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Cystic fibrosis: Exploiting its genetic basis in the hunt for new therapies

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Abstract

Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel expressed in epithelial cells throughout the body. In the lungs, absence or dysfunction of CFTR results in altered epithelial salt and water transport eventuating in impaired mucociliary clearance, chronic infection and inflammation, and tissue damage. CF lung disease is the major cause of morbidity and mortality in CF despite the many therapies aimed at reducing it. However, recent technological advances combined with two decades of research driven by the discovery of the CFTR gene have resulted in the development and clinical testing of novel therapies aimed at the principal underlying defect in CF, thereby ushering in a new age of therapy for CF.

Keywords

Cystic fibrosis; CFTR; Gene therapy; Corrector; Potentiator; Transcriptional read-through

1. Introduction

Cystic fibrosis (CF) is a genetic disease of abnormal ion transport. Specifically, abnormalities in the expression and function of the cystic fibrosis transmembrane conductance regulator (CFTR) result in abnormal salt and water transport across epithelial surfaces in the gastrointestinal and hepato-biliary systems, respiratory tract, reproductive system and sweat glands. With the exception of the sweat glands, abnormal salt and water transport eventuate in end-organ damage causing significant morbidity and severely shortening life-span. Currently available therapies for CF such as supplemental pancreatic enzymes, antibiotics, mucus thinners, and non-specific anti-inflammatory agents address the consequences of CFTR deficiency rather than the underlying cause. However, decades of research have culminated in the recent testing of therapies that address the basic defect and hold promise for significant clinical benefit. This review attempts to place these recent discoveries in historical context, highlighting how research into the function of CFTR based on the knowledge of the gene prepared the CF community to harness technological advances for the benefit of all CF patients.

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2. History of CF

2.1. Determining the underlying defect

The first description of CF as a pathological entity in the United States was published in 1938 by Dorothy Andersen, M.D., a pathologist at The Babies & Children's Hospital of Columbia University in New York City. Her paper entitled "Cystic fibrosis of the pancreas and its relation to celiac disease" firmly established cystic fibrosis of the pancreas as a diagnosis separate and apart from celiac disease (Andersen, 1938). It was not until more than a decade later, however, that the connection was made between salt transport and cystic fibrosis of the pancreas. In 1951, Kessler and Andersen reported on 12 children admitted to Babies' Hospital with heat prostration who were in relatively good health before a heat wave, and who presented acutely with vomiting and signs of shock without evidence of infection. All of these children, except for one who died, responded quickly to rehydration (Kessler & Andersen, 1951). In patients for whom laboratory data were available, serum electrolyte analyses showed low Cl^- and high HCO_3^- concentrations that were reversed with therapy. These findings supported an etiological hypothesis that "fibrocystic disease is associated with widespread abnormality of epithelial glands (Kessler & Andersen, 1951)."

Following these observations, Paul di Sant'Agnese, M.D., also at Columbia University, prospectively studied sweat electrolyte levels in 43 CF patients and 50 control patients. His results demonstrated that Na^+ , K^+ and Cl^- all were elevated in the sweat of CF patients, with sweat Na^+ and Cl^- levels being markedly elevated (Di Sant'Agnese et al., 1953). The authors also demonstrated that the elevated sweat Na^+ and Cl^- levels were not the secondary result of pancreatic dysfunction, pulmonary disease, adrenal dysfunction or renal disease, and concluded that the increased susceptibility to dehydration in CF was due to increased salt loss from sweat glands. These findings led directly to the development of the sweat test as a diagnostic test for CF (Gibson & Cooke, 1959).

By the early 1980's, observations in the sweat gland, pancreas and respiratory tract began to suggest that CF was, at its essence, a disease of altered anion transport. Multiple studies confirmed the findings of di Sant'Agnese that sweat electrolyte concentrations were abnormal in CF. In separate studies using different techniques, Quinton (P. M. Quinton & Bijman, 1983) and later Fromter (Bijman & Fromter, 1986) concluded that CF sweat glands had decreased ductal Cl^- permeability and reduced secretion in response to adrenergic stimulation. Similarly, studies of pancreatic HCO_3^- secretion in CF patients concluded that abnormal pancreatic secretion in CF could be attributed at least in part to altered Cl^- secretion (Kopelman et al., 1988). Knowles and colleagues at the University of North Carolina at Chapel Hill reported that the electrical potential across the nasal epithelia (the nasal potential difference or NPD) of CF patients was more electronegative than control patients (Knowles et al., 1981) and that the apical plasma membrane of nasal epithelial cells in CF patients with CF was impermeable to Cl^- (Knowles et al., 1983). It seemed, then, that the basic defect in three different organ systems could be attributed to altered Cl^- permeability.

2.2. Finding the CF gene

Armed with the knowledge that CF was a disease of altered Cl^- transport, researchers began to search for the affected gene. In 1985, two laboratories using different markers for linkage analysis localized the gene to the long arm of chromosome 7 (Knowlton et al., 1985; Wainwright et al., 1985). In 1989, in a collaborative international effort, Tsui, Riordan, Collins and colleagues discovered the gene responsible for CF (Kerem et al., 1989) and found that in the majority of CF patients the gene was missing three nucleotides that resulted in the in-frame deletion of a phenylalanine residue at position 508 of the polypeptide chain (ΔF508) (Riordan et al., 1989). They designated the protein the cystic fibrosis transmembrane conductance

regulator, or CFTR (Riordan et al., 1989). In doing so, the group recognized that if CFTR was not itself a chloride ion channel, then the protein would almost certainly function as a regulator of Cl^- channel activity.

