Gene Therapy for Autoimmune and Inflammatory Diseases
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# Gene therapy for cystic fibrosis lung disease

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## Abstract

Cystic fibrosis (CF) is characterised by respiratory and pancreatic deficiencies that stem from the loss of fully functional CFTR (CF transmembrane conductance regulator) at the membrane of epithelial cells. Current treatment modalities aim to delay the deterioration in lung function, which is mostly responsible for the relatively short life expectancy of CF sufferers; however none have so far successfully dealt with the underlying molecular defect. Novel pharmacological approaches to ameliorate the lack of active CFTR in respiratory epithelial cells are beginning to address more of the pathophysiological defects caused by CFTR mutations. However, CFTR gene replacement by gene therapy remains the most likely option for addressing the basic defects, including ion transport and inflammatory functions of CFTR. In this chapter, we will review the latest preclinical and clinical advances in pharmacotherapy and gene therapy for CF lung disease.

## Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder among Caucasians. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene identified by positional cloning in 1989 [1–3], which encodes a chloride ($Cl^-$) channel expressed in the epithelia of many tissues. The gene encodes a single polypeptide chain of 1480 amino acids, with a predicted molecular weight of around 168 kDa [1]. The CFTR protein is embedded in the apical membrane of epithelial cells and is made up of distinct structural domains, including two membrane spanning domains (MSDs), two nucleotide binding domains (NBD) containing conserved motifs for ATP binding and hydrolysis, and a regulatory domain (R) [4]. The CFTR protein, a member of the ABC family of proteins, normally functions as a cAMP-activated $Cl^-$ channel [5], and has been shown to interact with other ion channels and transporters, such as the amiloride-sensitive epithelial sodium channel, ENaC (recently reviewed by Berdiev et al. [6]) and the outwardly rectifying chloride channel (ORCC) (reviewed by Kunzelmann, [7]).

## CFTR mutations

The Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca) currently lists 1604 mutations located throughout the gene and affecting all domains of the protein. The most common mutation ΔF508, is found in 70% of disease alleles [2], and is caused by a deletion of three consecutive base pairs, resulting in the loss of a phenylalanine (F508) [1]. Mutations in the CFTR gene are grouped into five classes, based on their effect on CFTR protein expression and/or function: Class I–III mutations commonly cause severe disease phenotypes, Class IV and V mutations tend to be associated with milder disease, although not systematically for lung disease which can be highly variable even within identical genetic backgrounds [8, 9].

## Pathophysiology of CF lung disease

Mutations in CFTR disrupt transport in the epithelium of several tissues, which results in the production of abnormally thick, sticky mucus. The main cause of morbidity in CF is lung disease [10], with deterioration of lung function and pulmonary failure being the cause of death for the majority (>90%) of patients. The production of thick, sticky mucus in the lumen of the lung impedes mucociliary clearance [11], a consequence of which is chronic inflammation and recurrent bacterial infections (typically *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex), in a self-perpetuating cycle that leads to the progressive destruction of lung tissue [10]. In severe cases, *P. aeruginosa* can form antibiotic resistant biofilms in the lumen of the airways, the presence of which correlates with a decline in lung function [12].

Airway epithelial cells (AEC) are covered in air surface liquid (ASL), made up of a mucus layer that traps potentially harmful particulate matter that is inhaled, and the periciliary liquid (PCL) layer. The PCL provides a less viscous layer for the cilia to beat and remove the mucus (containing the trapped particles) from the airways by mucociliary clearance. The PCL also acts as a lubricant between the mucus layer and the mucins tethered to the cell surface to facilitate cough clearance [13, 14]. Finally PCL contains antimicrobial peptides and proteins (e.g., defensins, lysozyme, lactoferrin and anti-microbial surfactant proteins) to fight pathogens [15]. In CF these processes are disrupted, causing dysregulation of liquid movement, and lung infection and inflammation [16].

## The low volume hypothesis to explain CF lung disease

The low volume hypothesis [17], also know as the ‘isotonic volume transport/mucus clearance’ theory, implicates reduced ASL volume as the initiating event in CF lung pathology. The airway epithelium is thought to regulate ASL

volume so that the height of the PCL layer is approximately the same as that of the extended cilia on the cell surface of the epithelium (around 7 μm) [18], allowing them to beat efficiently. In CF airways, the ASL volume and hence PCL height is decreased, making the mucus layer sticky and harder to move [18]. The resulting flattening of the cilia prevents them from beating [19], causing mucus to adhere to cells [14]. Together these events lead to defective mucociliary clearance, initiating the chronic cycle of infection and inflammation characteristic of CF lungs (reviewed by Boucher, 2007 [20]).

