**Gene therapy for cystic fibrosis: recent progress and current aims**

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**Abstract**

Introduction: Since identification of the disease causing gene over 25 years ago, cystic fibrosis (CF) has been at the forefront of gene therapy research. Despite initial optimism, CF gene therapy has proven considerably more challenging than initially anticipated. However, research conducted over the past two decades has clarified the strength and weaknesses of viral and non-viral gene transfer agents for CF gene therapy. Methodology: The older literature related to CF gene therapy has been reviewed in many publications and we will, therefore, restrict this review to a brief description and discussion of the key lessons learnt, instead focusing on more recent progress in the field which was identified through literature searches. This review will summarize research leading up to the recent pivotal proof-of-concept study showing that non-viral gene therapy can stabilize the decline of lung function in CF patients and also highlight recent advances in viral vector development which may overcome problems related to loss of efficacy on repeated administration. Expert opinion: The demonstration that gene therapy can stabilize CF lung disease is an important milestone in gene therapy.

**Keywords**

Cystic fibrosis, gene therapy, lung, viral vector, non-viral vector.

1. **Introduction**

Cystic Fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The disease is most common in Caucasians affecting approximately 1 in 2500 (although this number various greatly in different populations) live birth and has long been thought of as a Caucasian disease. However, improved awareness and diagnostic tools now lead to increasing numbers of CF patients being diagnosed in India for example {2016 1770 /id} CF is a multi-organ disease, affecting many organs including the lung, intestine, pancreas, reproductive organs and sweat glands. In the developed world most morbidity and mortality is due to CF lung disease, because effective drugs to treat pancreas and intestinal disease are available. In the underdeveloped world pancreatic and intestinal disease contribute significantly to premature mortality.

CFTR is best known as a cAMP-dependent chloride channel which acts in the apical membrane of many epithelial cells throughout the body. However, the protein has several other functions and has also been shown to transport bicarbonate and regulate other ion channels such as epithelial sodium channels (ENaCs). CFTR therefore plays an important role in epithelial ion transport and fluid homeostasis.

Over the past decades several hypotheses have been postulated to explain how mutations in CFTR might cause CF. The ‘low-volume’ hypothesis postulates that, in addition to a reduction in chloride transport, the lack of functional CFTR also leads to sodium hyperabsorption through disinhibition of ENaC, and subsequent increased water absorption into the tissue, leading to reduced airway surface liquid and impaired mucociliary clearance {Boucher, 2007 430 /id}. More recently it has been suggested that reduced bicarbonate secretion through CFTR alters the pH on the airway surface which may affect airway defense mechanisms {Stoltz, 2015 1773 /id} and alters mucus properties {Shamsuddin, 2014 1774 /id}.

Approximately 2000 mutations/gene alterations in the *CFTR* gene have been described {2016 1786 /id} and the “*Clinical and Functional Translation of CFTR (CFTR2)*” initiative aims to identify and classify the disease causing mutations{2016 1787 /id} . To date mutations have been grouped into six classes based on the effect they have on CFTR expression and function (**Table 1**). The genotype/phenotype correlation for pancreatic disease is very strong with class I-III mutations generally leading to pancreatic insufficiency and class IV and V mutations being associated with pancreatic sufficiency {Wang, 2014 1675 /id}. In the lung the genotype/phenotype correlation is less strong likely because environmental factors, treatment compliance and modifier genes significantly contribute to disease severity {Green, 2012 1775 /id}. The most common mutation, F508del (previously known as ΔF508), accounts for approximately 70% of CF alleles world-wide (although the exact frequency is population dependent) and is an example of a Class II processing-defect mutation {Wang, 2014 1675 /id}.

Over the past decades, advances in patient care have led to significant improvements in survival in the developed world; latest data from the UK CF Registry show that the median age of survival is now ~ 40 years {2015 1614 /id}. However, the treatment burden is high and patient compliance and adherence to treatment remains a significant problem {Riekert, 2015 1776 /id}.

Most recently the improved understanding of CF pharmacogenetics has led to licensing of drugs that correct the molecular defect of certain CFTR mutations {Wang, 2014 1675 /id}. Kalydeco (also known as ivacaftor or VX770) targets class III gating defect mutations (which are present in ~4% of patients). The drug leads to rapid and very significant improvements in lung function, weight gain and reduced pulmonary exacerbations {McKone, 2014 1777 /id}. Orkambi (a combination drug consisting of ivacaftor and lumacaftor, which targets the processing defect of class II mutations) has achieved modest improvements in lung function and, maybe more significantly, reduced pulmonary exacerbations in F508del homozygotes (but not compound heterozygotes) {McKone, 2014 1777 /id}. The results of these trials has led to licensing of Orkambi by the FDA andEuropean agencies. The search for more potent drugs to combat CF lung disease, therefore, has to continue. In contrast to the small molecule drugs described above, which generally have only been tested in primary airway epithelial cells, before progressing into trials, the assessment of gene transfer agents, which are biologics, require an intact organism to assess immunological and inflammatory effects, as well as extra- and intracellular barrier to pulmonary gene transfer.The use of pre-clinical animal models can, therefore, not be bypassed.