3. The CFTR protein

3.1. Normal CFTR

Even before the CFTR gene was cloned, it was known that cAMP-stimulated Cl^- secretion was defective in CF epithelial cells (Frizzell et al., 1986). Shortly after the CFTR gene was identified, data emerged that the defective cAMP-mediated Cl^- secretion could be corrected by expression of normal CFTR, but not by expression of ΔF508 CFTR. These data supported the hypothesis that CFTR was a Cl^- channel, but still left open the possibility that CFTR was functioning as a positive regulator of another Cl^- channel (Rich et al., 1990), (Drumm et al., 1990). In 1991, Anderson and colleagues at the University of Iowa expressed recombinant CFTR in three different cell lines and conferred on those cells a cAMP-activated Cl^- conductance that was not found in cells expressing ΔF508 CFTR (Anderson Rich et al., 1991). These results were independently confirmed in other cell lines (Rommens et al., 1991), (Kartner et al., 1991). The demonstration that mutating specific amino acids in CFTR altered the anion selectivity of the ion permeation pathway conferred on cells in which CFTR was heterologously expressed also strongly suggested that CFTR was a Cl^- channel (Anderson Gregory et al., 1991). Finally, Bear and colleagues purified the CFTR protein, expressed it in isolated planar lipid bilayers, and demonstrated that it had ion permeation and gating properties identical to those of CFTR heterologously expressed in cell culture (Bear et al., 1992). When studied by standard electrophysiological techniques in either native or heterologous systems, CFTR has a characteristic biophysical profile. It is an anion-selective channel with a single channel Cl^- conductance of 6–10 picosiemens (pS) in ~120 mM Cl^- and a permeability selectivity sequence $\text{Br}^- \geq \text{Cl}^- > \text{I}^- > \text{F}^-$ (P. M. Quinton, 1999; Sheppard & Welsh, 1999). CFTR can also conduct HCO_3^- (Poulsen et al., 1994; J. J. Smith & Welsh, 1992). When studied by patch clamp electrophysiology in symmetrical Cl^- -containing solutions, CFTR channels demonstrate a linear current-voltage relationship (AndersonRich et al., 1991). The opening of the anion permeation pathway in CFTR requires phosphorylation of the channel, particularly by cAMP-dependent protein kinase A (Cheng et al., 1991), as well as the presence of ATP (AndersonBerger et al., 1991).

CFTR is a unique member of the ATP-binding cassette family of transporters (ABC transporters), which ordinarily use energy from ATP hydrolysis to pump substrates actively across the membrane (David C. Gadsby et al., 2006). CFTR has seven domains: cytoplasmic amino and carboxyl termini, two membrane-spanning domains that each contain six membrane-spanning segments, two nucleotide binding domains (NBD1 and NBD2) and an R, or regulatory, domain (Riordan et al., 1989) (Fig. 1). Although the crystal structure of NBD1 has been elucidated (Lewis et al., 2005), a high-resolution structure of full-length CFTR has not yet been determined. However, homology modeling based on crystal structures of bacterial ABC transporters has provided insights about CFTR's possible three-dimensional structure in cell membranes (Serohijos et al., 2008). Nonetheless, functional studies have revealed how each domain plays a role in the function or regulation of the channel. The putative twelve transmembrane helices provide the anion permeation pathway and contain the gate that controls transmembrane anion flux (reviewed in (Linsdell, 2006)). The two nucleotide binding domains of CFTR bind and/or hydrolyze ATP to modulate channel activity in a manner that is not yet completely determined, but likely involves dimerization of the two NBDs (Vergani et al., 2005). Nucleotide binding and/or hydrolysis induces conformational changes of the NBDs that are somehow communicated to the channel gate in the transmembrane domain resulting in its opening and closing. This communication may be mediated by extensions of the transmembrane helices that interact with the NBDs. The R-domain of CFTR, unique among

ABC transporter family members, is rich in consensus phosphorylation sites, mainly for protein kinases A and C (D. C. Gadsby & Nairn, 1999). However, other kinases can also phosphorylate CFTR (Picciotto et al., 1992). Phosphorylation of CFTR is necessary for its activation (AndersonBerger et al., 1991), and CFTR channels are deactivated upon dephosphorylation carried out by protein phosphatases (Berger et al., 1993; Reddy & Quinton, 1996). The amino and carboxyl terminal regions of CFTR have specific amino acid residues that allow it to bind to intracellular proteins (W. B. Guggino & Banks-Schlegel, 2004). For example, the carboxyl terminus interacts with the scaffolding protein NHERF-1 (Hall et al., 1998), which modulates channel gating (Raghuram et al., 2001) and enables CFTR to interact with other proteins (Sun et al., 2000).

