; *CFTR*, cystic fibrosis transmembrane conductance regulator gene; CF, cystic fibrosis; ENaC, epithelial sodium channel; iFABP, intestinal fatty-acid binding protein promoter; $[Ca^{2+}]_I$, intracellular Ca^{2+}; KO, knockout; LPS, lipopolysaccharides; MI, meconium ileus; MUC5AC, mucin 5AC; MUC5B, mucin 5B; MCC, mucociliary clearance; PI, pancreatic insufficiency, PS, pancreatic sufficient; PD, potential difference; RE, respiratory epithelia; I_{sc}, short-circuit current; SMGs, submucosal glands, Vt, transepithelial voltage; VIP, vasoactive intestinal peptide; WT, wild type.

Respiratory failure is the major cause of death but as a multiorgan disease, CF also causes PI, intestinal obstruction and malabsorption, biliary cirrhosis and congenital bilateral absence of the vas deferens (Durie and Forstner, 1989; Wong, 1998; van der Doef et al., 2011; Wilschanski and Novak, 2013; Lavelle et al., 2016).

Animal models are essential tools for understanding and comprehensively investigating CF pathophysiology. Each model is unique and variably resembles human CF manifestations with variable ortholog CFTR protein identity (**Table 1**). This review describes the availability of various animal models for CF and how useful they are for studying disease manifestation and progression in the lungs, intestines and other affected organs.

MURINE MODELS IN CYSTIC FIBROSIS RESEARCH

The first genetically modified mouse models of CF disease were created 3 years after the identification of the *CFTR* gene by homologous recombination in embryonic stem cells through targeting a mutation to a specific site in the murine genome (Clarke et al., 1994; Gosselin et al., 1998). Cloning of the mouse homolog of the human *CFTR* gene, which is located on mouse chromosome 6, revealed a 78% AA sequence homology to the human CFTR protein (Tata et al., 1991; Ellsworth et al., 2000). To our knowledge, 14 more mouse models have been described in the literature to date with different characteristics, grouping them in two categoriess (**Table 2**) (O'Neal et al., 1993; Ratcliff et al., 1993; Dorin et al., 1994; Colledge et al., 1995; Hasty et al., 1995; van Doorninck et al., 1995; Zeiher et al., 1995; Delaney et al., 1996; Rozmahe et al., 1996; Fisher et al., 2012). The first category consists of those models in which the *CFTR* gene was disrupted using a "replacement strategy" or an "insertional strategy." A replacement strategy causes an interruption of the *CFTR* gene and generates complete nulls ("knockout": KO) with no normal CFTR protein production, while with the "insertional strategy" a low amount (∼10%) of normal mouse CFTR mRNA is produced (Davidson and Dorin, 2001; Scholte et al., 2004). The models generated by an "insertional strategy" method are also referred as "residual function" models (Davidson and Dorin, 2001). Phenotypic differences are observed between the absolute nulls and the residual function models due to the low amount of normal *CFTR* expression in the latter.

TABLE 1 | Amino acid identity in CFTR orthologs.

Species	CFTR aa Identity vs. humans	Reference
Mouse	78%	Tata et al., 1991
Pig	93%	Ostedgaard et al., 2007
Ferret	92%	Fisher et al., 2012
Rat	75.5%	Trezise et al., 1992
Zebrafish	55%	Liu et al., 2017
Sheep	91%	Harris, 1997

Percentage of amino acid identity of CFTR orthologs from animal models described in this review, and compared to human CFTR.

The second category contains mouse models that replicate known clinical mutations in CF, such as the Class II F508del (*Cftr*^{tm1Eur}, *Cftr*^{tm1Kth}, and *Cftr*^{tm2Cam}) and Class III, G551D (*Cftr*^{tm1G551D}) (Delaney et al., 1996; Lavelle et al., 2016) utilizing a replacement-gene targeting strategy or a double homologous recombination method, also known as a "hit and run" procedure (Hasty et al., 1991; Davidson and Dorin, 2001). In this category, survival rates, disease severity and pathology vary from model to model. The variations are attributed to different types of mutations, levels of mRNA expression, environmental factors and genetic backgrounds that can be responsible for various levels of activity of modifier genes in the mouse strains used (Tarran et al., 2001; Scholte et al., 2004). Moreover, because CF mutant mice are less fertile than normal mice, breeding CF mutant mice from heterozygous animals is strongly recommended to avoid phenotypic abnormalities and experimental variability (Zeiher et al., 1995). Lastly, gender and age are factors that should be considered especially for studying the progression of lung disease.

In a recent study, McHugh et al. (2018a) generated a G542X CF mouse model with CRISPR/Cas9 gene editing method. 40.9% of founder mice expressed the G542X mutation and this new clone was generated in approximately 3 months, whereas traditional methods using embryonic stem cells, would necessitate 1–2 years to obtain the desired mouse colony. These mice have decreased CFTR expression and a lack of CFTR function in the airways and intestine; they have low survival rates due to intestinal obstruction. Pharmacological read-through of the G542X mutation in this mouse model enables functional CFTR protein production, making this model very interesting for further investigation of non-sense mutations (Than et al., 2016; McHugh et al., 2018b).

Do Cystic Fibrosis Mice Develop Spontaneous Lung Disease?

Progressive lung disease with respiratory failure is a major clinical concern that causes 95% of the morbidity and mortality in patients with CF. Lung disease develops over recurrent exacerbations of pulmonary infections caused by a large spectrum of bacterial species including *Staphylococcus aureus*, *Haemophilus influenza*, *Pseudomonas aeruginosa* and the *Bcc* (Tarran et al., 2001; Festini et al., 2006). *P. aeruginosa* is persistent in human lungs with CF and chronic pulmonary infection is inevitable, despite antibiotic therapies, with biofilm-forming cells of a mucoid phenotype in more severe advanced infections. Spontaneous colonization with the above pathogens has not been detected in mice models unless a pathogen challenge was applied.

Kent et al. (1997) designed a model from a single genetic background (congenic), C57BL/6J *Cftr*^{tm1Unc}/*Cftr*^{tm1Unc}, that under specific pathogen-free conditions, developed spontaneous and progressive lung disease (Guilbault et al., 2005, 2006). Loss of mucociliary transport, post-bronchiolar hyperinflation of alveoli, and parenchymal interstitial thickening with indication of fibrosis and inflammatory cell recruitment characterize the lung disease in this congenic model (Kent et al., 1997). It also showed reduced control of the *P. aeruginosa* infection. Acinar and alveolar over inflation, reflect small airway obstructions that

TABLE 2 | Murine models used in cystic fibrosis research.

CF mice models	Cftr mRNA	Survival to maturity	Reference
Knockout models (replacement method)			
Cftr$^{tm/Unc}$	No WT mRNA detectable	<5%	Snouwaert et al., 1992
Cftr$^{tm/Cam}$		<5%	Ratcliff et al., 1993
Cftr$^{tm/Hsc}$		25%	Rozmahe et al., 1996
Cftr^{tm3Bay}		40%	Hasty et al., 1995
Cftr^{tm3Uth}		25%	Wilke et al., 2011
Residual function models (insertional method)			
Cftr$^{tm/Hgu}$	10% of WT	90%	Wilke et al., 2011
Cftr$^{tm/Bay}$	<2% of WT	40%	O'Neal et al., 1993
F508del models (replacement-gene targeting method)			
Cftr^{tm2Cam}	30% of WT	<5%	Colledge et al., 1995
Cftr$^{tm/Kth}$	Decreased levels of mutant mRNA in intestine	40%	Zeiher et al., 1995
F508del models (Hit and Run method)			
Cftr$^{tm/Eur}$	Normal level of mutant mRNA	90%	Rozmahe et al., 1996
Models with other mutations			
Cftr^{tm2Hgu} (G551D- replacement-gene targeting method)	50% of WT	65%	Wilke et al., 2011
Cftr^{tm3Hgu} (G480C- Hit and Run method)	Normal level of mutant mRNA	90%	Wilke et al., 2011
Cftr^{tm2Uth} (R117H- replacement-gene targeting)	5–20% of WT	95%	Wilke et al., 2011
Transgenes			
Tg (FABPCFTR): correction of intestinal obstruction	–	1.5–8.5 months	Zhou et al., 1994
Tg (CCSPSsnn 1b): βENaC over-expression (model of CF-lung disease)	–	40%	Wilke et al., 2011
VIP-KO: CF-like phenotype in lung and intestine	Normal level of WT	Normal	Alcolado et al., 2014

Levels of Cftr mRNA expression, compared to WT Cftr mRNA, are indicated when known. Survival rates at maturity refers to after weaning.

are observed in the early stages of CF in humans (Guilbault et al., 2005). Later on, more studies boosted our knowledge with significant observations in CF pulmonary pathology using mice that were not exposed to pathogens. Thus, excessive inflammation was noticed in *Cftr*^{tmr1Hgu} mice, MCC dysfunction in *Cftr*^{tm1Hgu} and *Cftr*^{tm1Unc} mice, goblet cell hyperplasia with ASL depletion in the nasal epithelium of *Cftr*^{tm1Unc} mice, and a distal extension of SMGs in *Cftr*^{tm1Hgu} and *Cftr*^{tm1G551D} mice (Cowley et al., 1997; Zahm et al., 1997; Tarran et al., 2001; Scholte et al., 2004). The *Cftr*^{tm1G551D} model demonstrated defective pulmonary clearance and a high sensitivity to *P. aeruginosa* (McMorran et al., 2001).

To date, many research groups using congenic mice are still trying to develop methods for *P. aeruginosa* inoculation that would mimic chronic infection with this persistent pathogen. Techniques such as intratracheal inoculation of bacteria-loaded agar beads or seaweed alginate must be used to reproduce the human lung phenotype with *P. aeruginosa*.

The fact that CF mice do not develop airway's infections led Shah et al. (2016) to investigate the reasons why mice have functioning airway's host defenses compared to humans and pigs with CF. This interesting study demonstrated that, in addition to the presence of Ca^{+}-activated anion channels, mice also express small amounts (1–10%) of the non-gastric H^{+}/K^{+} adenosine triphosphatase ATP12A transcripts, which catalyzes the hydrolysis of ATP coupled with the exchange of H^{+} and K^{+} ions across the plasma membrane. Consequently, mouse airways, including those in CF models, secrete low levels of H^{+} maintaining the pH of the ASL unaltered. The authors showed that the over-expression of ATP12A in CF mice airways caused acidification of the ASL, impairment of airway host defenses as well as increased amount of bacteria. These data suggest ATP12A as a potential therapeutic target for CF disease treatment (Shah et al., 2016).