There is now a significant amount of evidence to support the low volume hypothesis in primary human cell culture and mouse models. Most recently, accelerated $Na^+$ absorption, leading to a decrease in PCL height and reduced mucociliary clearance was demonstrated *in vivo* in the ENaCβ over-expressing mouse (reviewed by Mall, 2008 [21]) and in primary air-liquid interface (ALI) cultures from CF patients [18, 19].

The recent availability of the ENaCβ over-expressing mouse, with its lung pathology that closely mirrors that of CF patients, has provided a powerful tool for understanding the links between CFTR deficiencies and the complex pathophysiology of CF. For example sterile inflammation is also observed in these mice [22], supporting the hypothesis that inflammation can occur independently of infection.

## Pharmacological approaches to treat CF lung disease

Pharmacological processes that restore effective ASL height and mucociliary clearance in CF patients could be targeted to upregulate CFTR activity, modify alternative channels (ENaC, Calcium-activated $Cl^-$ channel (CaCC)), or rehydrate the ASL with hyperosmotic agents such as inhaled mannitol and hypertonic saline [23, 24]. Current approaches to pharmacological correction of CFTR include: 1) drugs that increase the level of CFTR protein synthesised, 2) CFTR correctors to increase trafficking out of the ER, and 3) CFTR potentiators that correct gating defects of CFTR at the membrane (Fig. 1).

## Drugs that increase CFTR protein levels

Several strategies to increase the amount of CFTR at the cell surface have been investigated, including increasing overall transcription levels with butyrate [25] or sodium 4-phenylbutyrate (4-PBA) [26], although neither has had an impact in clinical trials. Several drugs that promote read-through of nonsense stop codons have been shown to produce full-length CFTR, including aminoglycoside antibiotics (e.g., gentamicin) [27, 28] or the recently developed PTC124 [29]. The latter appears capable of improving electrophysiological features of nasal epithelium and several clinically relevant outcome measures (FEV1, FVC, circulating neutrophils) [30–32]. Interestingly it has been

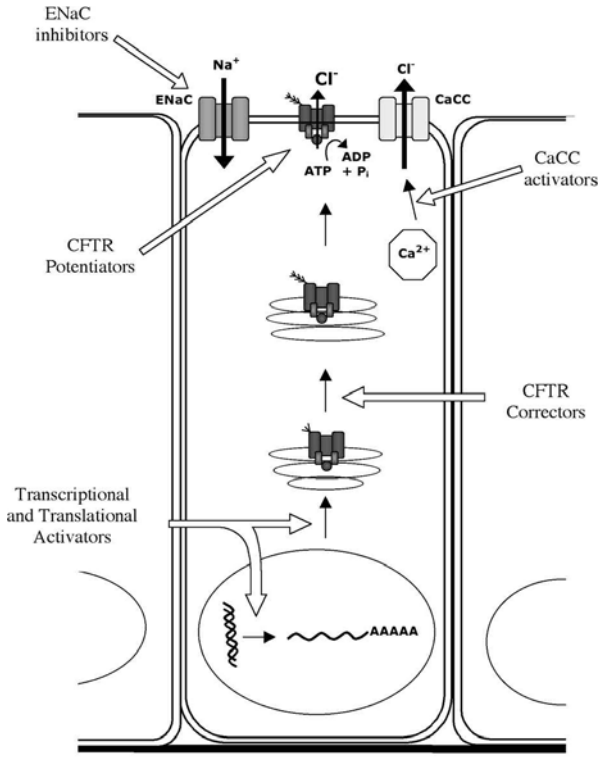


Figure 1. Schematic representation of sites of action for pharmacological correction of CFTR deficiency. Pharmacological drugs that are being investigated for the correction of CFTR deficiency may act by increasing the level of mRNA by enhancing transcription or translation ('Transcriptional and Translational Activators'), correcting the trafficking defect through the endoplasmic reticulum ('CFTR Correctors'), increasing activity of CFTR at the membrane ('CFTR Potentiators'), or regulating other ion channels such as Epithelial $Na^+$ Channel (ENaC) ('ENaC inhibitors') or $Ca^{2+}$-activated Cl channel (CaCC). Sites of action of these drugs in an airway epithelial cell are indicated by the white arrows, relative to CFTR biosynthesis and ATP-dependent channel activity, and relative to the pathways for activation and inhibition of CaCC and ENaC respectively.

reported that a drug such as PTC124 may not be effective in all cases of premature stop codons, due to exonic skipping which removes the early stop codon from the mature mRNA [33].

