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Progress towards gene therapy for cystic fibrosis

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A decade ago it was widely anticipated that cystic fibrosis would be one of the first diseases to be treated by gene therapy. The difficult hurdle of cloning the responsible gene had been accomplished, its function was established and the lung appeared readily accessible for gene replacement. Since the first clinical trials for cystic fibrosis lung disease in the early 1990s it has become increasingly apparent that successful lung-directed gene therapy is significantly more complex than was first envisioned. Numerous obstacles including vector toxicity, inefficient transgene expression and limited vector production have delayed progress. An increased understanding of vector biology and host interaction has led to the development of novel strategies to enhance the efficiency and selectivity of gene delivery to the lung. Although significant challenges remain, there is now a realistic prospect of a clinically effective treatment in the next 10 years.

Keywords: adeno-associated virus, adenoviral, animal model, cystic fibrosis, gene therapy, liposome

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1. Introduction

Cystic fibrosis (CF) is the most common recessively inherited lethal disease among the Caucasian population, affecting around 1 in 2500 newborns with a median life expectancy of over 30 years. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a chloride channel situated on the apical surface of epithelial cells [1]. The mechanism by which defective CFTR leads to severe lung disease in CF has recently become clearer [2,3]. Abnormal respiratory epithelial ion transport appears to cause depletion of airway surface liquid volume, delayed transport of concentrated mucus and impaired bacterial clearance. This process stimulates airway inflammation and predisposes to repeated and chronic bacterial infection leading to persistent lung injury and progressive bronchiectasis, eventually causing death from respiratory failure in more than 90% of patients.

In theory, CF is an ideal disorder for lung-directed gene therapy. CF is a monogenic disease, heterozygotes appear to be phenotypically normal, expression of CFTR is normally low (1 – 2 mRNA copies per cell) and the target epithelial cells lining the airways are directly accessible via noninvasive, clinically available techniques (aerosol, nebulisation).

The feasibility of CF gene therapy became apparent when the *CFTR* gene was cloned in 1989 [4-6]. This was rapidly followed by studies demonstrating proof of principal for *CFTR* gene transfer *in vitro* and in CF knockout mice [7,8].

So far, 30 clinical trials have been published, using three main gene transfer agents (GTAs): adenovirus, adenoassociated virus (AAV) or various cationic liposomes [101]. The results have generally been disappointing mainly due to transient and low level expression of the CFTR transgene.

Successful gene therapy for CF will necessitate efficient delivery of CFTR transgenes to the respiratory epithelium, transfection of appropriate resident cells and expression of biologically relevant products. Although these interlinked aspects have