**2. The history of CF gene therapy**

The *CFTR* gene was identified in 1989 and in the following decade CF gene therapy was at the forefront of gene therapy research in academia and industry (see **Table 2** for key publications), due to being a monogenic disease with insufficient treatment options and having a target organ that is non-invasively accessible. However, gene transfer to the lung proofed to be far more challenging than initially anticipated, causing interest to wane and many academics and industry to chase easier disease targets. After the turn of the century CF gene therapy was pursued by only a small, but highly motivated, number of groups and important progress (see below) has recently been made.

The older literature related to CF gene therapy has been reviewed in many publications (see for example {Griesenbach, 2015 1778 /id}) and we will, therefore, restrict this review to a brief description and discussion of the key lessons learnt, instead focusing the review on more recent progress in the field.

 3. **Key lessons learnt**

*3.1. The lung is a difficult target organ*

As mentioned above the lung was initially, and naively, classified as an “easy” non-invasively targetable organ. However, this was rapidly disproven. CF gene therapists had to learn painfully that intra- and extracellular barriers that have evolved to protect us from viruses, bacteria and other inhaled particles also “protect” against inhalation and uptake of gene transfer agents (GTAs). Extracellular barriers include airway mucus and mucociliary clearance, which evolved to trap and remove inhaled particles as well as humoral and cellular immune responses, evolved to combat inhaled microorganisms (see {Xia, 2014 1779 /id} for more detailed discussion). In addition, sputum, which is a hallmark of CF lung disease, presents an additional extracellular barrier, particularly in older patients. {Stern, 2003 166 /id;Yonemitsu, 2000 35 /id;Schuster, 2014 1701 /id}

In a clinical setting it would presumably, therefore, be preferable to give gene therapy to younger patients as a preventative treatment, although in reality it is neither ethical nor practical to treat such patients in early phase clinical trials.

*3. 2. Adenoviral vectors are currently not suitable for CF gene therapy*

Adenoviruses have a natural tropism for the lung and it is, therefore, not surprising that adenoviral vectors were the first gene transfer agent to be used in CF clinical trials. Only four years after cloning of the *CFTR* gene Zabner *et al* conducted the first ever CF gene therapy trial. Increasing doses of an adenoviral vector carrying the *CFTR* cDNA (2e6, 6e6 or 5e7 infectious units, n=1 patient/dose) were delivered to the nasal epithelium of three CF patients. Despite using a comparatively small amount of virus the authors suggested that the treatment corrected the CF ion transport defect. A further 8 trials administering adenovirus either to the lung or nose of CF patients were performed between 1993 and 2001 and did not consistently show evidence of efficient gene transfer {Zabner, 1993 223 /id;Crystal, 1994 224 /id;Knowles, 1995 225 /id;Hay, 1995 226 /id;Zabner, 1996 227 /id;Bellon, 1997 228 /id;Harvey, 1999 70 /id;Zuckerman, 1999 229 /id;Joseph, 2001 130 /id;Perricone, 2001 230 /id}. In addition, a pivotal trial conducted by Harvey *et al* showed that transduction efficiency of the vector further reduces after repeated administration {Harvey, 1999 70 /id}. To date the research community largely agrees that (a) adenoviral vectors are comparatively inefficient in the human lung because the receptor required for virus entry into airway epithelial cells is located on the basolateral membrane and therefore difficult to access after topical administration and that (b) acquired immune responses to the virus prevent efficacy on repeated administration, a problem which has not yet been overcome despite many attempts at transiently inhibiting humoral and cellular immune responses and the generation of helper-dependent “gutted” adenoviral vectors in which all viral genes have been deleted.