Durie et al. (2004) described a promising long-living congenic *Cftr*$^{-/-}$ model bred in the C57BL/6 background that displays the human lung pathology with signs of inflammation, presence of macrophages, tissue damage, and acinar dilation (Durie et al., 2004). Defective bacterial clearance, increased levels of pro-inflammatory factors, and inflammation was demonstrated using *Cftr*^{tm1Unc} and *Cftr*^{tm1G551D} mice (Heeckeren et al., 1997; McMorran et al., 2001; Van Heeckeren et al., 2006). Macrophages are the most commonly found immune cells in the lung that play a significant role in bacteria eradication from the airways through phagocytosis. The input of macrophages polarization and plasticity in CF disease progression is still undefined. Studies in CF mice have shown that macrophages are unable to degrade internalized pathogens such as *P. aeruginosa* and *Bcc* (Bruscia and Bonfield, 2016; Li et al., 2017). More specifically, *Cftr*$^{-/-}$ mice challenged with *P. aeruginosa* had increased IL-8, IL-6, and TNF-alpha levels and decreased IL-10 level, indicating that the absence of CFTR can cause excessive lung inflammation (Bruscia and Bonfield, 2016). Additionally, the number of both alveolar and peritoneal macrophages was high in F508del mice treated with LPS, illustrating their adaptability in the airway environment (Bruscia and Bonfield, 2016). CF

mice may not be able to develop acute lung disease as patients with CF do, but they have been successfully used to demonstrate a defective autophagy mechanism which can lead to lung hyper-inflammation (Luciani et al., 2010). An increasing amount of literature on CF macrophages is becoming available (Andersson et al., 2008; Bruscia et al., 2009, 2011; Meyer et al., 2009; Abdulrahman et al., 2011; Sorio et al., 2016; Li et al., 2017).

Zhou et al. (2011) generated a transgenic "β-ENaC" mouse model to recapitulate the pathophysiology of the CF lung. This model, which over-expresses the β subunit of the ENaC with increased Na^+ absorption, presented with ASL depletion, reduced MCC with airway mucus obstruction, goblet cell hyperplasia, chronic lung inflammation, and high mortality related to lung disease (Zhou et al., 2011). Nonetheless, even this interesting model failed to replicate the spontaneous bacterial infection that develops in patients with CF (Zhou et al., 2011).

Overall, while the above-mentioned methods and murine models further our understanding of CF lung abnormalities, they do not provide any information on how the disease starts and the development of early stages of infection and inflammation. Furthermore, the general notion that mice do not show "spontaneous" lung pathology could be misleading. Many factors play a role in this. The short life span of mice and clean or sterile environment under which the experiments are conducted are not comparable with the conditions for human lungs with CF that are continuously challenged with various pathogens along their lifetime. In addition, diverse genetic background is an important factor, while low levels of residual CFTR protein expression in some models affect the disease severity. Moreover, the size of the airways and the presence of a non-CFTR calcium-activated Cl^- channel (CACC) in parallel with CFTR and other dissimilarities in lung disease between humans and mice models (Clarke et al., 1994; Gosselin et al., 1998; Lavelle et al., 2016) can account for differences in resistance to bacteria.

Submucosal Glands to Study Fluid Secretion Defects

Submucosal glands lining the cartilaginous airways are responsible for maintaining sterility in the airways by secreting antimicrobial defenses, such as lysozyme, lactoferrin, defensins, bicarbonate, and others (Salinas et al., 2005; Ianowski et al., 2007). Moreover, they secrete mucus when stimulated with Ca^+ -elevating agonists, for example acetylcholine (ACh) or carbachol (CCh), and/or cAMP agonists, such as forskolin (FSK) or VIP, and contain serous cells that express high levels of CFTR (Borthwick et al., 1999). In CF airway glands, the synergy between cAMP and calcium signaling is lost, and glands do not respond to VIP or FSK. In humans, SMGs extend into the bronchi, while, in mice they are confined to the trachea. Congenic mice models, especially those with a C57BL/6 genetic background, have been used to study SMG dysfunction since these mice show increased airway abnormalities and are prone to infection with *P. aeruginosa*. Grubb and Boucher (1999) mentioned that mice have very few SMGs (Grubb and Boucher, 1999). However, in a study of tracheal SMG secretion in $Cftr^{-/-}$ mice, Ianowski et al.(2007) observed that the upper trachea contains only 15–20 airway glands in wild-type (WT) mice and an abundance of glands in CF-KO mice (De Jonge, 2007; Ianowski et al., 2007). Furthermore, they demonstrated that glands from an inbred congenic $Cftr^{-/-}$ mouse strain responded to CCh but not to VIP or FSK similar to human CF glands (Ianowski et al., 2007). This study also documented that glands from CF mice did not secrete when they were exposed to chili oil, a treatment that produces secretion through stimulation of sensory nerves and the release of tachykinins, such as substance P. Additionally, it was demonstrated for the first time, that on tracheal SMGs of $Cftr^{-/-}$ mice, the substance P-stimulated fluid secretion requires CFTR (Ianowski et al., 2007).

The neuropeptide VIP is abundantly secreted around all exocrine glands including SMGs and sweat glands. It is well known to work in synergy with ACh to regulate secretions (Lundberg et al., 1980; Heinz-Erian et al., 1985, 1986). Interestingly, VIP KO mice generated in the C57BL/6 background demonstrated, *in vivo,* the central role of VIP in CFTR regulation (Chappe and Said, 2012; Alcolado et al., 2014). Originally developed to study airway diseases, such as bronchial asthma (Hamidi et al., 2006; Szema et al., 2006; Said, 2009), VIP-KO mice were used by Alcolado et al. (2014) to demonstrate the molecular link between VIP and CFTR regulation in the airways and small intestines, and observed a CF-like phenotype. The absence of VIP resulted in CFTR intracellular retention. A subsequent loss of CFTR-dependent chloride current was measured in functional assays with an Ussing chamber analysis of the small intestine *ex vivo*. In the lung, lymphocyte aggregation, increased airway secretion, alveolar thickening, and edema were observed. Inflammation and tissue damage observed in VIP-KO mice were attributed, at least in part, to the lack of CFTR-dependent secretions that rely on VIP stimulation of CFTR function. These observations support early findings of a lack of VIPergic innervation of human CF glands.

Nasal and Tracheal Epithelium

Murine CF nasal epithelium exhibits two important characteristics of human CF airways: Na^+ hyper-absorption and a defect in cAMP-mediated Cl^- secretion (Grubb and Boucher, 1999; Tarran et al., 2001). Hyper-absorption of Na^+ was demonstrated *in vivo* with an increased baseline PD across the epithelium that was largely decreased in the presence of the ENaC blocker, amiloride (Grubb et al., 1994; Grubb and Boucher, 1999). In addition, the decreased level or absence of cAMP-stimulated Cl^- conductance was noticed in all murine models except for F508del $Cftr^{tm1Eur}$. As mentioned above, the ∼10% residual normal CFTR expression in this model, may allow for a residual Cl^- permeability. In addition, nasal epithelium from $Cftr^{tm1Unc}$ and $Cftr^{tm1Hsc}$ demonstrates a Cl^- secretory response, which may be due to calcium dependent chloride channel (CACC) (Grubb and Boucher, 1999). The CACC is the prevalent Cl^- channel in

murine tracheal epithelium (Rock et al., 2009). The gene that encodes this "pseudo" Cl^- channel is *TMEM16A*, and it was shown to be necessary for maintaining regular ASL levels. Studies on tracheas from *Tmem16a*$^{-/-}$ mice demonstrated more than 60% lower CACC activity and reduced MCC with mucus accumulation (Ousingsawat et al., 2009; Rock et al., 2009).

Despite bioelectric similarities, the murine nasal mucosa is composed of ∼40% olfactory epithelia and 60% RE, while the human nasal cavity is lined by more than 95% RE. However, the dominant cell type is the cilia cell, as in humans (Harkema et al., 1991; Tarran et al., 2001). In the *in vivo* analysis of *Cftr*^{tm1Unc}, the nasal septal mucosa demonstrated goblet cell hyperplasia and enlargement correlated with reduced ASL height rather than changes in salt composition (Tarran et al., 2001). A study using *Cftr*^{Hm1G551D} and *Cftr*^{tm1Unc}–*TgN*$^{(FABRCFTR)}$ mouse models was conducted to examine whether the structural changes in nasal mucosa were related to infection and inflammation. Interestingly, they detected features that are comparable with human CF tissues, such as thickening of the RE with an increased number of mucus cells. The conclusion of the study suggested that the changes observed were not a consequence of initial inflammation (Hilliard et al., 2008). Another study from Grubb et al. (2007) using *Cftr*^{tm1Unc} mice reported that an ion transport defect in CF results in progressive morphological and structural changes in the olfactory epithelium of the nasal cavity (Grubb et al., 2007).

The nasal cavity has been used extensively to investigate Cl^- permeability in response to corrector drug treatment by measuring the PD across the nasal epithelium. The murine nasal mucosa appears to offer an excellent model for investigating the abnormal electrophysiological profile of CF epithelial cells and testing potential corrector drugs. The murine trachea has also been used for studying cartilaginous rings alterations in *Cftr*$^{-/-}$ and F508del-CFTR mice. Structural modifications were observed in both newborn and adult CF mice independently of the CFTR absence or protein dysfunction and those changes could be indigenous (Bonvin et al., 2008).

Although the murine nasal cavity closely resembles that of humans and is easily accessible, most studies have rather used murine tracheal SMGs to examine MCC abnormalities and airway submucosal inflammation (Zahm et al., 1997; Grubb et al., 2004; Ianowski et al., 2007). Additionally, data from other animal models reported significant differences in size, density, secretory capacity and fluid secretion defects between nasal and tracheal glands. Ianowski et al. (2007) suggested that the murine trachea is an appropriate tissue for studying airway glands abnormalities in CF (Ianowski et al., 2007). Airway SMGs are found in the proximal trachea of mice. In the cartilaginous rings, CFTR expression is found at the highest levels in SMG serous tubules as reported by Engelhardt et al. (1992). Furthermore, it is well known that serous cells secrete antimicrobial proteins, such as lysozyme and lactoferrin, indicating the importance of tracheal glands in CF disease (Engelhardt et al., 1992). Nevertheless, in murine trachea, the CACC exhibits "CFTR-like" activity and protects CF mice from having the severe lung phenotype that is observed in humans. Consequently, one must ask whether murine tracheal glands are appropriate for studying secretion abnormalities in CF. Until now, there has been no consensus about it.

Cystic Fibrosis Mice Develop Severe Postnatal Intestinal Disease

One of the earliest disease manifestations in CF is the MI in 15–20% of CF newborns and distal intestinal obstruction syndrome in 25% of adults (Zielenski et al., 1999). Moreover, CF mice are characterized by severe intestinal obstructions, including mucus accumulation, goblet cell hyperplasia, and crypt dilatation and elongation, symptoms similar to the human disease manifestations (Grubb and Boucher, 1999; Norkina et al., 2004), which develop postnatally and appear to be the hallmark for all the CF mouse models (Grubb and Gabriel, 1997; Keiser and Engelhardt, 2011) with the exception of only two mouse models (*Cftr*^{tm1HGU} and *Cftr*^{tm1Eur}). The severity of the disease and the survival rate differ among genotypes (Grubb and Gabriel, 1997; Durie et al., 2004; Canale-Zambrano et al., 2007; De Lisle and Borowitz, 2013). The neonatal death rate is ∼60% for CF null mice and only 35% for F508del-CFTR. However, this percentage of MI is still high compare to only 10% in the human disease. The *Cftr*^{tm1Eur} and *Cftr*^{tm2Hgu} models, carrying the F508del and G480C mutations, respectively, exhibit intestinal alterations that are not as severe as those in the *CFTR*-KO mice (De Lisle and Borowitz, 2013). The *Cftr*^{tm1G551D} has an approximate 70% survival rate with less severe pathology than the null models (Scholte et al., 2004). A murine F508del model was generated with a replacement-gene targeting method by Colledge et al. (1995) and was suggested to be an accurate model for testing therapeutic drugs. It demonstrated severe meconium obstruction, reduced cAMP-stimulated Cl^- secretion, and the temperature-dependent trafficking defect that is described associated with this mutation in the human disease (Colledge et al., 1995). Tissue sections of the colon and duodenum revealed huge crypt dilation with increased mucus accumulation. As previously mentioned, the genetic background variation and low residual level of expression of normal CFTR proteins have been proposed to explain dissimilarities between models.