In addition to regulation of resident CFTR channels by intrinsic factors such as nucleotide binding, phosphorylation, and protein interactions, the amount of CFTR in the apical plasma membrane is also regulated. A first level of regulation occurs in the endoplasmic reticulum (ER) where CFTR undergoes extensive quality control and abnormally folded mutant CFTR or abnormally glycosylated wild-type CFTR is retained in the ER and degraded by a ubiquitin/proteasome-dependent process known as ER-associated degradation (ERAD) (Seng H. Cheng et al., 1990; Denning et al., 1992; Farinha & Amaral, 2005; P. J. Thomas et al., 1992; Vembar & Brodsky, 2008; Ward et al., 1995; Y. Yang et al., 1993). There are also biochemical and functional data supporting a second level of regulation in the apical plasma membrane where the amount of CFTR is regulated by trafficking and recycling of the protein in and out of the apical plasma membrane from specific sub-cellular pools (Bertrand & Frizzell, 2003; Bradbury et al., 1994; William B. Guggino & Stanton, 2006; Prince et al., 1994; Swiatecka-Urban et al., 2007).

3.2. Abnormal CFTR

CF is an autosomal recessive disorder, meaning that a person must have two abnormal CFTR genes to manifest the abnormal epithelial ion transport characteristic of the disease. Knowledge of the CFTR gene and of the more than 1,400 known disease-causing mutations (www.genet.sickkids.on.ca/cftr/) allows for classification of CFTR mutations based on the resulting cellular phenotype. Initially, five classes of mutations were proposed (Zielenski & Tsui, 1995). Currently, there are six classes of CFTR mutations (Table 1).

Class I mutations are nonsense mutations that result in premature truncation of the nascent CFTR polypeptide and, therefore, little or no protein expression. Class II mutations are those that result in the synthesis of CFTR protein that is not adequately expressed at the apical plasma membrane. For $\Delta F508$ CFTR, the prototypical class II mutation, if the protein reaches the apical plasma membrane, it also demonstrates abnormally short membrane residence time (Lukacs et al., 1993) and abnormally low open probability (P_O) (Dalemans et al., 1991). Class III mutations alter CFTR gating and result in lowered Cl^- transport, despite expression of full-length protein at the apical plasma membrane of epithelial cells. Class IV mutations are missense mutations that result in normal expression of full-length CFTR with reduced Cl^- permeability. Class V mutations are those that cause reduced expression of normal CFTR. Finally, class VI mutations result in a protein that has abnormally short residence time at the apical plasma membrane (Haardt et al., 1999). Although genotype-phenotype correlations in CF are imprecise (Castellani et al., 2008), a CF patient's clinical phenotype will usually reflect either full loss of CFTR ion transport function or some fraction of CFTR ion transport if there is residual ion transport function afforded by one of the mutant CFTR alleles. For example, patients who have a single R117H CFTR allele (a class IV mutation) have less severe reduction of apical plasma membrane anion permeability (Reddy & Quinton, 2003; Sheppard et al., 1993) and generally have milder disease than CF patients where CFTR function is absent, such as those homozygous for a class I or II mutation.

The pathophysiology of end-organ damage in CF patients differs from one organ system to the next; however, the basic defect – a lack of apical plasma membrane Cl^- and HCO_3^- permeability in epithelial cells – remains the same. Whereas CF pancreatic and pulmonary disease are characterized by luminal obstruction and fibrotic parenchyma, the sweat gland is unique in that it does not demonstrate any macroscopic pathological defects such as luminal obstruction or scarring. This may be because the sweat gland does not secrete significant amounts of protein or mucus that need to be flushed from its lumen, as is the case in other tissues affected in CF. That the physiology of the sweat gland is abnormal in CF despite it not being a mucus-secreting epithelium strongly supports the notion that the basic defect in CF is confined to abnormal ion transport rather than extending to basic abnormalities in mucus production or secretion (Paul M. Quinton, 2007).

4. Therapies for CF

CF is sometimes referred to as “the most common fatal (or lethal) genetic disease of Caucasians,” and it has an appreciable, though smaller, prevalence in African Americans, Hispanics, and Asians (O'Sullivan & Freedman, 2009). Despite this ominous description, median predicted survival for patients with CF has risen from less than a few years in 1938 to more than 37 years presently. This remarkable success has many underlying factors, including the use of therapies not directed at the underlying ion transport defect.

4.1. Pancreatic enzyme replacement therapy (PERT)

The early association of CF with celiac disease highlights the severe protein and fat malabsorption associated with pancreatic insufficiency, which occurs in approximately 90% of CF patients. With the use of exogenous pancreatic enzymes, early nutrition in CF improved as evidenced by improvements in the body's nitrogen balance (Harris et al., 1955). Exogenous pancreatic enzyme formulations and dosing have changed over the years (reviewed in (Ferrone et al., 2007)), though the principle remains the same: replace the amylase, lipase, and protease that the CF pancreas is unable to produce adequately. Recent data further highlight the critical importance of good nutrition and its effects on long-term health in CF patients (McPhail et al., 2008).

4.2. Therapies for CF lung disease

Survival of infancy and early childhood because of better nutrition resulted in lung disease rather than malnutrition being the most common cause of mortality in CF. Typically, lung disease in CF is described as a vicious cycle of mucus retention, infection, inflammation, and tissue damage, and there are many therapies designed to treat each part of the cycle (Fig. 2).