*3.3. Adeno-associated vectors are also unlikely to be suitable for CF gene therapy*

In the 2000s, adeno-associated virus (AAV) began to supercede adenoviral vectors. Between 1999 and 2007 an AAV2 vector was assessed in six CF clinical trials using either the nose, maxillary sinus or lung as the target organ. In the pivotal trial ~50 CF patients received 3 doses of AAV2-CFTR 30 days apart {Moss, 2007 141 /id}. The trial did not meet its primary endpoint of statistically significant improvement in lung function and the AAV2 CF programme was stopped. Several reasons may explain the lack of efficacy: (a) We now know that the AAV2 serotype is comparatively inefficient in transducing airway epithelial cells, (b) the limited packaging capacity of AAV (~5 kB) meant that the 4.7 kB *CFTR* cDNA barely fits and led to the use of a weak AAV2 promoter which is located in the long-terminal repeat (LTR), (c) efficacy on repeated administration was likely affected by the induction of humoral and cellular immune responses to the virus, (d) two doses delivered 30 days apart followed by assessment of lung function 30 days after the second dose may have been too short to improve a period or stabilise lung function.

Several of the factors that may have limited efficacy in the AAV2 trial are beginning to being addressed.

a. Serotypes that are more efficient in transducing airway epithelial cells have been identified using pre-clinical models {Limberis, 2006 91 /id;Zabner, 2000 1771 /id}, but their efficacy remains to be proven in man.

b. Strategies have been developed to package the *CFTR* cDNA and appropriate regulatory elements into AAV. For example a truncated CFTR construct devoid of the regulatory R domain (CFTR/R) has been shown to retain the ability to transport chloride {Ostedgaard, 2002 1772 /id}. However the R-domain is a central part of the protein and it is currently unclear whether all other function of CFTR are retained by CFTR/R; at face value deletion of a central part of the protein appears a risky strategy. Other approaches are based on dividing the *CFTR* cDNA into two AAV construct s which when both transcribed in the same cell may trans-splice and reconstitute a full length *CFTR* mRNA {Song, 2009 317 /id}. This approach adds another layer of complexity which is likely to significantly impact on efficiency of CFTR expression and function. Neither of these strategies has been assessed in trials yet.

c. The impact of pre-existing and acquired immune responses to AAV has been recognised as a significant challenge in diseases such as haemophilia B. Only patients without pre-existing neutralising antibodies to AAV8 were included in the trial {Nathwani, 2014 1703 /id} and the induction of cellular and humoral immune responses after intravenous AAV8 injection was clearly demonstrated.

Although AAV-mediated gene expression in the lung persists for prolonged periods of time {Vidovic, 2016 1780 /id} and AAV appears to be able to transduce lung progenitor cells {Liu, 2009 269 /id} the need for repeated vector administration to the lungs of CF patients remains. The literature reporting efficacy of repeated administration of AAV to the lung is conflicting. The AAV2 trial mentioned above lacked efficacy on repeated administration. These data were corroborated by a pre-clinical study which showed that transduction efficiency of AAV 5 was markedly reduced after the second administration and effectively abolished after the third dose {Sumner-Jones, 2007 239 /id}. In contrast, Limberis *et al* and Vidovic *et al* state that repeat administration of AAV2/9 and AAV2/5 is feasible in mouse models. However, it is important to note that both studies only assessed two doses and that at least in the latter study gene expression of the second dose was ~1 log lower than after the first dose {Vidovic, 2016 1780 /id;Limberis, 2006 91 /id}. The lack of convincing pre-clinical data and the clinical data available from other diseases lead us currently to suggest that repeat administration of AAV to the lung may not be feasible unless the development of humoral and cellular immune responses to the vector can be prevented. Further, studies that assess efficacy after repeated administration, should have undertaken three or more administrations.

*3.4. Lentiviral vectors are currently the most promising viral vectors for CF gene therapy*

Lentiviral vectors are able to transduce dividing and non-dividing cells and may, therefore, be suitable for targeting differentiated cells in the lung. Several groups have investigated lentiviral vectors for airway gene transfer. Although integrating vectors have an inherent risk of inducing insertional mutagenesis, it is important to discriminate between the early gamma oncoretroviral vectors that have been shown to cause leukemia in some recipients when used for bone marrow transduction {Rivat, 2012 1781 /id}, from the more advanced lentiviral vectors that have not shown evidence of insertional mutagenesis in clinical trials {Aiuti, 2013 1708 /id;Biffi, 2013 1559 /id;Palfi, 2014 1706 /id}.