## CFTR correctors, drugs to increase trafficking of CFTR

Enhancing trafficking of CFTR using chemical chaperones can increase CFTR levels in the membrane in preclinical studies [34–36]. Recent efforts to identify more CFTR-specific correctors have involved high-throughput screening [37–40]. The phosphodiesterase-5 (PDE-5) inhibitor, sildenafil, shows promis-

ing correction of the CF defect *in vitro* [41] and is currently in Phase I/II clinical studies expected to conclude in 2010. KM1160, a much more potent analogue of sildenafil, is in early stages of development [42]. A different corrector, Miglustat, functions through inhibition of α-1,2-glucosidase, thus preventing rapid degradation of ΔF508 in the ER, and has shown promise in multiple preclinical studies [43–47]. Miglustat is now in a Phase II trial (Actelion). VX-809 is a ΔF508 CFTR corrector that was discovered through a collaborative programme, between Vertex and Cystic Fibrosis Foundation Therapeutics Inc. It recently entered a Phase II safety/efficacy study [48]. Results of both these studies are eagerly awaited.

## CFTR potentiators, drugs that increase conductance at the apical membrane

Other potential drug therapies have been based on increasing the activation of CFTR, by increasing the level of cyclic nucleotides in the cell using phosphodiesterase (PDE) inhibitors such as milrinone [49, 50], or by direct activation with curcumin [51], genistein or 8-cyclopentyl-1,3-dipropylxanthine (CPX) [52]. The most promising advances involve Vertex compound VX-770 [53], a drug that increases the open probability and $Cl^-$ conductance of CFTR; it has completed a Phase IIa trial [54] and entered the FDA registration programme in 2009.

## Modulation of other ion channels

Upregulation of CaCC ($Ca^{2+}$-activated $Cl^-$ channel) by Denufosol and Moli1901 (Lancovutide®) was recently demonstrated to be a safe and tolerable way of inducing some $Cl^-$ transport via non-CFTR channels [55–58]. Further evaluation of efficacy in CF patients for both drugs is ongoing.

Alternatively, ENaC activity could be inhibited in order to decrease the elevated $Na^+$ absorption seen in CF airways. The Parion compound 552-02 [59] is a recent improvement on the ENaC inhibitor, amiloride, and has already entered clinical trials, including one for CF, which is currently under way.

Inhibition of serine proteases can prevent activation of ENaC, but most candidates have not yet proceeded beyond preclinical studies [60]. A recent small-molecule inhibitor of proteases involved in ENaC regulation, Camostat, was tested with some success in sheep aerosol studies, improving mucociliary clearance for several hours after administration [61]. Partial correction (75% towards normal) of the $Na^{2+}$ transport defect in the nose of CF patients was reported [62], confirming that this is a promising candidate for evaluation in the clinic for CF lung disease.

Although these data are encouraging, the majority of these new drug treatments have only modest ability to correct the CF defect, do not act on all

CFTR mutations, and even if successful, may only benefit a small proportion of patients depending on their genotype. It is also likely that the pharmacological approaches discussed here could require use in combination with each other for optimal correction of the CF ion transport defects, for example a corrector with a potentiator, and simultaneously adjusting ENaC activity with a third component; this may limit their use in the clinic and will certainly take time to evaluate in trials.

## Gene therapy clinical trials

The basic concept of gene therapy involves introducing a gene into target cells to prevent or slow the progression of a disease. CF is a good candidate for this technology as it is primarily caused by mutations in a single gene, a normal copy of which could be delivered to patients via topical delivery to the lung, without invasive techniques or surgery. Moreover, a gene complementation approach would directly target the cause of the disease and could correct many aspects of the complex lung pathology. A single therapy to treat the underlying defect could greatly reduce the high therapeutic burden that CF patients currently have to endure. In addition, one therapy might be suitable to treat subjects with a wide variety of mutations, meaning that a single treatment strategy could be relevant to all patients. Proof of principle for both viral and non-viral *CFTR* gene transfer was quickly established in CF patients [63] and to date, 25 trials have been completed involving approximately 450 CF individuals (see Griesenbach, 2009 [64]).