4.2.1 Therapies to improve mucociliary clearance—Although there is some debate about how patients enter the cycle of mucus retention, infection, inflammation, and tissue damage, there is consensus that augmented airway clearance techniques (e.g., chest percussion with postural drainage or high-frequency chest wall oscillation) are beneficial for CF patients (P. A. Flume et al., 2009; J. Thomas et al., 1995). These physical techniques are aimed at loosening mucus from the airway wall and assisting in its clearance from the lower airways. In addition, there are a number of pharmacological therapies designed to make the mucus itself more amenable to clearance (e.g., thinner) or to hydrate the airway surface so that the mucus is more easily cleared.

Airways mucus in CF is made viscous in part by the presence of DNA released by neutrophils in the airway. Dornase alfa (Marketed as Pulmozyme® by Genentech) is a recombinant human DNase that acts as a mucolytic by degrading this DNA. Pulmozyme therapy improves lung

function in patients with CF greater than 5 years of age with an FEV1 greater than or equal to 40% (Fuchs et al., 1994).

Hyperabsorption of Na^+ from the airway surface liquid (ASL) and subsequent depletion of ASL volume contributes to the pathogenesis of CF airways disease (Boucher, 2004; Boucher et al., 1988; Knowles et al., 1983; Matsui et al., 1998) (Fig. 3). There are two strategies to counteract this depletion: 1. Reduce Na^+ /fluid absorption to prevent ASL depletion and 2. Increase Cl^- /fluid secretion to increase ASL volume. Hypertonic saline (HTS, 7% NaCl) by inhalation is used as an osmotic agent to counteract the ASL depletion by pulling water into the lumen of the airways. HTS acutely improves mucociliary clearance and lung function, and over the long-term it reduces the frequency of pulmonary exacerbations in CF patients 6 yrs of age or older with an FEV1 greater than or equal to 40% (Donaldson et al., 2006; Elkins et al., 2006). An alternative osmotic agent, dry-powder mannitol (Marketed as Bronchitol, Pharmaxis) has been shown to improve lung function in a 2-week, Phase II study (Jaques et al., 2008) and is currently being evaluated in a Phase III clinical trial.

The ability of the airways to secrete Cl^- , and therefore fluid, in a CFTR-independent fashion is thought to occur through a Ca^{2+} -activated Cl^- conductance that appears to include the recently identified TMEM16A (Caputo et al., 2008; Schroeder et al., 2008; Y. D. Yang et al., 2008). Non-CFTR dependent Cl^- secretion can be elicited by activation of purinergic agonists, such as Denufosal tetrasodium (Inspire Pharmaceuticals, Durham, NC), a P2Y2 receptor agonist that has shown promise in Phase II clinical trials (Deterding et al., 2007) and is currently in Phase III clinical trials. In addition to stimulating CFTR-independent Cl^- secretion, UTP (and, likely, UTP analogs such as Denufosal) also inhibits Na^+ transport by the epithelial Na^+ channel, ENaC (Devor & Pilewski, 1999). A second pharmacological compound aimed at stimulating non-CFTR dependent Cl^- secretion is duramycin (marketed as Moli-1901 by Lantibio, AOP Orphan Pharmaceuticals AG). Duramycin acts by increasing intracellular Ca^{2+} concentration in a purinergic receptor-independent manner (Cloutier et al., 1990; Cloutier et al., 1993).

4.2.2. Anti-inflammatory therapies—The hallmark of CF lung disease is mucopurulent bronchitis characterized by neutrophilic infiltration of the airways. In addition, it is likely that T-cell-mediated immunity also plays a role (Banner et al., 2009). Anti-inflammatory medications such as ibuprofen (Konstan et al., 1995), a non-steroidal anti-inflammatory drug, and prednisone (Auerbach et al., 1985; Eigen et al., 1995), a corticosteroid, both improve lung function in CF patients. Significant side-effects including growth failure (Lai et al., 2000) have limited the use of long-term prednisone as therapy for CF, and the use of chronic oral corticosteroids is not currently recommended (Patrick A. Flume et al., 2007). The widespread use of ibuprofen has also been limited by concerns about side effects (Oermann et al., 1999), although recent comprehensive reviews of the available literature and data suggests that the benefits of ibuprofen therapy may outweigh the risks for CF patients with mild-to-moderated lung disease (Patrick A. Flume et al., 2007; Konstan, 2008). Currently, the macrolide antibiotic azithromycin (marketed as Zithromax by Pfizer) is in use as an anti-inflammatory medication that has been shown to improve lung function in CF patients 6 yrs of age or older who are chronically infected with *Pseudomonas aeruginosa* (Saiman et al., 2003).

4.2.3. Antimicrobial therapy—There is little question that the recognition of chronic pulmonary infections in children with CF and the treatment of these infections with antibiotics has been a major factor in improving survival in CF. Antibiotics for pulmonary infections in CF may be administered orally, intravenously, or by inhalation and are generally targeted at the major pathological organisms found in a CF patient's sputum. Antimicrobial therapy may be administered acutely for treatment of pulmonary exacerbations, or may be administered chronically for CF patients who are chronically infected with *Pseudomonas aeruginosa*.