Lentiviral vectors have no natural lung tropism and, therefore, require pseudotyping with appropriate envelope proteins to facilitate lung gene transfer. The vesicular stomatitis virus G (VSV-G) protein is commonly used for pseudotyping lentiviral vectors and works well for bone marrow transduction *ex vivo*. However, for transduction of airway epithelium it is necessary to pre-condition the tissue with detergents which damage the epithelium and allow access to the basolateral membrane via intercellular spaces {Cmielewski, 2010 364 /id}. This approach raises safety concerns for translation into clinical trials, particularly in CF patients with chronic lung infections. As a result, several groups, including our own, have investigated the use other envelope proteins including the baculovirus protein GP64 {Sinn, 2012 1555 /id}, proteins from Ebola or Marburg filoviruses {Kobinger, 2001 282 /id}, the HA protein from influenza virus {Patel, 2013 1554 /id}and the F and HN protein from Sendai virus {Kobayashi, 2003 160 /id;Mitomo, 2010 339 /id;Griesenbach, 2012 1768 /id}, which are viruses that either have a broad tissue tropism (baculovirus) or a natural tropism for the lung (influenza and Sendai virus). We, and others, have shown that a single dose of lentivirus leads to life-long stable gene expression in the lung (~ 2 years) and that repeated administration of the vector (10 daily doses or three times one monthly doses) is feasible {Mitomo, 2010 339 /id;Griesenbach, 2012 1768 /id;Sinn, 2012 1555 /id}. To date there has been no report of insertional mutagenesis or other untoward toxicity in lungs of mice. A direct comparison between the lead non-viral vector GL67A which was used in a recently completed Phase IIb CF gene therapy trial (see below) and the F/HN-pseudotyped lentiviral vector indicates that the virus is several log orders more efficient in transducing airway epithelial cells, which are the target cells for CF gene therapy. In addition to the envelope proteins optimisation of promoter/enhancer elements that drive recombinant protein expression require careful optimisation. We have recently shown that the hCEF regulatory element, consisting of the elongation factor 1α promoter coupled to the human CMV enhancer leads to highest levels of gene expression in murine lungs and human air liquid interface cultures (manuscript in preparation). The efficiency, duration of expression, lack of toxicity and uniquely efficacy on repeated administration support progression of the F/HN-pseudotyped lentivirus into a first-in-man phase I/IIa CF clinical trial in mid-2017.

We have also investigated this vector for the production of secreted proteins. Alpha-1-antitrypsin is an inhibitor of neutrophil elastase, a protease implicated in progressive destructive lung damage seen in late-stage cystic fibrosis and chronic obstructive pulmonary disease (COPD) {de, 2014 1782 /id}. We have recently shown that F/HN-pseudotyped lentivirus can produce therapeutic levels of secreted alpha-1-antitrypsin in the lungs of mice two years after a single vector administration (manuscript in preparation). Interestingly, we have also shown that this lentiviral vector administered to the lung results in secretion of proteins into the serum, suggesting that the lung could be used as a ‘factory’ for the production of secreted proteins for diseases such as haemophilia (manuscript in preparation).

*3.5. Non-viral gene therapy can slow progression of CF lung disease*

Between 1999 and 2004 nine CF gene therapy trials used non-viral gene transfer agents (GTAs) commonly administering a single dose to the nasal epithelium which was used as a surrogate to allow easier assessment of gene transfer (reviewed in {Griesenbach, 2015 1778 /id}. The first phase I/IIa single dose lung trial was reported by Alton *et al* in 1999 using the cationic lipid formulation GL67A {Alton, 1999 4 /id}. Combined, these studies presented a scattered picture with some studies detecting vector-specific mRNA and some partial correction of the chloride transport defect, whereas other did not. Similar to the viral gene therapy trials described above the non-viral studies also highlighted that gene transfer in the CF lung is comparatively inefficient. However, three arguments warranted further development of, and investment in, a non-viral gene therapy programme for CF. (1) In contrast to viral vectors the simpler composition of non-viral GTAs is less likely to lead to activation of the immune system. Proof-of-concept for efficacy of repeated administration (3 doses) of a non-viral vector to the nasal epithelium of CF patients was established by Hyde *et al* {Hyde, 2000 39 /id}. (2) Pre-clinical studies, as well as the lack of lung disease in patients with *CFTR* mutations that retain a small amount of residual CFTR expression support the argument that a small amount of CFTR expression after gene therapy may be sufficient to improve or stabilise lung function (see below for more in depth discussion). (3) The molecular efficacy endpoints (vector-specific mRNA and correction of ion transport) which had been used in previous single-dose trials may not predict the effect of gene transfer on lung disease severity (see below for more in depth discussion).