### Adenovirus and Adeno-Associated Virus

Two DNA viral vectors, adenovirus (Ad) and adeno-associated virus (AAV), have been evaluated in CF gene therapy clinical trials. Trials with Ad vectors have been disappointing, compared with preclinical studies, both in terms of persistence of gene expression and the level of gene transfer in the human airways. Gutless adenovirus (also referred to as helper-dependent Ad), in which all of the viral genome is removed (apart from the inverted terminal repeats (ITRs) and the viral packaging sequence) [65] have shown extended duration of transgene expression, with reduced toxicity and immunogenicity in mice compared with previous generations of Ad [66], but re-administration remains problematic due to the presence of viral capsid proteins [67].

An alternative viral vector that has been investigated in CF clinical studies is the non-pathogenic AAV. Phase I and II clinical trials administering a single dose of AAV2 expressing CFTR to the nose [68–71] and lungs [72, 73] of CF patients were deemed safe and resulted in consistent detection of vector-derived DNA for between 30 days and 10 weeks after delivery. CFTR mRNA was very rarely detected in the trials, although two studies reported transient

correction of the $Cl^-$ conductance defect in the nose for up to 2 weeks after delivery [69, 70]. Treatment did not result in any detectable clinical benefit in lung function [71] and neutralising antibodies against the vector were detected in the serum [72, 73]. Two trials to re-administer the virus have been performed, with a similar lack of clinical benefit [74, 75]. Once again, this has been disappointing in comparison with preclinical studies in mice in particular, but is partly attributable to a paucity of AAV2 receptors on the apical membrane of human cells [76].

Thus, alternative serotypes with potentially improved tropism for airway epithelial cells are being investigated. AAV5 and AAV6 appear to transduce airway epithelial cells more efficiently than AAV2 [77, 78], and up to 90% transduction efficiency was recently reported in mouse airways with AAV6 using the hybrid chicken β-actin/rabbit β globin promoter/intron with the human CMV immediate early enhancer (CAG) [79].

The lack of efficiency in the repeat administration clinical studies [74, 75] is in keeping with some preclinical studies showing an inability to re-administer AAV2 and AAV5 in airways of animals, unless genotypes were switched for re-administration [80–83]. Interestingly, it has been demonstrated that AAV9 serotype virus could be re-administered successfully to murine airways 1 month after the initial dose [84].

In addition to difficulties with tropism and immune responses, the utility of AAV for CF has been hampered by the limited packaging capacity of most rAAV vectors (<5 kb). Advances in this field have included development of miniCFTR genes that may be packaged more efficiently [85–88], including safety studies in non-human primates [89] and functional studies in mice [90], and the discovery that AAV2/5 could in fact package large genomes (up to 8.9 kb) with a reasonably yield compared with other rAAV pseudotypes (1 to 4 and 7 to 9) [91]. Together with evidence suggesting that AAV vectors may be able to target progenitor cells of the mouse lung [92], thus avoiding the need for repeat administration, this work continues to make incremental improvements.

## Negative strand RNA viruses

The murine parainfluenza virus type 1 [or Sendai virus (SeV)], the human respiratory syncytial virus (RSV) and the human parainfluenza virus type 3 (PIV3) are negative strand RNA viruses whose life cycle is completed in the cytoplasm. They have all been shown to transfect AECs efficiently via the apical membrane [93, 94], and express functional CFTR channels *in vivo* [95], but elicit an immune response that currently inhibits repeated administration [96]. Although such viruses may be useful for acute diseases that require only transient gene expression, in the context of CF their use is for now restricted to preclinical proof of principle studies, until the immunological barriers to repeat administration can be resolved.