TOBI™ (marketed by Novartis Pharmaceuticals) is a preservative-free tobramycin solution for inhalation that significantly benefits CF patients who are chronically infected with *Pseudomonas aeruginosa* (MacLusky et al., 1989; Ramsey et al., 1993; Ramsey et al., 1999).

In addition to the therapies reviewed above, there are numerous therapies aimed at altering ion transport, reducing inflammation, or reducing infection that are not mentioned but are currently being investigated. The Cystic Fibrosis Foundation website (www.cff.org/research) is a recommended resource for information regarding these therapies. The fact that lung disease remains the most common cause of morbidity and mortality in CF despite the many therapies aimed at reducing it highlights the need for specific interventions aimed at the basic defect in CF: absence of functional CFTR. The accumulated knowledge of two decades of research into CFTR has positioned the CF community to take advantage of the technological and scientific advances that have led to novel therapies addressing this basic defect.

5. CFTR-specific therapies

There are two main approaches to correct the underlying defect in CF. First, gene therapy attempts to replace the missing function by introducing part or all of the CFTR gene into the target epithelial cells in the lungs. Second, pharmacological compounds attempt to correct or potentiate abnormal CFTR. Correction of abnormal CFTR is the process of enabling mutant $\Delta F508$ CFTR to escape the cell's quality control machinery and be expressed in the apical plasma membrane. Potentiation of abnormal CFTR is the process of improving the Cl^- transport properties of mutant CFTR that is already in the apical plasma membrane.

5.1. Gene therapy

Attempts at gene therapy for CF began shortly after the identification and cloning of the gene. Some of the early studies on CFTR function were *de facto* gene therapy experiments demonstrating that replacing absent CFTR restored airway cell Cl^- secretion. Why then has there not been successful therapeutic gene transfer of CFTR to the lungs? Limitations to successful gene therapy include (but are not limited to) gene, vector, and host factors (reviewed in (Griesenbach & Alton, 2009; Mueller & Flotte, 2008)).

Innate host factors play an important role in gene therapy for CF. The lungs have sophisticated innate defense mechanisms against inhaled pathogens, including mucociliary clearance, cough clearance, and secreted antimicrobial peptides (Bartlett et al., 2008). These barriers must be overcome or avoided for the target epithelial cells to receive the CFTR gene. In CF, where there is mucus adherent to the surface epithelium (Boucher, 2004) and inflammation in the presence and possibly in the absence of infection (Armstrong et al., 2005; Brennan et al., 2009; Rosenfeld et al., 2001), successful gene transfer to all target epithelial cells is likely to be even more difficult. The target cells themselves, the airway epithelial cells, are terminally differentiated. This implies that these cells have a limited life span, and, therefore, gene transfer will need to be repeated on a regular and potentially life-long basis. However, the lungs and airways have a strong adaptive immunity to respiratory viruses (reviewed in (Kohlmeier & Woodland, 2009)). This response appears to be intact after administration of replication-deficient viral-based vectors as evidenced by the detectable antibody response to repeated administration of viral gene transfer vectors (Flotte et al., 2003; Kaplan et al., 1996; Moss et al., 2004; Y. Yang et al., 1995; Yei et al., 1994).

5.1.1. Viral-based gene therapy—The first attempts at gene therapy for CF were made using replication-deficient adenovirus-based vectors. Adenovirus was a logical choice because of its ability to infect the respiratory epithelium. However, the receptor for adenoviral entry into the cell is found predominantly on the basolateral surface of epithelial cells, limiting the efficacy of gene delivery by aerosolized adenovirus vector (Walters et al., 1999). Furthermore,

adenoviral vectors elicited a dose-dependent inflammatory response in non-human primates and humans (Brody et al., 1994; Joseph et al., 2001; Simon et al., 1993). Vecotr administration in humans demonstrated inefficient gene transfer (Harvey et al., 1999; Joseph et al., 2001) and progressive decreases in gene transfer with repeat administration that did not correlate with the appearance of adenovirus vector-specific neutralizing antibodies (Harvey et al., 1999).

Because of the limitations encountered with adenoviral-mediated gene delivery, alternative viral and non-viral (see below) vectors were investigated. Adeno-associated virus (AAV) is a good candidate for viral gene delivery because it has wide tissue tropism (Wu et al., 2000), it efficiently transduces non-dividing cells (Malik et al., 2000), and it has not been directly associated with clinical disease in humans (R. H. Smith, 2008). Nonetheless, significant problems have presented themselves in the use of AAV for CF gene therapy. For example, the CFTR cDNA has a length of 4.4 kB, and the packaging capacity of AAV is approximately 4.7 kB (Nahreini et al., 1992). Therefore, it is difficult to insert CFTR, a strong promoter, and necessary untranslated region (UTR) sequences into AAV vectors to generate an optimal AAV gene therapeutic. To circumvent this problem, multiple investigators have used truncated forms of CFTR and have shown correction of electrical abnormalities both *in vitro* (Ostedgaard et al., 2002; Zhang et al., 1998) and *in vivo* (Ostedgaard et al., 2002). More recently, Song and colleagues used segmental trans-splicing, a multiple vector transfection technique (Pergolizzi et al., 2003), to successfully transfer CFTR with part of its 3'-UTR into human airway epithelial cells *in vitro* (Song et al., 2009).