In response to an initiative by Rosie Barnes (then the CEO of the CF Trust) the UK CF Gene Therapy Consortium was founded in 2001, consisting of the three groups in Oxford, Edinburgh and London who had previously conducted CF gene therapy trials, with the explicit aim to share expertise and funding to assess whether repeated administration of a non-viral gene transfer agent can change the progression of CF lung disease. Our translational research pathway (which is briefly summarized below but reviewed in more detail in {Griesenbach, 2015 1778 /id} led to a recently completed Phase IIb multi-dose trial (see below). In brief:

a. Following an extensive screening programme we determined that the cationic lipid formulation GL67A, first used in the 1990s, remained the most potent GTA for airway gene transfer some two decades later {McLachlan, 2011 518 /id}.

b. First generation plasmids used in previous trials contained a large number of immune-stimulatory CpG dinucleotides which may have contributed to the mild flu-like symptoms noted in the previous two single dose lung trials {Alton, 1999 4 /id;Ruiz, 2001 63 /id}. We improved the plasmid (termed pGM169) by removing the CpG islands, codon-optimised the *CFTR* cDNA and generated the novel regulatory element, hCEFI, consisting of the elongation factor 1α promoter coupled to the human CMV enhancer {Hyde, 2008 241 /id}.

c. Regulatory-compliant multi-dose toxicology studies were performed in mice {Alton, 2014 1587 /id} and sheep {Alton, 2013 1588 /id} and supported progression into a multi-dose clinical trial. Interestingly, repeated aerosolisation of pGM169/GL67A to mice led to dose-related expression showing a cumulative effect on repeat dosing reaching 94±19% of endogenous murine Cftr levels after 12 deliveries (**Figure 1**). Thus, these data further supported progression into a multi-dose clinical trial.

d. A single dose, dose-escalation (5, 10 and 20 ml of pGM169/GL67A) Phase I/IIa safety trial showed that despite CpG-depletion of the plasmid, patients receiving the 10 and 20 ml dose still developed mild flu-like symptoms including a fever {Alton, 2015 1783 /id}. The likely explanation is that both volume administered to the lung, as well as CpG sequences contribute to the inflammatory response. The 5 ml dose (containing ~12.5 mg plasmid DNA) was therefore chosen for the multi-dose trial.

e. The multi-dose trial was double-blinded and placebo-controlled. Patients (12 years or older with moderate or mild lung disease) received 5 ml of nebulised pGM169/GL67A or 5 ml 0.9% saline every month for 12 months. The primary endpoint was a change in lung function measured as a relative change of % predicted forced expiratory volume in one second (FEV1). Data from 116 patients (who received 9 or more doses) was analysed {Alton, 2015 1769 /id}. The treatment was well tolerated and, excitingly, the trial met its primary endpoint showing a stabilisation of lung disease severity after gene therapy (**Figure 2a)**. Pre-specified subgroup analysis showed that patients with more severe lung disease at the start of treatment responded better than patients with milder lung disease at the start of treatment (**Figure 2b+c**). Reasons for this are currently unknown and various hypotheses have to be tested. One simple explanation may relate to the amount of material deposited in the bronchial airways, which is likely higher in patients with more severe lung diseases due to sputum restricted diffusion into the smaller airways.

Importantly, this trial showed for the first time that the significant effect on lung function was not supported by robust changes in the molecular assay (detection of vector-specific mRNA and correction of ion transport). This discordance may relate to assay sensitivity, the site of measurement and/or the relatively small area of airways assessed when using molecular assays and further questions the use of these assays as go-no-go decision points in the development of CF gene therapy.

The outcome of the trial raises a number of questions.

*a. Could the dose be increased?*

We now know that the 5 ml dose is well tolerated when administered repeatedly and a follow on trial might include a higher dose.

*b. Was the right dosing-interval chosen?*

Although animal studies have shown that gene expression persists for more than a month, it is conceivable that more frequent administration may further increase efficacy. However, moving from monthly to fortnightly or weekly dosing will significantly increase the treatment burden and may affect patient recruitment.

*c. Was the right placebo used?*

We are frequently asked to justify the use of saline as placebo. It is important to first consider the alternatives. Lipid only as a placebo is a poor choice because charge, pH, tonicity and chemical composition are very different compared to lipid/DNA complexes. The alternative could have been to use an empty plasmid or a plasmid carrying a mutant CFTR sequence. However, both these strategies are risky as we would not be able to rule out expression of an immunologically active peptide or novel non-coding RNA molecule with deleterius biological functions. For these reasons, ethical considerations (multiple doses of plasmid DNA/liposome had not previously been administered to CF patients) and pragmatic financial issues, we decided that 0.9% saline, which has not been shown to negatively affect lung function, was the best choice.

*d. What is the best primary endpoint?*

Spirometry is a variable and effort dependent measurement and, therefore, less than ideal. However, we spent approximately 2 years studying the longitudinal progression of countless validated and more novel markers of disease severity in about 200 patients (“Run-in” Study, manuscript in preparation) and were unable to identify a more advantageous endpoint.