Studies have shown that the expression of modifier genes may also be responsible for this intestinal variability. A genetic modifier of MI was detected in chromosome 19 in humans and on mouse chromosome 7 and was shown to be involved in the disease progression in KO mice (Rozmahe et al., 1996; Zielenski et al., 1999; Gyömörey et al., 2000). Norkina et al. (2004), using a DNA microarray, observed the presence of some gene expression associated with inflammation and presence of innate immune cells, such as mast cells and neutrophils, similar to those observed in the intestine of patients with CF (Norkina et al., 2004). Amelioration of the intestinal obstruction and an increase in the survival rate was achieved by the expression of human CFTR cDNA in the intestinal track of *Cftr*^{tm1Unc} mice, under the control of the rat intestinal fatty-acid-binding protein (iFABP) gene promoter. This *Cftr*^{tm1Unc}–*TgN*$^{(FABPCFTR)}$

model enabled long-term studies in the intestine (De Lisle and Borowitz, 2013).

In a recent study, McHugh et al. (2018b) investigated the efficacy of linaclotide in treating intestinal pathophysiology in CF mice carrying the F508del mutation or *Cftr*-KO mice. Linaclotide is a drug that has been approved by FDA to treat chronic idiopathic constipation. This study demonstrated that this drug elevates the amount of fluid in the intestinal lumen and improves intestinal transit, thus prompting the authors to recommend linaclotide as a therapeutic option to treat CF intestinal manifestations (Than et al., 2016; McHugh et al., 2018a).

Electrophysiological studies have confirmed the ion transport abnormalities in CF mice with a similar phenotype to that of the human CF intestine: low or absence of cAMP-mediated Cl^- transport (Grubb and Gabriel, 1997). Moreover, they display a significant reduction in the basal PD and short-circuit current (I_{sc}) (Grubb, 1999).

In summary, the CF murine intestine exhibits many pathophysiological similarities with that in humans with CF. However, it is important to categorize the different mouse models and background strains, the various techniques by which they were generated, the pathogen exposure, and the husbandry conditions during the research before evaluating the intestinal phenotype.

Cystic Fibrosis Mice Develop Mild Pancreatic Disease

In contrast to patients with CF in whom exocrine PI is a major condition, CF mice exhibit only moderate pancreatic changes (Snouwaert et al., 1992). Durie et al. (2004) demonstrated that a long-living CF-KO mouse bred into a congenic C57BL/6 background develops pancreatic pathology similar to humans with presence of inflammatory cells and macrophages and increased acinar volume at an older age (Durie et al., 2004). Surprisingly, mice with the F508del or G551D mutation, do not show any apparent pancreatic disease (Dorin et al., 1992; Colledge et al., 1995; van Doorninck et al., 1995; Delaney et al., 1996). The milder pancreatic disease in mice is attributed first to the lower level of normal CFTR protein expression in the murine pancreas, while high CFTR expression level has been shown in humans (Gray et al., 1995), and second to differences in the CACC channel expression. The CACC is detected in both WT and CF mice alleviating the pancreatic disease progression and perhaps explaining the mild murine pancreatic pathology (Marino et al., 1991; Clarke et al., 1994; Gray et al., 1995; Keiser and Engelhardt, 2011).

Mice Sweat Glands – An Underestimated Tissue in Cystic Fibrosis Research

Increased NaCl reabsorption abnormalities from sweat have been broadly studied with the sweat concentration being the most reliable pathophysiological marker. In mice, sweat glands are present in the palmoplantar skin (paw) (Lu and Fuchs, 2014). This tissue has largely been underutilized in CF research. In humans, sweat glands are controlled by adrenergic, cholinergic, and peptidergic innervation (Heinz-Erian et al., 1985). Heinz-Erian et al. (1985) demonstrated a reduction of VIP, in the sweat glands of patients with CF (Heinz-Erian et al., 1985). They proposed that, since VIP activates the opening of the CFTR channel, enabling the movement of water and Cl^- across epithelium, the lack of VIP innervation could cause low levels of water, Cl^- impermeability, and abnormalities involving other ions that manifest in CF disease. Sato et al. (1994) investigated changes in cholinergic and β-adrenergic responsiveness during the postnatal life and reported that the mouse sweat glands are mainly under cholinergic control and express CFTR. Sweat secretion can be monitored in response to cAMP elevating agonists with activation of either K^+ channels, Cl^- channels, or both. The secretion rates are higher in older animals (over 6-weeks old) but are independent from glandular enlargement. Additionally, Chappe et al. (personal communication) examined VIP expression in sweat glands of F508del mice and found support for the murine sweat glands as a valuable tissue for studying CF disease progression. Sweat glands remain free of infection, do not exhibit morphological alterations and are easy to collect. Accordingly, their use should be further evaluated for studying CF disease development and progression.

PORCINE MODELS IN CYSTIC FIBROSIS RESEARCH

Cystic Fibrosis Pigs Develop Lung Pathology Gradually

The expression of the CACC channel in parallel with the CFTR channel in some mouse organs and the inability of mice to develop "spontaneous" lung disease led investigators to examine the pathology of the CF lung using other animal models. Rogers et al. (2008b) recommended porcine airways as a possible model for CF because of their similar lung anatomy and morphological similarities with humans and identical bioelectric ion properties. Welsh et al. (2001, 2009) (Rogers et al., 2008b; Fisher et al., 2011) developed the first CF pigs with homologous recombination, utilizing adeno-associated virus (rAAV) vectors to target the *CFTR* gene in fibroblasts from fetal pigs. A somatic cell nuclear transfer of these cells into oocytes was made to generate a heterozygous male pig and through heterozygous × heterozygous breeding, $Cftr^{-/-}$ piglets were born (Rogers et al., 2008b).

At birth, the lungs of the $Cftr^{-/-}$ piglets, like those of human CF infants, are normal with no signs of pathology (Rogers et al., 2008c). However, in contrast to control animals, BAL fluid from neonatal CF piglets revealed a large number of different bacterial species (Rogers et al., 2008c; Welsh et al., 2009; Stoltz et al., 2010) and a rapid lung infection, which shows a more severe lung pathology than in patients with CF (Stoltz et al., 2010; Wine, 2010). When lungs from newborn WT and CF piglets were specifically challenged with *S. aureus*, the most common lung pathogen in infants and children with

CF, only the CF piglets had more *S. aureus* than normal (Stoltz et al., 2010). That could be due to abnormalities in the pH of the ASL caused by reduced CFTR-dependent bicarbonate transport as reported by Pezzulo et al. (2012). Newborn $Cftr^{-/-}$ pigs were also used to examine macrophage dysfunction and to determine if their defective polarization and function are due to the absence of CFTR, or a consequence of the chronic inflammation and infection. Data from this study suggested that defective macrophages function is present at the onset of the disease and is the cause of inflammation (Paemka et al., 2017). Since the newborn CF pigs do not present signs of lung inflammation at birth, they are considered a good model for studying macrophages dysfunction prior to the beginning of the chronic inflammation.

Importantly, neonatal CF piglets have not been examined after a challenge with *P. aeruginosa*, and hence we do not know if CF pig airways are able to eradicate these bacteria. These data suggest that lungs from newborn CF pigs lack inflammation at birth but are unable to sterilize their lungs, suggesting that infection initiates inflammation (Wine, 2010).

The CF piglets revealed tracheal abnormalities, including narrowed proximal airways with thicker and more discontinuous cartilage, enlarged SMGs, and hyperplasia. Alterations in tracheal smooth muscle of F508del homozygote piglets were milder because of the residual CFTR function (Ostedgaard et al., 2011). These congenital tracheal alterations are similar to airway structural changes that occur in CF infants (Meyerholz et al., 2010a; Stoltz et al., 2015). It is suggested that the loss of CFTR produces tracheal abnormalities that may contribute to CF disease development during postnatal life (Rogers et al., 2008c; Welsh et al., 2009). However, the specific mechanisms for these abnormalities and how the absence of CFTR during development induces these defects are still unknown (Meyerholz et al., 2010a).

While mucociliary transport plays a significant role in maintaining the airways sterile, it is still unknown if in CF it is defective from birth and contributes to the beginning of the disease or if it becomes defective later, contributing to disease progression. A study from Hoegger et al. (2014a), established a new *in vivo* mucociliary transport assay (X-ray-computed tomographic-based method) with newborn pigs, which provides important insight into the pathophysiology of CF, as well as other respiratory diseases, at their early stages. The authors analyzed the movement of particles in bulk, and individually, in the large airways. They found that cilia orientation directs particles to the ventral area of the trachea and to the larynx, while the rate of individual particle's movement was largely heterogynous. They speculate that different mucus properties may account for this heterogeneity (Hoegger et al., 2014a). Moreover, using 350 μm diameter tantalum microdisks, they found that CF SMGs secrete dense mucus strands that remained tethered and prevent the movement of those dense disks. The authors conclude that impaired mucus detachment from SMGs could be an early defect in CF (Hoegger et al., 2014b).

The development of lung disease in CF pigs is not easy to investigate because most of them die from MI that occurs in 100% of the piglets (Rogers et al., 2008c; Welsh et al., 2009; Meyerholz et al., 2010b; Stoltz et al., 2010, 2013; Olivier et al., 2015). A study from Stoltz et al. (2010), performing an ileostomy on CF pigs that survived approximately 2 months or more, demonstrated the presence of structural, bacteriological and inflammatory markers of CF. Part of the airways were characterized by the presence of macrophages, neutrophils and bacteria, similar to those in human airways. Ostedgaard et al. (2011) demonstrated that, despite residual CFTR expression, older $Cftr^{F508del/F508del}$ pigs exhibited lung disease similar to humans, rendering the minimal CFTR activity observed (∼6% to WT function) insignificant for lung pathogenesis. A study from Shah et al. (2016) demonstrated that CF pigs, like humans, have ASL acidification in contrast to mice. This is thought to be due to ATP12A in absence of CFTR chloride channels. Acidification of the ASL impairs airway defense while ATP12A inhibition, which increases the pH, improves host defense properties of the ASL in CF pigs (Shah et al., 2016).

Although CF pigs recapitulate most of the human manifestations of CF, only a small number have been used for studying CF pathology, mostly due to the difficulty of keeping them alive long enough. Even after an ileostomy or cecostomy to cure MI, 60% of the piglets still had to be euthanized for gastric ulcers (Stoltz et al., 2010; Wine, 2010; Olivier et al., 2015). Long-term research still needs to be conducted to validate the data.