## Lentiviruses

Lentiviruses are retroviruses that transduce non-dividing cells including terminally differentiated AECs. The viral dsDNA genome stably integrates into the genome of transduced cells after its RNA has been reverse transcribed, so expression is likely to last for the lifetime of the cell (approximately 17 months for AECs, [97]). VSVG-pseudotyped HIV-derived lentivirus carrying the CFTR gene transiently and partially corrected the $Cl^-$ defect in CF knockout mouse nose for up to 46 days, although pre-treatment with the tight junction opener lysophosphatidylcholine was necessary [98]. There have been attempts to improve the tropism of lentivirus by pseudotyping with envelope glycoproteins from the filoviruses Ebola or Marburg [99], GP64 of baculovirus [100], the spike envelope glycoprotein of the SARS virus [101] and the F and HN proteins of SeV [102]. This latter vector, F/HN-SIV, was able to transduce polarised epithelial cells from both the apical and basolateral side and importantly, murine AEC *in vivo* without the need for pre-conditioning, with gene expression *in vivo* persisting for at least 17 months, i.e., the lifetime of AECs [97, 103]. This is consistent with gene expression for up to 12 months in mouse nose with GP64-FIV [100]. As with other viruses, it will be important that researchers address the challenge of multiple repeat administrations without loss of efficacy, or find ways of targeting stem cell populations of the airways, to treat the chronic aspects of CF lung diseases.

## Non-viral vectors for CF gene therapy

The need for effective long-term repeated administration to treat CF lung disease had led to the investigation of non-viral vectors, which take the form of circular plasmid DNA (pDNA) delivered to cells as naked pDNA in diluents such as PBS, saline or water, or complexed with agents such as lipids or polycations as protection from extracellular degradation and to aid cellular entry. The relative lack of efficiency compared with viral vectors is counterbalanced by reduced safety concerns regarding integration, more flexible and easier production methodology, extended storage and an unlimited packaging capacity [104, 105].

Non-viral Phase I clinical trials to deliver pDNA expressing CFTR began in the mid 1990s, with a variety of cationic liposome formulations delivered to the nose and/or lungs of CF patients. In general, gene transfer was well tolerated and evidence of *CFTR* gene transfer (as measured by vector-specific mRNA or CFTR-mediated chloride transport) has been established in some, but not all, studies (reviewed in Rosenecker et al., 2006 [106]).

One side effect of lipid formulations has been the transient mild flu-like symptoms reported by Ruiz and Alton in the lung trials of GL67:DOPE:DMPE-PEG/pDNA [107, 108]. This inflammation may be related to the stimulation of the Toll-like receptor 9 by bacterially-derived CpG dinucleotides in the formu-

lation [109, 110]. In a bid to reduce this response, the UK CFGT Consortium has generated a CpG-free pDNA [111], for CF clinical trials now under way. The deletion of CpG motifs is one of the latest aspects of plasmid development programmes in the field of CF gene therapy. A common feature of previous trials has been the transient nature of any correction that was measured. Persistence of expression has been improved in preclinical studies by swapping viral promoters such as CMV (cytomegalovirus) for human promoters including UbC (polyubiquitin C) and EF-1α (elongation factor 1-α) [112, 113]. Similarly specificity has been improved in mice using the human cytokeratin (K18) and FOXJ1 promoters which both directed epithelial cell-specific transgene expression in mice [114, 115]. Clinical trial data will confirm whether the modifications to the DNA construct have improved the duration and tolerability of gene transfer in the nose and lungs of CF patients. Table 1 summarises the features of recent non-viral vector developments in the context of airway gene therapy.

## Cationic polymers as mediators for CF plasmid DNA gene therapy

A particular polycation that has shown promise for lung gene therapy is polyethylenimine (PEI), the most commonly used forms of which are 25 kDa branched PEI and 22 kDa linear PEI. Although not used in lung gene therapy clinical trials to date, PEI/pDNA complexes have led to successful gene delivery in a clinically relevant model of aerosol gene delivery [116], and work towards improving the formulation by concentrating the particles has shown promising results in murine airway studies [117]. Following comparison of over 25 different non-viral formulations, concentrated 25 kDa PEI has been selected as a ‘Wave 2’ product by the UK CFGT Consortium. Additional improvements may also come from the field of integrases, with the recent demonstration that mice treated with integrase-encoding and reporter constructs complexed with PEI expressed the reporter protein longer than those treated with a non-integrase-encoding construct [118].

The only cationic polymers used in clinical trials for CF gene therapy to date have been compacted DNA nanoparticles, which consist of a single molecule of pDNA compacted with a 30-mer lysine polymer covalently linked to polyethylene glycol (PEG) [119]. The advantage of this gene transfer agent is the identification of its receptor for cell uptake, nucleolin [120] and nuclear translocation bypassing the endosomal pathway. A Phase I study resulted in detection of vector-derived DNA 3 days after dosing in nasal epithelium of CF patients, and partial to complete correction of the $Cl^-$ transport defect in some patients [121].