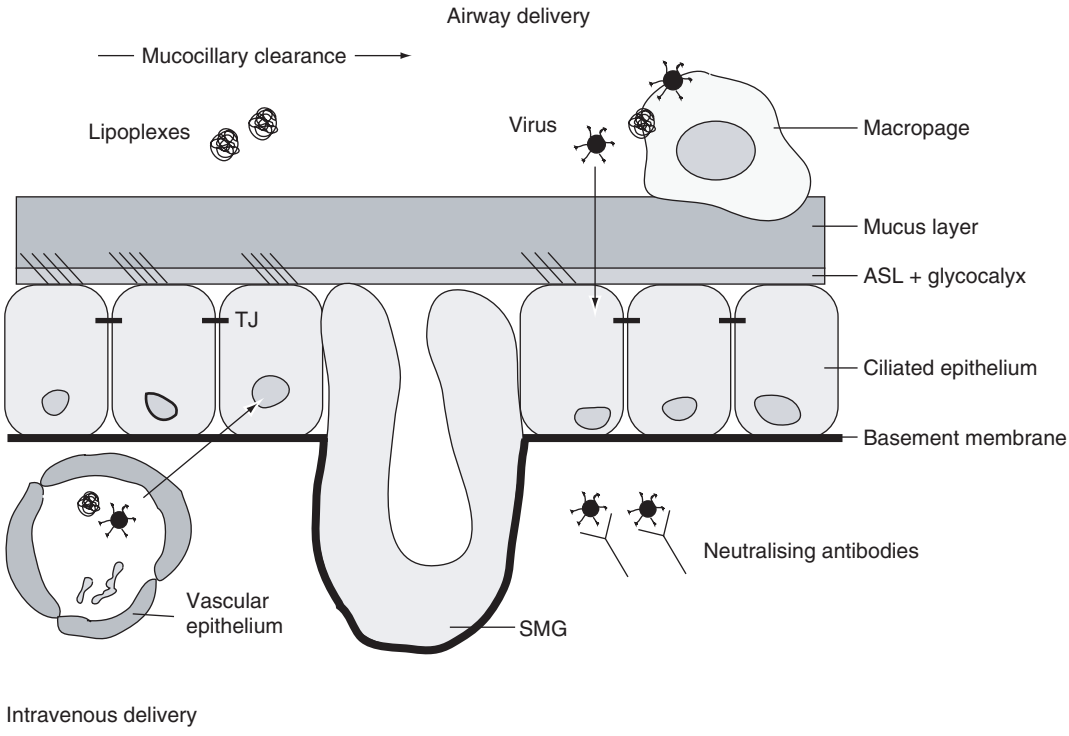

Figure 1. Extracellular barriers to airway gene therapy. Vectors delivered to the airway lumen encounter several obstacles including the mucous layer, mucociliary clearance, scavenging macrophages and the glycocalyx. Following intravenous delivery the vascular endothelium and the basement membrane of the epithelial cells are the main barriers.
ASL: Airway surface liquid; SMG: Submucosal gland; TJ: Tight junction.

been the subject of concerted research over the past 10 years, significant obstacles have become apparent and the promise of gene therapy for CF as a practical reality has yet to be realised.

This review will focus on the current barriers to successful lung-directed gene therapy and the vector engineering strategies aimed at overcoming them. The progress in preclinical animal model testing and pertinent clinical trials will also be covered.

2. Barriers to airway gene therapy

If CF gene therapy is to be effective, GTAs need to overcome a number of extra- and intracellular barriers to efficiently deliver the therapeutic transgene to the nucleus of the target cells.

2.1 Extracellular barriers

The human respiratory tract, and the CF airway in particular, represents a formidable challenge to the success of lung-directed gene therapy [9]. Following any method of airway delivery a GTA must get past a number of innate defence mechanisms specifically evolved to prevent the entry of foreign particles (**Figure 1**). The mucous layer lining the airways is a natural barrier against viral and bacterial infection that can trap vectors, which are then removed by mucociliary clearance or engulfed by macrophages. Beneath the mucous layer the complex glycocalyx (composed of glycoproteins, glycolipids and proteoglycans) can physically obstruct vectors and prevent binding to cell surface receptors. Finally, if vectors reach the apical cell membrane there is a paucity of viral and growth/tropic receptors to facilitate cell entry. Epithelial tight junctions further prevent vectors from reaching the basolateral membrane where receptors are present in greater abundance.

In the CF airways this situation is exacerbated by heightened inflammation and excess viscid, purulent secretions, containing cellular debris: notably DNA and actin that can alter the integrity of a lipoplex and limit vector access to the airway epithelium. These extracellular barriers seem to have a greater effect on transfection with synthetic vectors than with viruses, but may be helped by the use of mucolytic agents or recombinant DNase [9].

2.2 Intracellular barriers

The intracellular trafficking of plasmid DNA and synthetic vectors is a complex and major barrier to gene therapy, which has recently been reviewed in detail [10]. Briefly, vectors can enter cells either by charge-mediated interactions with proteoglycans on cell membranes or by receptor-mediated endocytosis by ligand–receptor binding interactions. Both methods result in uptake into vesicular compartments that