*e. Were the right patients selected for the trial?*

The outcome of the subgroup analysis comparing patients with better or more severe lung function at the start of treatment was certainly surprising. If possible, future trials should ensure that subgroup analysis is sufficiently powered, rather than preferentially including patients with poorer lung function.

**4. Other outstanding questions**

*4.1. How much CFTR expression do we need?*

As mentioned above patients with certain “mild” CF mutations who retain approximately 10% of residual CFTR expression per cell do not suffer from lung disease, although other organs are affected {Chu, 1993 389 /id}. *In vitro* cell mixing experiments have shown that ~10% of non-CF cells restore CFTR-mediated chloride secretion when mixed with 90% of CF cells {Farmen, 2005 71 /id}. In a separate study it was shown that CFTR has to be expressed in at least 25% of cells grown in a monolayer to restore mucus transport {Zhang, 2009 331 /id}. However, these studies do not address whether complete correction of CFTR expression in 10% cells is equivalent to a low level (~10%) of CFTR expression in all cells. We currently also do not know whether gene therapy is more likely to achieve the former or the latter.

*4.2. Which cells do we need to target?*

CFTR is expressed in various cell types in the lung including submucosal glands, ciliated epithelial cells and goblet cells. It is currently unknown which cells express the CFTR transgene following gene transfer. However, in our opinion it is unlikely that gene transfer agents when applied topically to the airways transfect submocosal glands whose ducts are filled with mucus. As mentioned above it has been postulated that CFTR is also expressed in macrophages and neutrophils. Whether this leads to an intrinsic defect in host defense in the CF lung is still widely debated {Stoltz, 2015 1773 /id} and needs to be resolved before deciding whether gene replacement in inflammatory cells may lead to therapeutic benefit.

*4.3. Should studies in CF models form a go-no-go decision point before progression into clinical trials?*

CF mice do not acquire spontaneous airway infections or develop CF lung disease, but the nasal epithelium shows the characteristic CF chloride and sodium transport defects {Griesenbach, 2008 246 /id}. However, the relevance of measurement of CFTR function in the murine nose (via *in vivo* potential difference) has been called into question by Ostrowski *et al* who showed that expression of human CFTR under the transcriptional control of a cilia-specific promoter did not correct ion transport in CF knockout mice {Ostrowski, 2007 205 /id}. In addition Grubb *et al* have suggested that the olfactory, rather than the respiratory, nasal epithelium mainly contributes to the ion transport defect in CF mice {Grubb, 2009 322 /id}. We have also shown that the CF mouse is of limited value as a stepping stone to human gene therapy trials. Although GL67A-mediated CFTR gene transfer partially corrected chloride transport in the human lung, we were unable to correct a panel of CFTR-specific endpoint assays in the murine nose, including ion transport, periciliary liquid height, and *ex vivo* bacterial adherence {Griesenbach, 2010 344 /id}. Our data are also consistent with an earlier study by Jiang *et al* {Jiang, 1998 62 /id}, who showed that GL67A-mediated gene transfer did not lead to correction of the ion transport defect in CF mice and our own report of successful correction of chloride transport in the human, but not in the murine, nose after transfection with DC-Chol/DOPE {Caplen, 1995 234 /id;Alton, 1993 6 /id}. Taken together these data suggest that the CF-knockout mouse may not be a representative model in which to assess gene transfer efficiency to human airway epithelial cells and that correction of ion transport in mice should not be used as a go-no-go decision point for progression into clinical trial.

Whether correction of lung disease in CF knockout pigs or ferret is a better model remains to be seen. Currently, these animals die shortly after birth due to intestinal disease and, therefore, are not yet available in large enough numbers to conduct meaningful studies. In addition, these models will only be useful if transduction efficiency of viral vectors is not affected by species-specific differences in transduction efficiency. Finally, whether the CF-like pathology is a close enough mimic of human disease to be used as a critical decision point for therapeutic development, is still unclear.

*4.4. Is there a role for genome editing in CF?*

Genome editing using Zinc finger nucleases, TALENS or the CRISPR/Cas9 system are revolutionising research and therapeutic strategies for many diseases. In the context of CF, genome editing has been used to repair CFTR mutations and restore protein function in cell lines {Lee, 2012 1713 /id}, intestinal organoids {Schwank, 2013 1711 /id} and CF patient-derived induced pluripotent cells (iPS) *in vitro* {Crane, 2015 1784 /id;Firth, 2015 1785 /id}. This technology will be very useful for developing mutation-specific cell lines for drug screening.

Wong *et al* have developed protocols to allow differentiation of human pluripotent stem cells into mature airway epithelium expressing functional CFTR protein {Wong, 2012 1532 /id} which may lead to speculation that genome edited iPS cells could be differentiated into airway epithelial cells or airway progenitor cells, which could then be used to replenish cells in the CF lung. However, there are currently no successful reports of cell therapy in the lung due to the structural complexity of the organ.