Host factors or barriers have also been identified by the initial clinical trials investigating AAV. Tissue expression following administration was not as durable as had been seen in animal models such as rabbits where transgene expression lasted up to 6 months (Flotte et al., 1993), and administration of AAV did lead to the development of a humoral immune response (Moss et al., 2004). A large, placebo-controlled clinical trial using AAV encoding CFTR did not demonstrate differences in lung function between controls and those receiving CFTR 30 days following administration (Moss et al., 2007). Nonetheless, administration of AAV to humans appears to be safe and well-tolerated; therefore, investigations into the use of AAV as a gene therapy vector continue. For example, there are novel AAV preparations that improve conducting airways tropism (Limberis et al., 2008) and that can be readministered despite the presence of neutralizing antibodies in the serum in mice (Limberis & Wilson, 2006).

5.1.2. Cationic lipids complexed with plasmid DNA—A method for overcoming both the lack of packaging capacity of AAV vectors and the immunogenicity of viral vectors in general for gene transfer is to use non-viral vectors for DNA delivery. These include cationic lipids complexed with plasmid DNA and compacted DNA nanoparticles.

The principles of lipofection were introduced in 1987 in a demonstration that synthetic cationic lipids spontaneously incorporated plasmid DNA that was effectively delivered to and expressed in mammalian cells (Felgner et al., 1987). Initial studies of cationic lipid-mediated DNA transfer to the nasal epithelium of CF patients were promising, but failed to demonstrate persistent correction of NPD abnormalities (Caplen et al., 1995; Goddard et al., 1997). Further investigation of lipid-based DNA transfer to the lower airways revealed an innate inflammatory response to inhalation of the complexes (Alton et al., 1999; Ruiz et al., 2001). Based on these results, further investigations have focused on reducing this response while preserving gene transfer efficiency (Yew et al., 2000).

5.1.3. Compacted DNA nanoparticles—Alternative non-viral vectors are cationic polymers. One such polymer is a polyethylene glycol-substituted poly-L-lysine 30-mer that complexes with DNA to form essentially charge-neutral DNA nanoparticles. These nanoparticles transfer genetic material to non-dividing cells by delivering the DNA to the

nucleus via nuclear envelope pores (Liu et al., 2003). Preclinical studies with DNA nanoparticles in CFTR-knockout mice showed transient, dose-dependent correction of NPD abnormalities (Ziady et al., 2002), suggesting effective gene transfer. A double-blind, placebo controlled dose escalation trial of DNA nanoparticles to the nasal epithelium demonstrated transient correction of nasal potential difference (NPD) abnormalities without evidence of local or systemic inflammation (Konstan et al., 2004).

5.2. CFTR-specific therapies

5.2.1. Drugs that promote read-through of CFTR with stop mutations—*In vitro* and *in vivo* studies have provided insights into the cell biology of the different genetic classes of CFTR mutations. Class I mutations that are caused by a genetic change resulting in the presence of a premature stop codon, result in little to no protein production. However, because the abnormal stop codons are not surrounded by the usual genetic milieu, they are not as efficient at terminating translation of the polypeptide chain as are normally positioned stop codons. If the premature stop codon is “read-through” by the ribosome, the resulting protein may retain normal function.

The first compounds shown to promote read-through by suppressing the normal proofreading ability of the ribosome were the aminoglycoside antibiotics (A. Singh et al., 1979) (Martin et al., 1989). The first demonstrations of aminoglycoside-based correction of CFTR came from Bedwell and colleagues (Howard et al., 1996) (Bedwell et al., 1997). In 2003, Wilschanski and colleagues performed a clinical trial applying the aminoglycoside antibiotic gentamicin or placebo topically in the noses of CF patients with premature stop codons and demonstrated that gentamicin partially corrected nasal potential differences in both patients that were heterozygous and those that were homozygous for premature stop mutations (Wilschanski et al., 2003). Unfortunately the side-effect profile of aminoglycoside antibiotics, which includes both ototoxicity and nephrotoxicity, is likely unfavorable for long-term administration.

A non-aminoglycoside compound, PTC124 (3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid, marketed as Ataluren by PTC Therapeutics) was identified by a combination of targeted high-throughput screening approach to identify compounds with UGA read-through ability and subsequent chemical and pharmacological optimization. Ataluren is an orally bioavailable candidate for therapeutic suppression of premature termination of translation (Hirawat et al., 2007). It restored translation of dystrophin with premature stop mutations in human muscle cells *in vitro* and *mdx* mice *in vivo* (Welch et al., 2007), and restored translation of human G542X CFTR in a transgenic CFTR $-/-$ mouse (Du et al., 2008). Phase I clinical trials of PTC124 demonstrated safety and tolerability in both men and women (Hirawat et al., 2007). In a prospective, Phase II clinical trial, Kerem and colleagues demonstrated significant improvements in NPD measurements with a 28 d administration of Ataluren to CF patients who carried at least one nonsense mutation receiving (Kerem, 2008). A Phase III clinical trial is scheduled to begin in 2009 (www.cff.org).