Porcine Submucosal Glands – Excellent for Studying Secretion

Porcine lungs highly express CFTR proteins in the serous cells of SMGs of the cartilaginous airways, and, as in humans, exhibit the same secretion abnormalities in CF animals, with no detected morphological changes (Rogers et al., 2008c; Welsh et al., 2009). Chen et al. (2010) demonstrated a lack of cAMP-mediated Cl^- transport and subsequently impaired bicarbonate (HNO_3^-) transport with an absence of sodium hyper-absorption or reduction in ASL depth (Chen et al., 2010). This finding in CF pigs, with no signs of inflammation at birth, contradicts the hypothesis that Na^+ hyper-absorption might trigger lung disease. The authors suggested that defects in Cl^- and HNO_3^- initiate lung pathology and not deviations in sodium transport (Chen et al., 2010; Stoltz et al., 2015).

Ion transport abnormalities were detected in nasal airway epithelia from CF piglets with a hyperpolarized baseline transepithelial voltage (Vt), while the addition of amiloride (ENaC inhibitor) decreased the Vt in all CF piglets (Rogers et al., 2008a,c; Welsh et al., 2009). Perfusion of the apical membrane with a solution containing low Cl^- concentration and isoproterenol (to elevate cAMP) hyperpolarized nasal Vt in WT and heterozygous but not in $Cftr^{-/-}$ pigs (Rogers et al., 2008a,c; Joo et al., 2010). Lastly, perfusion with ATP to stimulate P2Y2 receptors and CACC channels produced further hyper-polarization while the CFTR inhibitor glybenclamide depolarized Vt in $Cftr^{+/+}$ and $Cftr^{+/-}$ but not in $Cftr^{-/-}$ piglets, demonstrating that a lack of CFTR channel function causes electrolyte transport abnormalities in airway epithelia (Rogers et al., 2008c; Welsh et al., 2009). The CFTR selective inhibitor CFTRinh-172 was found to be less effective with the porcine

protein compared to the human CFTR (Rogers et al., 2008a). Liu et al. (2007) have reported species-specific differences in airway epithelial electrolyte transport and CFTR function (Liu et al., 2007).

Nasal vs. Tracheal Submucosal Glands

Human CF SMGs only secrete minimally in response to agonists that increase intracellular cAMP alone (VIP and FSK) or slightly to agonists that elevate $[Ca^{2+}]_i$ (substance P and CCh) (Choi et al., 2007). The combination of these two types of agents produce a synergetic response that is lost in CF. Accordingly, Joo et al. (2010) showed that tracheal SMGs from CF piglets did not respond to FSK but did respond to CCh, while substance P proved to be more efficacious in CF pigs than in humans. In contrast to human SMGs, *Cftr*$^{-/-}$ pigs exhibited synergistic secretion which was attributed to CFTR-independent pathways (Joo et al., 2010). Future studies on the specific mechanisms are necessary and would clarify whether this tissue is appropriate for studying fluid secretion defects in CF disease.

Interestingly, a study from Ostedgaard et al. (2017) demonstrated that tracheal SMGs from CF pigs produce mucin 5B (MUC5B) while goblet cells produce mainly mucin 5A (MUC5AC) and moderate MUC5B. The same study suggested that these two types of mucins have different morphologic structures that account for a more effective MCC transport (Ostedgaard et al., 2017).

Cho et al. (2011) described morphological and fluid secretion differences between nasal and tracheal glands in CF piglets. In contrast to tracheal glands, nasal glands were found to be smaller and more numerous than in humans (Cho et al., 2011) with no response to FSK and a moderate response to CCh, while the synergy was almost lost (Joo et al., 2010). These findings are of high interest because, as Rogers et al. (2008c) showed, CF piglets have abnormal ion transport in nasal epithelium (Rogers et al., 2008c) which could contribute to lung pathology in CF when combined with defective fluid secretion from nasal glands.

Intestinal Obstruction Is Fatal in Cystic Fibrosis Piglets

Meconium ileus is 100% fatal for CF pigs and a surgical ileostomy must be performed right after birth so as they can survive longer (Welsh et al., 2009; Meyerholz et al., 2010b; Stoltz et al., 2013). Accordingly, to suppress intestinal obstruction, Stoltz et al. (2013) used the rat intestinal fatty-acid-binding protein promoter (iFABP) to express WT porcine CFTR proteins (pCFTR) in *Cftr*$^{-/-}$ "gut-corrected" pigs (Stoltz et al., 2013). Their data supported the hypothesis that approximately 20% of CFTR expression was adequate to partially restore CFTR-mediated Cl^- transport. Similar to *Cftr*$^{-/-}$ and *Cftr*$^{F508del/F508del}$ pigs, these transgenic animals developed pancreatic, liver, and lung disease and therefore failure to thrive (Stoltz et al., 2013). The expression of CFTR proteins in parts of the intestine, other than in ileum, might be important for improving the MI defect. Gut correction was tested only in neonatal CF pigs and hence it is not known yet if the required amount of CFTR function during adult life would be the same. Nonetheless, while surgical gut correction is not always successful, this transgenic model would be another method for making these animals live long enough (up to 12 months) to study disease progression.

Interestingly, while one would expect less disease severity in *Cftr*$^{F508del/F508del}$ pigs compared to null animals, due to residual function of CFTR (∼6% of WT), they still show 100% penetrance of MI, as was reported by Ostedgaard et al. (2011). In general, MI in CF pigs closely resembles the disease in humans, while distinct anatomy or physiology in combination with the differences in genetic background between pigs and humans may account for the dissimilarity of penetrance rate.

Porcine Pancreatic Pathogenesis Is Closely Similar to Humans

Cystic fibrosis pigs closely recapitulate pancreatic pathogenesis of humans CF with varying levels of disease severity among genotypes (Rogers et al., 2008c; Meyerholz et al., 2010b). After surgical correction of MI, PI develops spontaneously in these animals (Keiser and Engelhardt, 2011). The exocrine pancreas in newborn *Cftr*$^{-/-}$ pigs, as in human CF neonates, is smaller than normal, with degenerative lobules containing loose adipose tissue, mild, scattered inflammation, and dilated ducts with eosinophils, neutrophils, and macrophages. The endocrine tissue is intact, spared from the surrounding exocrine destruction with fewer acinar cells and zymogen granules (Rogers et al., 2008c; Welsh et al., 2009; Meyerholz et al., 2010b). Insulin-immunoreactive islets are mostly localized on the intact lobular tissue without alterations in the amount of insulin produced or glucagon cells. The volume and pH of pancreatic fluid are reduced in CF neonatal pigs, as in humans, with increased protein concentration (Gibson-Corley et al., 2016).

Interesting data from Abu-El-Haija et al. (2012) in fetal and newborn *Cftr*$^{-/-}$ and *Cftr*$^{F508del/F508del}$ pigs demonstrated that tissue damage starts *in utero* and pro-inflammatory, complement cascade, pro-apoptotic, and pro-fibrotic pathways are involved (Abu-El-Haija et al., 2012). They noticed elevated activation of the apoptotic caspase-3 pathway in the CF pig pancreas whereas the α-smooth muscle actin, a myofibroblast marker and activator of early fibrosis pathways, was observed in both fetal and newborn pigs. Tissue remodeling with mucus accumulation and duct cell proliferation was observed in the neonatal and not in fetal CF pigs, indicating that these changes do not initiate disease pathogenesis but follow pancreatic damage over time. Compared to *Cftr*$^{-/-}$, *Cftr*$^{F508del/F508del}$ pigs exhibit slightly less severe exocrine pancreatic pathogenesis that may be attributed to the residual CFTR function (Ostedgaard et al., 2011). Future studies that investigate how much CFTR chloride channel function is necessary for the correction of pancreatic disease would be a fundamental knowledge for the progression of CF pathogenesis. Studies in adult CF pigs would be useful and would enhance our understanding of pancreatic damage in humans.

FERRET MODELS IN CYSTIC FIBROSIS RESEARCH

Comparable Lung Pathogenesis With Cystic Fibrosis Patients

The lungs of ferrets share many similarities with human lungs in terms of function and architecture, and like CF pigs, CF ferrets develop spontaneous lung infections soon after birth (Sun et al., 2010, 2014a; Keiser et al., 2015). Similar to pigs, CF ferrets were produced using homologous recombination. The first genetically designed CF ferret was described by Sun et al. (2008), utilizing rAAV-mediated targeting of the *CFTR* gene in fetal fibroblasts followed by nuclear transfer cloning (Sun et al., 2008). The *Cftr*$^{-/-}$ ferrets develop severe lung pathology within the first weeks of life, and like piglets, they are unable to eliminate bacteria. Data from the BAL fluid of newborn CF ferrets revealed various bacterial species including *Streptococcus*, *Staphylococcus* and *Enterococcus* genera while three species of *Pseudomonas* (*P. fluorescents, P. putida* and *P. fulva*) were found in low amounts (Sun et al., 2010, 2014a). In addition, macrophages from CF ferrets produce high levels of cytokines after being challenged with pathogens such as *P. aeruginosa*, *Bcc* or challenged with LPS (Bruscia and Bonfield, 2016). Interestingly, a study from Keiser et al. (2015) demonstrated differences in BAL fluid composition from CF and WT animals (Keiser et al., 2015). Levels of lactoferrin and lysozyme were especially increased in the naturally born CF ferrets in contrast to those delivered by C-section, while α_1-antitrypsin and apolipoprotein A1 were reduced following natural birth but remained unaltered in animals delivered by C-section (Keiser et al., 2015). Moreover, proteins related to neutrophil activity were raised in naturally born CF ferrets but absent in those delivered by C-section. The authors measured levels of inflammatory cytokines (IL-1β, IL-8, and TNF-a). They did not find any alteration between C-sectioned CF and non-CF ferrets while the newborn CF animals exhibited lower levels of IL-1β, but IL-8 and TNF-α were higher, similar to infants with CF.

Thus, they concluded that inflammatory dissimilarities in CF ferrets may start *in utero* and result in excessive inflammation during birth, following bacterial exposure (Keiser et al., 2015). These data suggest that innate immunity and inflammatory pathways change under sterile and non-sterile conditions, making these animals a valuable model for studying the early onset and progression of CF disease.

Adult CF ferrets do not show abnormal inflammatory response. However, they have other similarities in lung pathology with humans, such as airway obstruction, thick mucus accumulation, and inflammation (Sun et al., 2014a).

Postnatal Development of Submucosal Glands

In ferrets, as in humans, the CFTR protein is highly expressed in serous cells of SMGs lining their cartilaginous rings. Newborn ferrets do not have ciliated cells or SMGs, but they develop them abundantly a few months after birth (Sun et al., 2014a; Keiser et al., 2015). A study from Keiser et al. (2015) on newborn *Cftr*$^{-/-}$ ferrets reported that tracheal MCC was similar to non-CF ferrets, while ASL height was decreased. Only 1–3 weeks after birth, when MCC rate slightly increases, CF related MCC abnormalities would have an impact on the lung pathology (Keiser et al., 2015). Additionally, Sun et al. (2014a) demonstrated a seven-fold decrease in MCC in adult compared to juvenile *Cftr*$^{-/-}$ ferrets that was attributed to an age-dependent ENaC hyperactivity (Sun et al., 2014a). The unique characteristic of ferret lungs to develop SMGs postnatally suggests the presence of CFTR-independent mechanisms that cause the early lung inflammatory profile and future investigations will be valuable.