The technology may also lead to proposals for genome editing of airway epithelial cells *in vivo*. However, there are several flaws with this. First, genome editing *in vivo* will require the delivery of the genome editing molecules as well as appropriate wild-type CFTR sequences required for homologous recombination-based repair of the mutant CFTR, which is yet more complex than standard gene therapy. Second, airway epithelial cells are terminally differentiated and do not undergo homologous recombination. Third, one might argue that *in vivo* genome editing of airway progenitor cells may be an alternative. However, these cells are buried beneath the surface epithelium and difficult to access with gene therapy vectors. In addition airway progenitor cells only divide very slowly, again making homologous recombination inefficient.

**5. Conclusions**

25 years of CF gene therapy have opened our eyes to strength and weaknesses of the available viral and non-viral gene transfer agent. After a 10 year lull, interest in CF gene therapy is now on an upward slope due to (a) proof-of-concept that the molecular defect is targetable using small molecule drugs, (b) the successful completion of a non-viral Phase IIb multi-dose trial showing that gene therapy can alter the progression of CF lung disease and (c) the success of gene therapy in other diseases including immune deficiencies and haemophilia.

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**6. Expert Opinion**

***What are the key findings and weaknesses in the research done in this field so far?***

The recent demonstration that non-viral gene therapy can alter CF lung disease progression has been a mile stone. One may also argue that too much time and effort has been spent on too many Phase I/IIa single dose trials assessing molecular surrogates. In addition, it has taken too long to acknowledge that most viral vectors are not suitable for CF gene therapy due to pre-existing and acquired immune responses. Lentiviral vectors are the exception and look promising in pre-clinical studies

***What are the key lessons for the industry?***

CF gene therapy is not a “low hanging fruit”, but a rather challenging target disease. Trials aimed at improving lung function are long and complex. This is, in part, due to the fact that lung function measurements are variable and good biomarkers are lacking.

***What potential does this research hold?***

Gene therapy will be suitable for all patients, regardless of what mutation class they carry. In addition, once successful, CF gene therapy will have smoothed the path for other lung diseases. Interestingly, the lung can also be used as “factory” for production of systemic proteins, which are released from the lung into the blood.

***What is the ultimate goal in this field?***

The ultimate goal is to develop a gene therapy-based treatment that will prevent lung disease. However, an intermediate goal is to reduce the burden of treatment, and increase both the quality and importantly life expectancy in CF patients worldwide.

***What research or knowledge is needed to achieve this goal and what is the biggest challenge in this goal being achieved?***

Successive Phase IIb studies focused on clinically relevant endpoints are needed to further improve efficacy. The development of new surrogate endpoints and biomarkers and their validation are urgently required. In addition, production methods for manufacturing of clinical grade lentiviral vectors in quantities sufficient for aerosol administration to the lung are urgently required. Funding is also a significant bottle neck. CF Phase IIb studies have to be large and run for months to assess effects on lung disease severity and are, therefore, expensive. In addition, production of viral and non-viral gene transfer agents is currently expensive. This, in part, explains why many academics and industry stopped to work on CF gene therapy, after realising that the lung is a more difficult target than initially anticipated. However, the recent success in CF and other disease indications, have significantly increased interest and enthusiasm in the field.

***Where do you see the field going in the coming years?***

A follow-on Phase IIb study to further improve efficacy of pGM169/GL67A should be initiated. In parallel proof-of-concept studies for lentivirus-mediated gene transfer in human airways will be conducted. Efficacy of lentivirus repeat administration will be assessed in CF patients

***Is there any particular area of the research you are finding of interest at present?***

Yes, the immense potential of lentiviral vector for a large range of diseases is very exciting and has be explored.

***How will this research on rare diseases and/or orphan drugs impact the management and treatment of patients in the long term?***

Gene therapy may improve life-expectancy in CF and reduce the overall treatment burden, thereby increasing quality of life. In addition, gene therapy may be suitable to effectively treat CF patients in less developed and under-developed nations perhaps allowing these countries to leap-frog the establishment of symptomatic treatments, which currently form the cornerstone of treatment in more developed communities.