Nonsense mutations resulting in premature termination of the nascent CFTR polypeptide account for only about 5% of all mutant CFTR alleles, so only a minority of CF patients will potentially benefit from drugs like PTC124.

The most common mutation, $\Delta F508$ CFTR, accounts for approximately 70% of all mutant CFTR alleles. This class II mutation causes misfolding of the nascent CFTR polypeptide chain in the ER (S. H. Cheng et al., 1990). The improperly folded polypeptide is recognized by the cell's quality control machinery and is targeted for degradation by the proteasome (Ward et al., 1995). The chaperone proteins and cellular machinery involved in $\Delta F508$ biogenesis and degradation are of great scientific interest because the $\Delta F508$ protein retains some, albeit reduced, Cl^- channel activity (Drumm et al., 1991b). Therefore, if $\Delta F508$ CFTR were rescued

from degradation and made to express at the apical plasma membrane, there would be a possible benefit in a majority of CF patients. Also, class III mutations, such as G551D, result in expression of CFTR channels with abnormally low sensitivity to cAMP activation. Therefore, if $\Delta F508$ CFTR or CFTR with a class III mutation were made to function more effectively in the apical plasma membrane, there would also be a potential benefit to many CF patients. There are compounds that accomplish both of these goals.

5.2.2. Potentiators—Compounds that improve the channel gating characteristics of mutant CFTR are called potentiators. The first recognized potentiator of CFTR was 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor that potentiated the cAMP-stimulated Cl^- current in *Xenopus* oocytes injected with either $\Delta F508$ CFTR or G551D CFTR (Drumm et al., 1991a) and in CFTR-transfected mouse mammary epithelial cells (Haws et al., 1996). Since the initial demonstration of IBMX as a potentiator, many other compounds have been identified.

The flavonoid compound genistein is a tyrosine kinase inhibitor that potentiates the gating of wild-type (Illek et al., 1995) as well as $\Delta F508$ CFTR (Hwang et al., 1997) and G551D CFTR (Illek et al., 1999). Genistein itself is an unlikely clinical candidate because at higher doses it inhibits CFTR gating (Wang et al., 1998). Apigenin, another flavonoid compound, also activates CFTR (Illek et al., 2000). Using combinatorial synthesis with genistein and a benzo [c]quinolinizinium potentiator, MPB-07, as lead compounds, Galiotta and colleagues identified 7,8-benzoflavones and fused pyrazolo heterocycles as potentiators of G551D CFTR (Galiotta et al., 2001). Using a fluorescence-based high-throughput screen, Pedemonte and colleagues identified sulfonamides as potentiators of $\Delta F508$ and identified phenylglycines as potentiators of $\Delta F508$, G551D, and other mutant CFTR (Pedemonte Lukacs et al., 2005). A similar high-throughput approach led to the identification of 4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)phenol (VRT-532) (Van Goor et al., 2006). Other potentiator compound families include benzimidazolones (Gribkoff et al., 1994; S. Singh et al., 2001), 1,4-dihydropyridines (Pedemonte et al., 2007; PedemonteDiena et al., 2005), and tetrahydrobenzothiophenes (H. Yang et al., 2003).

In general, potentiators are believed to function by increasing the amount of time that the CFTR channel is open, allowing for more Cl^- to pass through the pore. Despite the discovery of many potentiators by high-throughput screening and subsequent medicinal chemistry, the exact mechanism of action remains unknown. Nonetheless, a shared mechanism of action may emerge (Wellhauser et al., 2009). Ai and colleagues proposed that capsaicin potentiated CFTR by stabilizing NBD dimerization (Ai et al., 2004). In Cos-7 cells, G551D CFTR responds differently to genistein than does G551D/G1349D CFTR, suggesting that both NBD sites play a role in the activity of genistein (Melin et al., 2004). 1, 4-Dihydropyridines appear to bind a hydrophobic pocket in NBD1 where they could also interact with NBD2 (Pedemonte et al., 2007), as was previously suggested by Al-Nakkash and colleagues (Al-Nakkash et al., 2001). Data in Fisher rat thyroid (FRT) cells transfected with different mutant CFTR cDNAs suggests that potentiators may bind to CFTR and cause conformational changes that promote CFTR being in the open configuration (Caputo et al., 2009).

The potential clinical benefits of CFTR potentiators are already coming in to focus. With support and collaboration of the CFF and Cystic Fibrosis Foundation Therapeutics (CFFT), Vertex Pharmaceuticals (Cambridge, MA) used high-throughput screening to identify and characterize potentiators and correctors of CFTR. VX-770, for example, increases the amount of time that wild-type CFTR, G551D CFTR, and $\Delta F508$ CFTR channels are open (Van Goor et al., 2009). VX-770 also reduces Na^+ absorption and increases Cl^- secretion *in vitro* in CF epithelial cells heterozygous for G551D and $\Delta F508$ CFTR mutations to approximately 50% of cells homozygous for wild-type CFTR (Van Goor et al., 2009). In 2008, Vertex and CFF

presented the results of a phase IIb clinical trial of VX-770, reporting that patients with the G551D mutation on at least one CFTR allele who took VX-770 orally for 14 or 28 d had improvement in lung function, NPD, and sweat Cl^- values, suggesting that VX-770 successfully addressed the underlying defect in these patients. Definitive phase III clinical trials are currently in progress for patients with at least one G551D CFTR mutation (www.clinicaltrials.gov). Additionally, there is a phase II clinical trial investigating the effects of a 16-week treatment course of VX-770 in patients homozygous for ΔF508 CFTR.