Ferret Trachea Is Appropriate for Studying Gland Secretion

Adult ferrets have an abundance of tracheal glands, and like humans, they depend on Cl^- and HCO_3^- for secretion (CFTR-dependent). In adult WT ferret tracheas, Cho et al. (2010) revealed a reduced secretion rate by blocking Cl^- or HCO_3^- in response to VIP or FSK. Additionally, CCh-induced MCC was highly decreased when blocking the Na^+-K^+-$2Cl^-$ (NKCC) co-transporter with bumetanide. The authors showed synergistic secretion when low concentrations of agonists that elevate $[cAMP]_i$ were combined with low concentrations of agonists that elevate $[Ca^{2+}]_i$. Neonatal ferret tracheas contain few cells that express high levels of CFTR since SMGs are not yet developed. Studies on newborn *Cftr*$^{-/-}$ ferrets have shown abnormal fluid secretion from their tracheas in response to FSK and CCh, with higher reduction to FSK stimulation, similar to data from human proximal CF airways (Sun et al., 2010).

As CF ferrets have a similar airway morphology to humans, they are a good model for studying CF pathogenesis. They develop human-like lung disease and CFTR-dependent secretion in trachea, albeit the amiloride-sensitive transepithelial PD was unaltered between CF and non-CF ferret tracheas. However, research in CF ferret fluid secretion is spare, and while it seems a promising animal model, our knowledge has been limited until now.

Intestinal Disease Is More Severe in Ferrets Compared to Humans

Newborn CF ferrets, like CF piglets, present a higher incidence of MI than seen in human infants with CF. Between 50% and 100% of CF ferrets develop MI and die within 36 h due to intestinal obstruction and sepsis (Fisher et al., 2011). Most of CF ferrets that survive MI still fail to thrive soon after birth because of malabsorption. Compared to other animals, they do not have caecum and therefore have less time to digest (Sun et al., 2010). A study from Sun et al. (2014b) in CF ferrets over a month old reported that the small intestine was characterized by a reduction in the height of the villi and mucus accumulation in the crypt with signs of inflammation within the lamina propria and into the epithelium (Sun et al., 2014b). A metagenomic deep

sequencing analysis revealed the exacerbation of *Streptococcus*, *Enterococcus,* and *Escherichia* in CF ferret feces, similar to what occurs in CF infants (Sun et al., 2014b). A gut-corrected transgenic model that expresses the *CFTR* gene under the control of the rat fatty acid-binding protein promoter (iFABP), is shown to correct MI in newborn $Cftr^{-/-}$ ferrets (Sun et al., 2010). The generation of a F508del-CFTR ferret would be useful to increase our knowledge of CF with the most common mutation.

Pancreatic Insufficiency Occurs the First Months of Life

Newborn $Cftr^{-/-}$ ferrets exhibit mild pancreatic pathology, similar to CF human infants, and are characterized by swollen acini and ducts with eosinophilic zymogen material, moderate alterations and pancreatic lesions observed in approximately 75% of $Cftr^{-/-}$ ferrets (Keiser and Engelhardt, 2011; Sun et al., 2014b). Olivier et al. (2012) demonstrated that neonatal CF ferrets exhibited elevated apoptosis in exocrine ducts and acini suggesting that pancreatic inflammation starts very early in life. Within the first month of life and before adulthood, the pancreas undergoes rapid tissue deterioration with destruction of acini, duct dilatation and evidence of inflammatory cells (neutrophils, macrophages and lymphocytes) (Olivier et al., 2012). Similar to patients with CF, 85% of adult CF ferrets present a loss of exocrine pancreas with fibrosis and islet remodeling. Interestingly, a small percentage of CF ferrets, like patients with CF, are born PS with light pancreatic pathology while they have normal growth and fecal elastase-1 (EL1) levels (Sun et al., 2014b). This finding demonstrates that, as in patients with CF, modifier genes could be responsible for this small percentage of PS. Additionally, adult CF ferrets exhibit disorganized islets, characterized by large clusters that contain an abundance of β and α cells as occurs in the pancreas of humans with CF (Olivier et al., 2012). In general, CF ferrets share many features of human pancreatic disease, and their ability to exhibit variability of disease severity at birth and in adulthood makes them an excellent model for testing potential therapies and examining the specific mechanisms for PI in CF.

LUNG AND INTESTINE OBSERVATIONS IN A RECENT CYSTIC FIBROSIS RAT MODEL

The first CF rat model was generated by Tuggle et al. (2014) with the use of the zinc-finger endonuclease technique. Rats are an appealing model for studying airway defects in CF because they share many characteristics consistent with humans. In contrast to mice, where SMGs are only located at the proximal trachea, rats SMGs extend to bronchi as in humans. The $Cftr^{-/-}$ rats, shortly after birth, exhibit congenital tracheal defects with a reduction in the cartilage and tracheal gland area (Tuggle et al., 2014). Nasal PD measurements demonstrated loss of Cl^- permeability in response to FSK, suggesting that the absence of CFTR chloride channels changes electrophysiological features in the rat (Tuggle et al., 2014). Additionally, young CF rats revealed reduced amounts of ASL in distal airways, similar to humans, while BAL fluid was free of inflammatory markers. The decreased ASL levels were not accompanied with Na^+ hyper-absorption suggesting that ASL regulation was CFTR and not ENaC dependent (Tuggle et al., 2014). Young CF rats did not show rapid pulmonary pathology progression, while the sterile environmental conditions under which they were kept may have had an effect (Tuggle et al., 2014). Future studies that will examine disease progression in adult CF rats housed in a natural environment will clarify the role of ASL depletion in airway obstruction. The presence of an increased number of dilated mucus cells was noticed in the proximal nasal epithelium, adding another similarity with the human pathology.

A study from Tipirneni et al. (2017) investigated nasal septal epithelial cultures from $Cftr^{-/-}$ rats and demonstrated that they would be a useful model for *in vitro* sinosal ion transport studies. The authors demonstrated that those cell cultures have comparable *Cftr* gene expression and nasal electrophysiological properties to nasal septal epithelial cultures from human and mice, while being more responsive to ion transport modulators (Tipirneni et al., 2017). Moreover, a recent study from Birket et al. (2018) demonstrated that the development of the SMGs is delayed in the $Cftr^{-/-}$ rats but only when the SMGs are fully developed, elevated mucus viscosity and impaired mucociliary transport were observed. Hyperacidic pH and changes in the periciliary layer, however, happen before SMGs develop. Importantly, blocking bicarbonate transport increased mucus viscosity. Additional studies on secretion abnormalities in tracheal glands using CF rats will add important information.

In addition, 70% of $Cftr^{-/-}$ rats mortality is due to intestinal obstruction between weaning and 42 days after birth in contrast to piglets and ferrets that suffered from severe intestinal obstruction at birth. Their small intestine was characterized by crypt dilation and mucus accumulation (Tuggle et al., 2014).

While CF rats do not spontaneously develop lung or pancreatic disease during their first 6-weeks of life, they are a good model for investigating bone growth and its direct link to CFTR, without the presence of other disease manifestations such as airway disease. Accordingly, Stalvey et al. (2017) examined the role of CFTR loss on bone growth using young $Cftr^{-/-}$ rats by measuring their femur length, bone histomorphometry and cartilaginous growth plates as well as the concentration of serum IGF-I. Deficiency in IGF-I can cause changes in cartilage growth. They found that in absence of the *CFTR* gene, rats show reduced growth and bone content. Also, IGF-I concentration in the serum of $Cftr^{-/-}$ rats (a marker also used in non-CF children with bone disease) is decreased, which may explain changes in the growth plate (Stalvey et al., 2017).

In general, the $Cftr^{-/-}$ rat is a promising model for long-term studies of CF disease manifestations. Although it is in the early stages, this model could be the base for generating CF rats with disease-causing mutations that would allow investigations of CFTR protein trafficking or gating defects and CFTR correctors and potentiators.

CYSTIC FIBROSIS ZEBRAFISH – A UNIQUE MODEL OF PANCREATIC DISEASE PROGRESSION IN ALL DEVELOPMENTAL STAGES

Zebrafish have been valued as an outstanding tiny research model for studying human diseases. They are inexpensive, and can be generated in large numbers, while requiring minimal housing and handling conditions (Phillips and Westerfield, 2014). Moreover, zebrafish CFTR chloride channels responds similarly to most agonists and antagonists of the human CFTR due to its high homology. Data have demonstrated that the absence of CFTR in zebrafish hinders the development of Kupffer's vesicle (KV), a basic ciliated organ, which determines lateralization in an early developmental zebrafish stage, emphasizing the importance of CFTR function in fluid secretion (Navis and Bagnat, 2015).

The first zebrafish CF model was produced by Navis and Bagnat (2015) using transgenic zebrafish mutant lines. They reported that CFTR was localized on the apical membrane of pancreatic ducts and investigated the absence of CFTR in zebrafish pancreas during various developmental stages suggesting comparable disease pathology as in humans. They reported that most of CF larval zebrafish started to die approximately 10 days post fertilization (dpf), suggesting that pancreatic dysfunction starts early in life. By 16 dpf, pancreatic tissue was partially damaged and neutrophil presence was slightly evident while by 20 dpf, the pancreas was remarkably disrupted. Pancreatic ducts contained mucus, and they were encircled by fibrotic tissue instead of acinar and were highly infiltrated with neutrophils. Additionally, the islets appeared widespread, abundant and diminished in size. The CF zebrafish model closely resembles pancreatic pathogenesis in human adults with CF suggesting that a mild PI may start early in life. Additional studies examining the specific mechanisms that cause pancreatic disease early in life, the role of the inflammatory factors in disease progression, and the ion transport abnormalities using the zebrafish model would be very valuable tools for pancreatic pathogenesis in CF.

SHEEP CF MODEL – A BRIEF DESCRIPTION OF AN ATTRACTIVE UNDERVALUED MODEL

The sheep model is not a commonly used model for studying CF disease compared to mice, pigs and ferrets, respectively. However, at the molecular level, the sheep *CFTR* gene is 91% homologous with the human *CFTR* while at protein level, they have 95% resemblance (Harris, 1997). Many researchers have used an ovine model for studying respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) since anatomically, functionally and electrophysiologically sheep airways parallels those of humans (Abraham, 2008; Allen et al., 2009). It has been utilized for examining secretion abnormalities, testing the effect of small molecules as a potential therapy for lung disease and for gene therapy (Cai et al., 2015). Apart from the lung pathology, CFTR protein expression in sheep pancreas mirrors human levels. As a large animal, in combination with its long lifespan, it could be appropriate for therapeutic trials. Generation of a CF ovine model utilizing somatic cell cloning was not successful in the past due to failure of homologous recombination in sheep fibroblasts. In 2018, Fan et al. generated the first $Cftr^{-/-}$ sheep model using the CRISPR/Cas9 genome editing and somatic cell nuclear transfer methods. The newborn $Cftr^{-/-}$ sheep demonstrated severe intestinal obstruction similar to the MI noticed in approximately 15% of human babies with CF. The pancreas was characterized by hypoplasia and fibrosis similar to humans with CF. This pancreatic hypoplasia was common in both KO and heterozygous sheep. Interestingly, lungs of newborn $Cftr^{-/-}$ sheep had minimal signs of disease which is an important feature that enables investigations of early disease progression (Fan et al., 2018). Production of a CF sheep model gave rise to a new chapter of large animals as useful models for novel therapeutic approaches.