**Article highlights**

* The success of the CFTR potentiator Kalydeco in patients with a class III gating mutations has provided proof-of-concept that the molecular defect in CF is drug targetable.
* The Phase IIb trial conducted by the UK CF Gene Therapy Consortium has, for the first time, shown that gene therapy can alter lung disease severity, but the effect needs to be enhanced before Phase III trials can commence
* In contrast to adenoviral and adeno-associated virus-vectors, lentiviral vectors retain efficacy on repeat administration in pre-clinical models.
* The first lentivirus CF trial will start in summer 2017

**Table 1: CFTR Gene Mutation Classes**

|  |  |  |
| --- | --- | --- |
| **Class** | **Effect on Protein** | **Key examples of disease causing mutations** |
| I | Defective protein production | G542X |
| II | Defective protein processing | F508del |
| III | Defective protein regulation | G551D |
| IV | Defective protein conductance | R117H |
| V | Reduced protein synthesis | A455E |
| VI | Reduced protein surface retention | c.120del23 |

CFTR=cystic fibrosis transmembrane conductance regulator gene. Of note: a large number of known CFTR mutations are currently unclassified with respect to mutation class.

**Table 2: Gene therapy milestone studies in the first decade after cloning the CFTR gene**

• Only one year after the cloning of CFTR was published Drumm *et al* established proof-of-principle that retrovirus-mediated gene transfer of CFTR can correct cAMP-mediated chloride conductance *in vitro* {Drumm, 1990 9 /id}.

• Three years after cloning of CFTR Rosenfeld *et al* provided evidence of successful *CFTR* mRNA and protein expression after adenovirus-mediated CFTR cDNA transfer into cotton rats {Rosenfeld, 1992 504 /id}.

• Four years after cloning of CFTR Hyde *et al* showed that non-viral CFTR cDNA transfer was able to partially correct the chloride transport in tracheal epithelium of CF knockout mice {Hyde, 1993 3 /id}.

• In the same year Zabner *et al* performed the first, albeit small and not placebo controlled, CF gene therapy trial in 3 patients. A first generation adenoviral vector carrying the *CFTR* cDNA was administered to the nasal epithelium and shown to partially restore cAMP-mediated chloride transport {Zabner, 1993 223 /id}.

• Five years after cloning of CFTR Crystal *et al* performed the first phase 1 dose-escalation CF gene therapy study. This was first and foremost a safety study and showed transient inflammatory responses at the highest dose (5x1e9 plaque forming units (PFU)/patient) {Crystal, 1994 224 /id}.

• Six years after cloning of CFTR Caplen *et al* provided first evidence that a non-viral gene transfer agent (DC-Chol:DOPE) complexed with *CFTR* cDNA could partially correct cAMP-mediated chloride transport in the nasal epithelium of CF patients {Caplen, 1995 234 /id}.

• Ten years after cloning of CFTR Alton *et al* demonstrated that a non-viral gene transfer agent (GL67A) complexed with a plasmid DNA carrying the *CFTR* cDNA could partially correct cAMP-mediated chloride transport in the lungs of CF patients {Alton, 1999 4 /id}.

• 26 years after cloning of CFTR Alton *et al* demonstrated that repeated administration of GL67A complexed with a plasmid DNA carrying the *CFTR* cDNA significantly, albeit modestly, stabilised lung function in CF patients {Alton, 2015 1769 /id}

**Figure legends**

**Figure 1: Repeated administration of pGM169/GL67A in mice**

Mice (n=10/group) received one, six or 12 deliveries of aerosolised pGM169/GL67A or air only. One day after the last treatment pGM169 mRNA was quantified in lungs using QRT-PCR. Data are expressed as a percentage of vector-specific pGM169/endogenous murine Cftr mRNA (\*\*\*=p<0.001 compared to control). pGM169 mRNA was detectable in a small number of air control animals (2/30) due to sample contamination during harvesting, processing or analysis, a common problem associated with PCR-based mRNA detection methods. This finding did not affect interpretation of the results. Each symbol represents one animal and the group median is shown as a horizontal bar. PBNQ=positive, but not quantifiable because lower than the standard curve.

**Figure 2: Stabilisation of lung function after repeated administration of the non-viral formulation pGM169/GL67A**

Cystic fibrosis patients were treated monthly for twelve months with either active drug or the placebo. Lung function (FEV1=forced expiratory volume in 1 second) was measured at each treatment visit before administration of study drugs. Data are expressed as relative change from baseline in % predicted FEV1. Error bars show the standard error of the mean. (**A**) All patients. There was a significant, albeit modest, treatment effect in the pGM169/GL67A group versus placebo at 12 months’ follow-up (3·7%, p=0·046). (**B**) Patients with “bad” lung function at start of treatment (Baseline FEV1 = 50-70%), (**C**) Patients with “good” lung function at start of treatment (Baseline FEV1=70-90%). The figure is adapted from [53] as part of a CCBY license.

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\* of importance: This study showed that repeat administration of adenoviral vectors to the CF lung is not feasible

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