5.2.3. Correctors—Compounds that facilitate the movement of ΔF508 CFTR (or other class II mutations) out of the ER and into the apical plasma membranes of epithelial cells are termed correctors. Early studies with non-pharmacological compounds such as dimethyl sulfoxide (Bebok et al., 1998) and glycerol (Sato et al., 1996) provided proof-of-principle that ΔF508 CFTR could be rescued from intracellular degradation to the same degree as low-temperature correction, though the exact mechanism of action remained elusive. The first identified pharmacological corrector of ΔF508 CFTR was 4-phenylbutyrate (Rubenstein et al., 1997; Rubenstein & Zeitlin, 1998), which was effective both *in vitro* and *in vivo*, though the *in vivo* effects were relatively small. Because of the potential clinical benefit to so many patients, many other compounds have since been identified as correctors of ΔF508 CFTR trafficking.

As for potentiators, high-throughput screening has proven a useful tool for identifying correctors. Some examples of correctors identified by high-throughput screening include the aminoarylthiazoles, quinazolinylaminopyrimidinones, and bisaminomethylbithiazoles (PedemonteLukacs et al., 2005), the sildenafil analog, KM11060 (Robert et al., 2008), and the quinazoline VRT-325 (Van Goor et al., 2006). Unlike the potentiators, it is less likely, though not impossible, that correctors will share a single mechanism of action because of the complexity and regulation of CFTR maturation and recycling at the apical plasma membrane. Some correctors may function as chemical chaperones that assist in stabilizing the nascent polypeptide chain and aiding its proper folding (Loo et al., 2008). Another mechanism may be stabilizing the cell surface expression of the mutant CFTR (Varga et al., 2008). In any case, it remains uncertain whether the residual ion transport afforded by the corrected mutant CFTR will be sufficient to ameliorate clinical disease. Therefore, investigations are on-going to identify molecules that are combined correctors and potentiators. Clinically, it may also be possible to administer both a corrector and a potentiator to achieve the desired outcome. Currently, a phase II, randomized, double-blind, placebo-controlled trial of the corrector VX-809 is enrolling patients who are homozygous for the ΔF508 CFTR mutation (ClinicalTrials.gov identifier: NCT00865904).

6. Conclusions

In summary, the identification of the CFTR gene led to an explosion of research that has provided invaluable knowledge about the pathogenesis and pathophysiology of CF. Armed with this knowledge, the CF community has been able to harness scientific and technological advances in order to generate novel genetic and non-genetic therapies that address the basic defect of CF. Because of these advances, there should be great optimism that novel, effective therapies for CF will be available to CF patients in the near future.

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Figure 1. CFTR model

The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ATP-binding cassette family of proteins. It consists of 7 domains: intracellular amino and carboxy terminal domains, 2 6-segment membrane-spanning domains, a regulatory R-domain, and 2 nucleotide-binding domains.

Figure 2. The vicious cycle of CF lung disease

Normal MCC

Normal CFTR Function

Impaired MCC

CF (Absent or dysfunctional CFTR)

Figure 3. Depletion of airway surface liquid in CF

In vitro and in vivo evidence supports the hypothesis that absence or dysfunction of CFTR in the airways results in hyperabsorption of sodium from the airway surface liquid. This results in osmotic absorption of water, causing depletion of the airway surface liquid. Depletion of airway surface liquid prevents normal ciliary beating, resulting in mucus retention.

Figure 4. Novel CFTR-directed therapies

Three novel types of CFTR-directed therapies are currently being investigated. **A.** For class I mutations resulting in premature termination of translation, there are promoters of ribosomal read-through such as PTC124. **B.** For class II mutations, including $\Delta F508$ CFTR, that cause abnormal protein folding and trafficking, there are small molecule correctors, such as VX-809, that increase surface expression of the mutant protein that retains some function as a chloride channel. **C.** For class III mutations such as G551D CFTR that have abnormal channel regulation, there are potentiators, such as VX-770, that increase the chloride conductance of the abnormal channel in the apical plasma membrane (Van Goor et al., 2009). VX-770 is also currently in clinical trials in patients with $\Delta F508$ CFTR to investigate whether it will potentiate the function of any mutant CFTR that reaches the apical plasma membrane.

Table 1

Classification of CFTR mutations

Class	Mutation Example	Cellular / molecular phenotype
I	W1282X	Absent CFTR production due to nonsense mutations, frameshift mutations, or abnormal mRNA splicing
II	Δ F508	Improper intracellular processing of CFTR with less than normal amounts of CFTR protein at the apical plasma membrane
III	G551D	Defective regulation of CFTR channels at the apical plasma membrane
IV	R117H	Defective permeation of anions through CFTR channels at the apical plasma membrane
V	3849+10KbC>T	Reduced synthesis of normal CFTR
VI	Q1412X	Altered apical membrane residence time of CFTR channels with truncated c-terminus