DISCUSSION

Despite recent research progress, our understanding of CF pathogenesis is still incomplete. Many questions remain unanswered and CF continues to be a fatal disease. Cell-culture models have been shown to be useful for pre-clinical trials but they lack the structural complexity of intact organs (Scholte et al., 2006; Rogers et al., 2008c; Cutting, 2015). Much of the knowledge on CF pathophysiology that we have today derives from animal models and especially from the wide variability of gene-targeted mouse models. Most of the studies repeatedly note that mice models, in contrast to pigs and ferrets, do not spontaneously develop lung disease as do humans. However, depending on the strain, such as congenic models with the C57BL/6 background, they have the advantage of surviving longer from intestinal disease and are susceptible to lung infection with pathogens, mainly the most persistent *P. aeruginosa*. Pigs and ferrets exhibit similar lung function and architecture with humans and at birth they lack inflammation, and they are unable to eradicate bacteria (Rogers et al., 2008c; Stoltz et al., 2010; Sun et al., 2010; Wine, 2010; Ostedgaard et al., 2011). The disadvantage of these models is the difficulty of keeping them alive long enough since, approximately 48 h after birth they die from intestinal obstruction and a surgical ileostomy is mandatory but not always successful. Consequently, while most of the studies report a lung pathology similar to that in patients with CF, they are only based on experiments that have been conducted in very few animals, mostly newborn (Rogers et al., 2008c; Joo et al., 2010; Stoltz et al., 2010; Sun et al., 2010, 2014a; Wine, 2010; Ostedgaard et al., 2011; Keiser et al., 2015).

Mice SMGs, contrary to those in rats that extend into the bronchi, are confined to the trachea, but they are abundant and identical in structure to those in humans. Despite the bioelectric similarities of murine nasal SMGs with those in humans, tracheal glands are widely utilized due to the highest expression levels of CFTR proteins, whereas studies on $Cftr^{-/-}$ mice have demonstrated secretion defects. Tracheal glands from

CF pigs also exhibit secretion defects but they do not completely lose synergy as mice or newborn CF ferrets do (Joo et al., 2010). Secretion defects are restricted only in newborn CF ferrets and CF pigs, in contrast to mice, while studies in CF ferret nasal glands have not been done yet (Sun et al., 2010, 2014a; Keiser et al., 2015). The dominance of the CACC channel in mice airways, intestine and pancreas, in parallel with CFTR proteins, alleviate the severity of the disease pathogenesis compared to other models. Intestinal pathology can be fatal for some CF mice, causing death a few days after birth or around the time of weaning (Davidson and Dorin, 2001). The disease severity varies among strains and although it is more severe than in humans, CF mice intestine still exhibit the same pathophysiological abnormalities, and they are considered a good model for studying human intestinal disease.

In addition, MI is fatal for both CF piglets and ferrets with a penetrance of 100% and 50–100%, respectively, while the occurrence in human infants with CF is only moderate at 15–20%. The generation of transgenic mice, pigs, and ferrets with tissue-corrected *CFTR* gene expression under the control of the iFABP gene promoter alleviates intestinal obstruction with no effects on the other organs. Interestingly, milder pancreatic disease is observed in CF mice, while CF pigs and ferrets that survived from MI demonstrated human-like disease. However, studies in adult pigs and ferrets are limited. The first CF zebrafish model enabled the examination of pancreatic pathogenesis in all developmental stages, demonstrating that a mild pancreatic disease starts early in life (Navis and Bagnat, 2015).

Although human CF is characterized by salty sweat, eccrine sweat glands are underestimated in the above CF animal models. Pigs use mud baths to cool their body (Rogers et al., 2008a) as they have very few sweat glands, which are in the nose, while ferrets do not sweat at all. Importantly, mice have sweat glands in their paws, this is a promising tissue for studying CF disease which should be further evaluated.

CONCLUSION

In conclusion, while various animal models present different advantages for studying different aspects of the disease, there is no single animal model available yet that completely replicates the complexity of CF in humans. Each model presents pros and cons and combining findings from various organs studied in different models provides important clues for our understanding of the CF disease pathogenesis and progression.

AUTHOR CONTRIBUTIONS

AS wrote the paper. All authors contributed to the review of appropriate literature and preparation of the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Emmanuelle Brochiero, Younes Anini, and Yassine El Hiani for their input and interesting discussions on this topic. AS was supported by CFF grant.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

REVIEW
published: 28 March 2019
doi: 10.3389/fphar.2019.00301

Trial Refresh: A Case for an Adaptive Platform Trial for Pulmonary Exacerbations of Cystic Fibrosis

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *27 September 2018*
Accepted: *11 March 2019*
Published: *28 March 2019*

Citation:
Schultz A, Marsh JA, Saville BR, Norman R, Middleton PG, Greville HW, Bellgard MI, Berry SM and Snelling T (2019) Trial Refresh: A Case for an Adaptive Platform Trial for Pulmonary Exacerbations of Cystic Fibrosis. Front. Pharmacol. 10:301. doi: 10.3389/fphar.2019.00301

Cystic fibrosis is a genetic disease typically characterized by progressive lung damage and premature mortality. Pulmonary exacerbations, or flare-ups of the lung disease, often require hospitalization for intensive treatment. Approximately 25% of patients with cystic fibrosis do not recover their baseline lung function after pulmonary exacerbations. There is a relative paucity of evidence to inform treatment strategies for exacerbations. Compounding this lack of evidence, there are a large number of treatment options already as well as becoming available. This results in significant variability between medication regimens prescribed by different physicians, treatment centers and regions with potentially adverse impact to patients. The conventional strategy is to undertake essential randomized clinical trials to inform treatment decisions and improve outcomes for patients with exacerbations. However, over the past several decades, clinical trials have generally failed to provide information critical to improved treatment and management of exacerbations. Bayesian adaptive platform trials hold the promise of addressing clinical uncertainties and informing treatment. Using modeling and response adaptive randomization, they allow for the evaluation of multiple treatments across different management domains, and progressive improvement in patient outcomes throughout the course of the trial. Bayesian adaptive platform trials require substantial amounts of preparation. Basic preparation includes extensive stakeholder involvement including elicitation of consumer preferences and clinician understanding of the research topic, defining the research questions, determining the best outcome measures, delineating study sub-groups, in depth statistical modeling, designing end-to-end digital solutions seamlessly supporting clinicians, researchers and patients, constructing

randomisation algorithms and importantly, defining pre-determined intra-study endpoints. This review will discuss the motivation and necessary steps required to embark on a Bayesian adaptive platform trial to optimize medication regimens for the treatment of pulmonary exacerbations of cystic fibrosis.

Keywords: adaptive trial, platform trial, Bayesian, cystic fibrosis, exacerbations, master protocol, response adaptive randomisation

BACKGROUND

Cystic fibrosis (CF) is a genetic disease typically characterized by progressive lung damage and premature mortality. Pulmonary exacerbations, or flare-ups of the lung disease, often require hospitalization for intensive treatment. Pulmonary exacerbations remain an important driver of progressive loss of lung function and premature death in CF. Up to 25% of patients do not recover their baseline lung function, typically measured as the forced expiratory volume in 1 s (FEV1), after an exacerbation (Sanders et al., 2010). Key to improving survival is the prevention of loss of lung function with each exacerbation. Reflecting this, when the James Lind Alliance recently canvassed research priorities from over 1,000 CF consumers from 23 countries, management of CF exacerbations, and specifically identification of the most effective/least toxic antibiotics, figured among their priorities (Rowbotham et al., 2018).

There are grounds for believing that better treatment of exacerbations may reduce the loss of lung function and thus improve survival. There is substantial variation in the rate of decline between individuals, even among those with the same primary genetic mutations. The demonstrated heterogeneity in outcomes among those with access to the same range of treatments (Stephenson et al., 2017), indicates that response may depend on individual patient characteristics or factors present during an exacerbation. Given these considerations, we identify three general questions that need to be addressed in order to improve the management of CF exacerbations: (a) What are the optimal interventions where multiple options exist; (b) How does optimal treatment vary by different patient characteristics; and (c) Do treatments have cumulative or antagonistic effects when used in combination.

Despite considerable research effort, answers to these questions have remained elusive. Antibiotics are a mainstay of the treatment of pulmonary exacerbations. A recent Cochrane review identified 40 studies of antibiotic treatment of CF exacerbations (Hurley et al., 2015). Most studies evaluated only two antibiotics and were small, inconclusive, never replicated, and completed a decade or more ago. The reviewers concluded that "No specific antibiotic combination can be considered to be superior to any other, and neither is there evidence showing that the intravenous route is superior to the inhaled or oral routes" (Hurley et al., 2015). The failure of this review of several decades of research to reach any meaningful conclusion is symptomatic of the challenges of conducting trials in CF. Continuing to undertake conventional trials may never usefully address this clinical uncertainty, and certainly not within a timeframe that will benefit many of the current generation of people with CF.

Recent advances in clinical trial methods can help to evaluate complex interventions for rare conditions like CF. These innovations allow for improved trial efficiency and may be more conducive to participation by patients and clinicians alike. These methods have recognized validity, having been used particularly in commercial cancer trials, accepted for regulatory purposes by both the Food and Drug Administration (Woodcock and LaVange, 2017) and the European Medicines Agency, and published in high impact journals. These methods are being applied in trials of the treatment of breast cancer (I-SPY2) (Park et al., 2016), severe community-acquired pneumonia (REMAP-CAP (NCT02735707)), and brain cancer (GBM-AGILE) (Alexander et al., 2018). Here, we describe the planning for BEAT CF, a Bayesian adaptive platform trial that aims to optimize the management of CF exacerbations.

BEAT CF aims to be an exemplar of the REMAP (randomized, embedded, multi-arm, adaptive, platform) trial approach (Angus, 2015). The key features of BEAT CF are the use of Bayesian statistical inference, flexible sample size, the comparison of several different treatment options simultaneously and in combination, response adaptive randomization, and the evaluation of treatment responses in different types of patient. Key to the successful implementation of BEAT CF will be its nesting within a treatment register, embedding of trial procedures in routine clinical care and a digital health approach that is dynamic to adapt to the informatics and data integration challenges (Bellgard et al., 2017). A core (master) protocol will allow the sequential introduction of new interventions over time, as initial questions are answered. Each of these features will be introduced below, contrasting REMAP trials with more traditional trial approaches, and highlighting the benefits, challenges and limitations inherent in establishing a REMAP trial for a complex clinical domain.