## Modifier genes and future directions

It is clear that the genotype of CF patients does not entirely predict the course of disease, particularly the rate of decline in lung function. A number of stud-

Table 1. Features of promoter/enhancer combinations for improved lung gene expression using plasmid DNA constructs

| | Construct description | Model | Feature | Ref. |
|---|---|---|---|---|
| pCUBI | 1.1 kb I/E hCMV enh, human ubiquitin B prom/intr | Lung instillation in mice | Duration for over 12 weeks vs pCF1 (4 weeks) (hCMV I/E enh/prom and hybrid int) | [112] |
| pUbLux | 1.2 kb of human polyubiquitin C prom, exon 1, int 1 and exon 2 sequences | Lung instillation in mice | >26 weeks vs viral enh/prom constructs: pCIKLux (hCMV I/E enh/prom and hybrid int), pRIKLux (hCMV IE enh, RSV 3' LTR prom) and pSIKLux (SV40 enh/early prom.) (<7 days, ±14 d, ±14d) | [113] |
| pEFLux | 1.3 kb of hEFI gene, including prom, exon 1, intr A and exon 2 sequences | Lung instillation in mice | >4 weeks vs viral enh/prom constructs as above | [113] |
| pG4-hCEFI | CpG-free I/E hCMV enh, hEF1 prom. to exon 2 fragment (from pEFLux) | Lung instillation in mice | Stable expression to >56 days, and no inflammatory response, compared with CpG-rich pG1-CMV (=pCIKLux) (<14d duration, high inflammatory response) | [111] |
| FoxJ1 | 1 kb of FoxJ1 genomic region and mouse transthyretin (TTR) gene intron | Transgenic mice | Tissue and cell specificity: ciliated cells of tracheal, bronchial and nasal epithelium | [115] |
| K18mLacZ | 2.2 kb of K18 5' promoter proximal flanking sequence, KRT18 promoter, and first intron/enhancer with mutated cryptic splice site | Transgenic mice | Cell specificity: restricted to the epithelium lining major airways, and submucosal glands in the lungs | [114] |

Abbreviations: enh, enhancer; prom, promoter; int, intron; I/E hCMV, immediate/early human cytomegalovirus; hEFI, human elongation factor 1α; K18/KRT18, cytokeratin 18; LTR, long terminal repeat

ies have attempted to identify modifier genes for different aspects of CF, as reviewed by Collaco and Cutting (2008) [122]. Lead candidates include inflammatory mediators and cytokines [122], and most recently IFRD1 [123], as well as gene polymorphisms that affect the response to bacterial infection (beta-defensins [124, 125], 8.1 ancestral MHC haplotype [126] and IL10 [127, 128]). In a disease such as CF where many factors influence the course of disease, with different clinical parameters to take into account (lung function, bacterial burden, inflammation), the identification of significant modifier genes will require large population studies. Ultimately this could provide new targets for anti-inflammatory drugs or gene silencing by RNAi strategies [129] to ameliorate disease.

Indeed it is likely that gene silencing therapies, including shRNA in pDNA constructs, will be evaluated that are not directly based on modifier genes, but on the general pathophysiology of CF. For example, a reduction in the transcription factor nuclear factor kappa B (NFκB), which regulates many pro-inflammatory cytokines and plays a central role in the exaggerated innate immune response in CF [130], or the ER-membrane protein BAP31, involved in blocking misfolded ΔF508 CFTR [131], may be beneficial. With further improvements in non-viral gene transfer, gene silencing may become a realistic treatment option.

## Conclusion

No single, novel, therapeutic approach to CF treatment has yet shown sufficient promise to stand out in the field. However as our understanding of the molecular processes in the CF lung deepens with better preclinical models to evaluate them, it is becoming clearer that early and broad intervention will be necessary to prevent the multifaceted defects that accompany CFTR mutations. Pharmacological approaches are heading into clinical trials at a regular pace, with some preclinical studies showing correction of several major defects. Although the challenges of finding a safe and effective formulation permit only slow progress, gene therapy still provides a great opportunity for an ‘all-round’ therapeutic intervention.

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