CORE PROTOCOL

A limitation of undertaking sequential disconnected trials is that the lack of standardized eligibility criteria, trial endpoints, subgroup definitions, and comparator treatments makes the aggregation of such data complex and uncertain. The core protocol of a REMAP trial aims to standardize these design elements so that treatment responses can be meaningfully aggregated across trials, across settings and over time. For BEAT CF, we propose to broadly involve clinicians and other domain experts, consumers and other stakeholders in this decision-making. Investment in the development of an overarching core protocol is also intended to prevent *reinventing the wheel* for each

trial, improving efficiency through the sharing of infrastructure, and reducing the time to commencement and completion. In addition to standardizing specific design elements, the core protocol can also set out an overarching governance framework, including how the safety of participants is monitored. A core protocol implemented across multiple centers might institute a platform for the ready identification of potentially eligible study participants, facilitating enrolments.

BAYESIAN STATISTICAL INFERENCE

Up until now, most clinical trials, and all trials in managing CF exacerbations, have been traditionally designed trials which have employed frequentist statistical inference. Frequentist statistical inference underlies the vast majority of clinical studies, although thanks to increasing computer processing capacity, there has been a steady resurgence in Bayesian statistical inference in recent years (Green et al., 2015). In brief, frequentist inference assesses the *likelihood* of an observation, such as the observed difference in treatment effect between an investigational and a control treatment if no true difference exists (Berry, 2006). If an observation is very unlikely under this "null hypothesis" (i.e., it would rarely occur just by chance), this is taken as evidence that, to the contrary, a true difference exists. Frequentist inference flips the question of the probability that a difference exists, into a question about the probability of a result if no difference exists. To apply frequentist inference, one needs to be able to enumerate all the possible ways that a trial could have unfolded (that is the possible number of treatment successes and failures across both arms), and this requires many aspects of the design to be fixed in advance (e.g., randomization probabilities, sample sizes, number of treatment arms). If these components are not fixed it may be impossible to enumerate the number of possible ways that a trial may have unfolded and therefore to calculate how "unlikely" an observation is. Fixing the design so as to confine the number of possible outcomes makes the frequentist analysis tractable, but it comes at the cost of lost flexibility. New methods allow the design to be adapted as data accrues according to established rules (Saville et al., 2014), and these rules can be designed to maximize efficiency (including the chance of a conclusive result), or to maximize the chance that participants receive optimal treatment, or both.

Whereas frequentist inference is based solely on the *likelihood of an outcome*, Bayesian inference is directly concerned with the actual question, i.e., the *probability of a difference in treatment effect*, or the most probable values of the true difference in treatment effects. Bayesian inference does this by combining the *likelihood* of the observation for the range of possible treatment differences with the baseline *probability* (or "prior") of those possible treatment differences (Dmitrienko and Wang, 2006). Bayesian inference provides a straight-forward mechanism for updating one's estimate of the most probable range of treatment differences as new observations are made, that is, as data accrues (Connor et al., 2013). Trial designs that are adaptive can unfold in any one of a nearly limitless number of ways, so estimating how "unlikely" a particular result is (that is a particular instance of treatment responses among those who receive an intervention or control) is very hard to determine. The ability to update the probabilities for a range of possible treatment differences as new data accrues therefore makes Bayesian inference very useful for adaptive studies, although it should be noted that adaptive designs based on frequentist inference have also been advocated.

RESPONSE ADAPTIVE RANDOMISATION

When we have surveyed Australian CF clinicians, we have found that, like colleagues in the United States (West et al., 2017), they use a wide range of antibiotics to treat pulmonary exacerbations. Sequentially comparing the relative efficacy of all currently used antibiotics two-at-a-time would take an unfeasibly long time, notwithstanding the complexity of antibiotic combinations. Furthermore, assessing all antibiotics contemporaneously in a multi-arm trial would require an unfeasibly large number of participants. REMAP trials aim to greatly improve the efficiency of multi-arm trials using response adaptive randomisation (RAR). RAR is the progressive, rule-based assignment of an increasing proportion of new participants to interventions which appear most promising, and the potential elimination of those which are demonstrated to be inferior to alternatives options (Berry et al., 2015; Cellamare et al., 2017) (**Box 1**). Rather than having a fixed ratio of treatment assignment across arms, the ratio is updated (or adapted) at predefined intervals based on evidence from accruing data (Connor et al., 2013). At each analysis we will estimate the probability that each treatment, or treatment combination, is superior to all other options for a given patient type. Future treatment assignments will be based on these probabilities such that probability of assignment to a treatment is proportional to the probability that treatment is better than all other options for a given patient type (Connor et al., 2013). Randomization to ineffective treatments may be suspended or entirely eliminated if pre-specified futility boundaries are met (Berry et al., 2015). By assigning progressively more randomized participants to the best strategies and dropping ineffective therapies, it is expected that RAR will produce better patient outcomes for patients who participate in the trial (Connor et al., 2013).

SUBGROUP EFFECTS

One challenge for clinicians when trying to apply clinical trial results to their clinical practice is in determining whether the results are applicable to their own patient population, or indeed to a specific patient at hand. In pulmonary exacerbations, for example, it seems highly plausible that exacerbations represent

BOX 1 | Response adaptive randomisation.
- The progressive, rule-based assignment of an increasing proportion of new participants to interventions which appear most promising.
- The potential elimination of interventions demonstrated to be inferior to available alternatives when pre-specified futility boundaries are met.

a range of pathophysiologic processes, with responses to certain treatments influenced by the predominant process and, in the case of exacerbations caused by infecting bacteria, the species, and susceptibility profile of those bacteria (Doring et al., 2012). However, most trials are only interested in measuring *average* treatment effects of an intervention over a study population, and implicitly assume individuals respond homogenously to different interventions or treatment combinations. If a difference in treatment effects between subgroups is expected, the trialist will need to confine the trial to only the patient group which is expected to respond, or will run parallel trials in each group. If a difference in treatment response is hypothesized but not expected, the trialist can choose to aggregate data across the subgroups unless a statistical test for heterogeneity confirms a difference in treatment effects across subgroups, in which case the results for the two groups are reported separately. The problem with the latter approach is that such tests are relatively insensitive for true differences which can lead to inappropriate pooling of results; the problem with splitting by patient subgroups is that the precision is diluted by ever shrinking sample sizes. This is a problem in CF where the range of potential subgroups and plausible treatment effect modifiers is very large, based on variation in sputum microbiology, disease stage, and prior and concurrent treatment history.

REMAP trials aim to estimate specific treatment responses across a range of predefined patient subgroups much more efficiently than traditional trials. Such subgroups could be defined by factors that plausibly impact on response to treatment, or more specifically, influence the probability of a better response to one treatment than another, for example baseline lung function and airway microbiology status in the case of pulmonary exacerbations. Bayesian modeling can be used to allow the observed treatment response in one group to inform the probable treatment effect in another. Bayesian prior distributions are carefully calibrated to control the amount of *borrowing* of information between respective sub groups, and are verified in the design stage via simulation (Berry and Berry, 2004). This differs from the extreme approaches of complete pooling (i.e., ignoring subgroups effects and estimating a single common treatment effect) or no pooling (i.e., independent estimation of treatment effects in each subgroup). The Bayesian modeling approach can be described as being in between these two extremes, with *partial pooling* of information from patient subgroups. For subgroups with small sample sizes, the estimated subgroup treatment effects tend to be closer to each other. As sample sizes increase within subgroups, estimation of treatment effects may grow apart depending on the observed data within the subgroups.

COMPARISON OF MULTIPLE DIFFERENT TREATMENT OPTIONS SIMULTANEOUSLY

Like most chronic diseases, the management of CF pulmonary exacerbations is complex and multimodal (Elborn, 2016; Waters et al., 2016; West et al., 2017). Traditional trial designs typically do not account for multimodality but instead focus on one or more treatments within a single therapeutic "domain," where domain refers to a set of mutually exclusive treatment options. In the management of pulmonary exacerbations for example, therapeutic domains include the choice and route of antibiotic (both primary/backbone and adjunctive) (West et al., 2017), the use of mucoactive therapies (including dornase alpha, hypertonic saline, and mannitol) (Bakker et al., 2014; Dentice et al., 2016), the use of immune modulators (steroids, non-steroidal anti-inflammatory drugs, and macrolides) (Ghdifan et al., 2010; Lands and Stanojevic, 2016), and type and intensity of airway clearance therapies. Trials that attempt to evaluate a complex multi-modal intervention typically evaluate them as a fixed "bundle"; the limitation of this approach is that it makes it impossible to know which components of the bundle, if any, are effective, which ineffective or deleterious, and whether any combinations are synergistic or antagonistic.

In the REMAP design, participants can be randomly assigned to one of two or more options across each of two or more therapeutic domains. Participants are therefore assigned to one of many possible treatment combinations. This gives rise to a multi-dimensional estimation problem. For example, in a REMAP with three therapeutic domains each with three treatment options, there are 3^3 (twenty seven) combinations and therefore 27 separate treatment effects to be estimated, ignoring any subgroup-specific effects. As for subgroup effects, this multi-dimensional estimation problem is made tractable by the use of Bayesian modeling which allows, for example, the observed treatment outcomes among patients receiving a backbone antibiotic together with one adjunctive antibiotic, to inform the estimated treatment effect of the same backbone antibiotic when given in conjunction with a different adjunctive antibiotic, and *vice versa* (Berry and Berry, 2004).

A PLATFORM TRIAL WITH A DECISION-BASED MASTER PROTOCOL

Most clinical trials are stand-alone, time-limited, and designed to answer a single efficacy or comparative efficacy question. Regardless of whether the trial is conclusive or not, any follow-up or completely new questions usually require the establishment of a new trial. Typically there is little or no transfer of study infrastructure between trials which is wasteful of resources. Also, trials of interventions for CF exacerbations have variously measured different but related outcome measures, such as the absolute or relative improvements in the predicted FEV1, or the proportion of patients returning to some fraction of their "baseline" FEV1, or the reduction in some composite measure of symptoms. Furthermore, trials have measured these endpoints at variable lengths of time after initiation of CF exacerbation therapy. Failure of concurrent and consecutive trials to adopt the same endpoints has made it impossible to compare, let alone aggregate, results across studies. More recently there have been efforts to establish consistent endpoints, or core outcome sets, for trials in cystic fibrosis and other trials (Stanojevic and Ratjen, 2016; West et al., 2017).

A REMAP trial aims to achieve greater efficiency through a core (or master) trial protocol. The core protocol sets out exactly what data are to be collected including the primary and any secondary endpoints, and the procedures around how data is captured and managed, including the trial governance arrangements. Any treatment options which are subject to random assignment are dealt with in a series of appendices to the master protocol, with a therapeutic domain and all treatment options within that domain covered by its own appendix.

This use of a core protocol facilitates consistency in trial endpoints and processes over time. The modular structure of the protocol allows for the domain-specific trial appendices to be modified over time without changing the core protocol; treatment options within a domain can be added or removed according to pre-specified rules and entire domains can be added or removed over time. Once the superiority of a treatment has been established over all other treatments within an existing domain, a new domain with unanswered questions could replace the existing domain (Berry et al., 2015). This circumvents the need for setting up an entirely new clinical trial. For example if the optimal combinations of backbone and adjuvant antibiotics for CF exacerbation have been determined for individual patient sub-groups, a new phase of the study might focus on optimization of immune modifiers, mucoactive agents, airway clearance strategies or other therapeutic domains.

Non-inferiority and equivalence findings can also be evaluated in a REMAP trial. In particular, the study would still result in improvement in care if it was able to eliminate one or more inferior treatment arms, while showing the remaining treatment arms are equivalent. In addition, some treatments may require an efficacy superiority margin to be favored relative to other treatments in the presence of differing toxicity/safety profiles. We are currently investigating various options for incorporating toxicity into the primary analysis and RAR algorithm.

EMBEDDING IN ROUTINE CLINICAL CARE

Despite the potential efficiencies gained through the use of adaptive processes, a REMAP trial must nonetheless enroll a large number and broad range of participants if it is to efficiently address the full range of management questions. For this to occur, a REMAP trial needs to be successfully embedded in routine clinical care. Embedding, in which trial participation occurs seamlessly with delivery of care and with minimal additional impost on either clinician or participant, requires extensive stakeholder involvement in the design and strong buy-in by clinicians and patients. To secure this, REMAP investigators must spend considerable effort eliciting clinician's expert understanding of the subject domain, as well as consumer input into identification of patient-centered study outcomes. Clinicians and patients may be more likely to engage with a trial for which there is prospect of a personal benefit from participation. Unlike traditional trials which are ethically predicated on no expectation of a personal benefit, response adaptive processes of REMAP trials are designed to improve the chances that a participant receives optimal therapy, and minimize the chance of inferior therapy.

Many traditional trials blind the participant, or the outcome assessor, or both, to the treatment assignment, usually through the use of matched placebos or sham treatments. Blinding helps to safeguard against bias that might arise, especially if the clinician, patient or assessor have preconceptions about the relative effectiveness of the treatment options. Complete blinding of participants is typically not feasible in a REMAP owing to the large number and range of therapeutic options evaluated, together with the desire to achieve successful embedding in routine care. In any unblinded study (regardless of equal or response adaptive randomization), there is the risk that patient's or physician's knowledge can affect outcomes. This can be minimized through the choice of objective outcomes such as change in FEV1, especially if the person performing the measurement is, herself, blinded to the treatment assignment. For unblinded studies incorporating RAR, there is additional risk of operational bias resulting from a site's perceived impression of adaptive randomization. Although it would be difficult to measure any such bias that might exist in this setting, we believe that knowledge of the best performing treatments at the site level would only be obvious in settings where there is a dominant treatment receiving greater randomizations across all or most subgroups. In such a setting, the risk of bias may be outweighed by the risk of continued randomization to under-performing arms.

DIGITAL HEALTH SOLUTION CONSIDERATIONS

The implementation of REMAP trials demands a rethink on approaches to data management, flow, sharing and the interfaces necessary to engage with participants, clinicians, and study coordinators. Whereas in conventional trials, paper-based records might be sufficient to manage the workflow and capture trial data, in a REMAP the need for continuous and iterative interaction between clinicians, patients, and statisticians demand digital solutions.

Any solution needs to ensure that data are captured electronically in order to support frequent pre-planned, scheduled analyses. Logic checks need to be built-in and designed to minimize data entry errors and the need for corrections which would otherwise delay analyses. All data needs to be held securely and privacy ensured – potentially identifiable information needs to be held at the site and not accessible to those who are not directly involved in patient care, including researchers. There should be facility for patients (or their parents) to directly enter symptom information, or patient reported outcomes (Napier et al., 2017). It is necessary for the randomisation procedure to accommodate the RAR process – that is, it is essential that any embedded randomisation process must be capable of being updated over time following each analysis. Static randomisation processes, for example those employing fixed randomisation lists, will not be fit-for-purpose.

Lessons learnt from the successful deployment of clinical registries offer insights into the types digital infrastructure required to implement REMAP trials (Bellgard et al., 2013; Lacaze et al., 2017). For instance, patient-centric registries allow patients to securely register through online registration with configurable online informed consent (Bellgard et al., 2012; Napier et al., 2017). Others have demonstrated the potential value of rich and well-collected patient registry data for improving patient decision-making in CF beyond simple rule-based algorithms (Alaa and van der Schaar, 2018). Online clinical reporting forms and participant questionnaires that can be configured by coordinators without software development skills enables the digital health solution to dynamically adapt to requirements (Bellgard et al., 2014). Longitudinal data capture and time-stamped ongoing patient assessments can be captured either by the patient themselves or by the clinician through automated notifications (Bellgard et al., 2015). In addition, patient registry platforms can have multi-lingual support and data elements can be derived allowing logic steps to be incorporated (Napier et al., 2017).

CONSENT

In traditional two-arm trials, once informed consent for participation has been obtained, recording that consent is relatively straight-forward. For a REMAP trial, where a participant may be offered randomisation across a number of therapeutic domains, capturing participant consent is more complex. For example participants might happily accept random assignment of treatment in one domain, but not in another. In some situations, specific treatment options, and possibly entire domains, may not be available in all centers. In the case of a REMAP in managing CF exacerbations, a participant might decline randomisation during one exacerbation, but accept randomisation during a subsequent exacerbation. Furthermore, a participant might never accept random assignment of treatment, but might nonetheless agree to have their treatment and outcome data collected in a treatment register. Consenting to a REMAP is not simply a binary choice to participate or not, and this complex and nuanced consent needs to be captured and efficiently and faithfully reflected in subsequent study processes.

Because of the complex nature of REMAP trial designs, it may not be possible to achieve adequately informed consent at the time of acute illness. It may be necessary to provide detailed education to prospective participants in the non-acute setting. In the case of a REMAP for managing CF exacerbations, for example, an option would be to obtain consent in a dynamic and stepwise fashion. In the first step, prospective participants may be invited to consent to enrolment in a prospective treatment register, in which patients simply agree to allow their treatment and outcome data to be captured to inform future best practice. This could occur in the outpatient setting, and could be supported by extensive education about the REMAP design and processes. Separately, those who are enrolled in the treatment register can then be invited to opt-in to randomized care at the time of pulmonary exacerbations. Because participants will have already received education in the outpatient setting, this additional consent to randomisation can be expedited so as not to unnecessarily disrupt the delivery of care. In this two-level consent process, participants may agree to have their treatment and outcome data captured in the treatment register, but not always (or perhaps never) further consent to receive randomized care of their exacerbations. Similarly, the treating clinician may decide against random treatment assignment for a patient during a given exacerbation. Having patients opting out of randomisation, or their clinicians deciding against random treatment assignment, is an issue found in nearly every randomized clinical trial. Patients who opt out of randomization are not included in the primary analysis. Hence, the generalizability of the primary analysis results is limited to a population of patients who are willing to be randomized. Whereas traditional clinical trials disregard data arising from unrandomized patients as inherently uninformative due to potential bias, it may be possible within the context of a REMAP to utilize this data for hypothesis generation, or possibly even for formal integration into treatment effect estimates.

ETHICS STATEMENT

Similar to all clinical trials BEAT CF will require approval by participating institutions' ethics and governance committees. Such committees will be confronted with a range of innovative features including the novel study design, complex Bayesian statistics, the absence of blinding to allocated treatment, and dynamic consent. Involvement of ethics and governance committees from the early planning stages may help to avoid roadblocks in the approval process. Evidence of extensive consumer involvement in study design and planning may also be looked upon favorably by these committees.

TRIAL EFFICIENCY AND COST

REMAP trials have the potential to be highly cost-efficient as multiple research questions can be addressed simultaneously in a single clinical trial (Berry et al., 2015). Thus, while there are significant initial costs associated with establishing the trial, these costs can be defrayed across many questions of interest. Additionally, the trial platform allows for efficiency with a single data capture and governance platform across multiple centers. If REMAPs are effectively embedded in clinical care the incremental costs of inclusion of additional study sites may be relatively small, and offset by improved effectiveness and cost-efficiency of care. The use of a core protocol allows new domains to be studied without the need to develop an entirely new protocol. While REMAP designs are motivated by the desire to greatly improve the efficiency of clinical comparative effectiveness research,

to date these efficiencies remain largely unproven and may only be achieved if a REMAP design aligns with the specific objectives of the study.

LIMITATIONS AND CHALLENGES

Whilst a Bayesian adaptive platform trial many potentially provide many benefits for the study of CF exacerbations, specific challenges still remain. Some of these challenges transcend statistical design. For example, the precise definition of an exacerbation remains controversial (Goss and Burns, 2007; Stenbit and Flume, 2011). The clinical decision to hospitalize for treatment of an exacerbation may vary from one treatment center to another (Johnson et al., 2003) but has been successfully used as a pragmatic definition in clinical trials. Furthermore, the decision to admit to hospital can potentially be standardized between participating study sites (Ferkol et al., 2006). Secondly, exacerbations may have multiple etiologies requiring different optimal treatments (Stenbit and Flume, 2011). However, similar to asthma, different etiologies that result in the same clinical phenomenon (i.e., exacerbation) can usefully be studied as a single entity. Thirdly, the optimal duration of treatment for exacerbations remain contentious (Szentpetery and Flume, 2018) and there is uncertainty about best outcome measure, and timing of the outcome measure, for studying treatment effect. Such challenges face all clinical trials of exacerbations but can be overcome in the CF community where collaborative effort is the norm.

Another challenge for platform trial is population drift that can influence results. This can be addressed with Bayesian modeling that accounts for changes in population over time. Logistical challenges include the need for rapid data accrual to inform adaptations. Digital solutions, discussed above, can be applied to facilitate timely data capture. Many of the abovementioned limitations are discussed elsewhere (Saville and Berry, 2016), alongside the statistical efficiencies obtained through adaptive platform strategies.

IN SUMMARY

There is a need to optimize the management of pulmonary exacerbations of CF, but the traditional clinical trial approach may not be a feasible approach for addressing the multitude of clinical questions. REMAP trial designs may offer a much more effective and efficient approach to finding answers to the many questions confronting CF patients and clinicians. Features include response adaptive randomisation, and the ability to compare multiple different treatment options simultaneously over a range of patient sub-groups. Once the original research questions have been answered, the platform design with a core protocol would facilitate the seamless transition to follow-up questions. For such a trial to be successful for the study of exacerbations of CF the trial will need to be embedded in routine clinical care and innovative digital solutions will be required for implementation. Overall, the challenges are large but the gains for CF could be considerable.

AUTHOR CONTRIBUTIONS

AS co-wrote the first draft of the manuscript and contributed to the writing throughout. JM contributed to writing the first draft. BS and MB wrote sub-sections of the manuscript and contributed the manuscript as a whole. RN, HG, and PM provided expert input into an advanced version of the manuscript. SB provided expert input and oversight. TS acted as senior author by writing large sections of the manuscript and providing oversight and leadership throughout.

FUNDING

BEAT CF is supported by a BHP Blue Sky Award, and funding from the MTPConnect Project Fund Program (PRJ2017-57) and the Australian Medical Research Future Fund (MRFF APP 1152376). The contents of the published material are solely the responsibility of the authors and do not reflect the views of the Australian Government. AS is supported by a National Health and Medical Research Council fellowship (APP1168022). JM is supported by a Telethon-Perth Children's Hospital Research Fund research capacity grant (CRIPTIC). BS and SB are supported by a Channel 7 Telethon Trust Major Beneficiary grant. TS is supported by a National Health and Medical Research Council fellowship (GNT1111657).

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