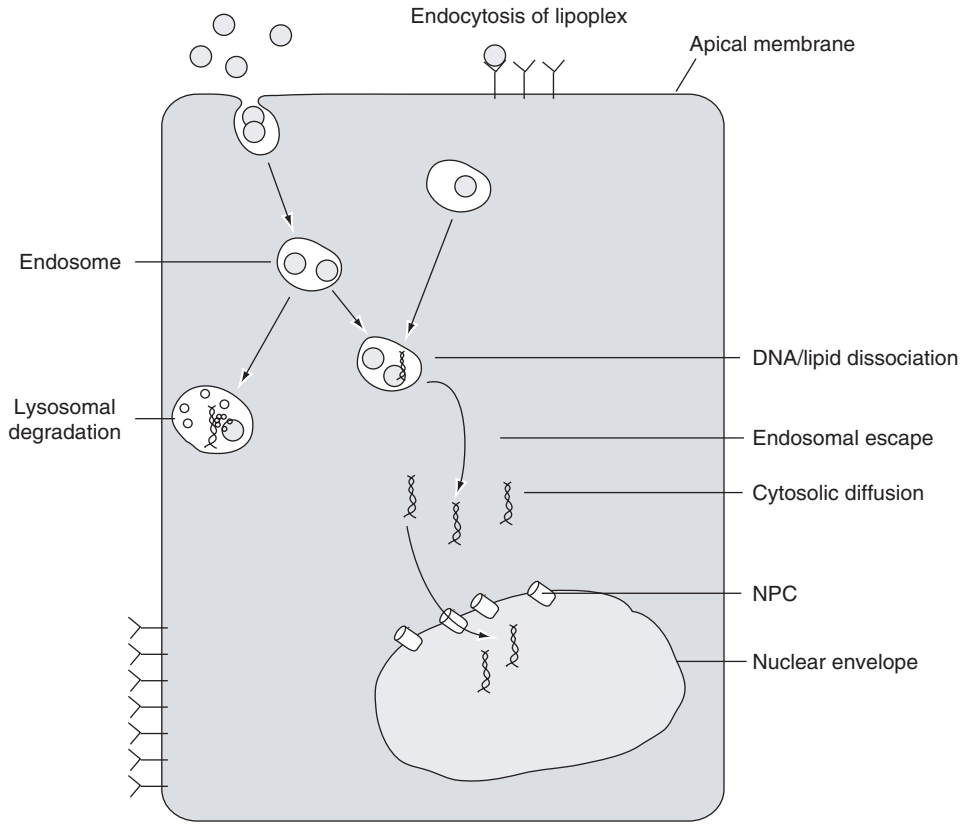

Figure 2. Intracellular barriers. Once a synthetic vector has been endocytosed at the cell membrane, barriers to reaching the site of transcription in the nucleus include escape from the endosome–lysosome degradative pathway, cytosolic trafficking/diffusion and the nuclear envelope. Unless the cell is dividing, the plasmid DNA must be transported into the nucleus via the NPC.
NPC: Nuclear pore complex.

ultimately deliver their contents to lysosomes for degradation. Effective transgene delivery to the nucleus, therefore, requires a specific sequence of events including escape of the vector from endosomes, DNA/lipid dissociation, cytoplasmic DNA diffusion and finally nuclear uptake (**Figure 2**). Viruses have evolved mechanisms to overcome many of these intracellular barriers, including cytoplasmic trafficking, and can escape from endosomes by exploiting the pH drop to trigger a cascade of events that ultimately allow for their exit [10]. The relative inefficiency of nonviral vectors in escaping from endosomes and in particular their poor nuclear uptake are thought to be major reasons for the poor transfection efficiency of these vectors.

3. Gene transfer agents

The exact cellular target for CF gene therapy is unclear, but given the known expression pattern of CFTR, correction of the CF phenotype will likely require persistent transgene expression in the terminally differentiated cells of the epithelium and submucosal glands of the small airways.

Critical to the success of this objective will be an appropriate vector to facilitate expression of physiologically relevant CFTR with minimal or no toxicity. Recombinant viruses that have been engineered to be replication deficient but carry a transgene are generally effective at transfection but highly immunostimulatory. Nonviral, synthetic vectors such as cationic lipids or polymers are less efficient at gene transfer, but generally less toxic and easier to produce in large quantity. There remains significant debate amongst gene therapists as to which class of vector will ultimately prove clinically superior.

3.1 Nonviral gene transfer agents

As electrostatic repulsion inhibits the entry of naked DNA into a cell, a synthetic carrier must be used to deliver the therapeutic gene. Nonviral GTAs are generated by complexing plasmid DNA with cationic lipids to form lipoplexes, cationic polymers to form polyplexes or both forming lipopolyplexes. When formulated with a positive charge, these particles interact with cell surfaces and are internalised by endocytosis.

3.2 Cationic lipoplexes

Cationic lipoplexes, as a delivery vehicle for DNA, have been extensively studied in preclinical animal models and CF patients. The mechanism of gene transfer with cationic lipoplexes is poorly understood, but several general features

appear to be important in the efficacy of gene expression with these agents including the lipid composition, the complex charge density determined by the lipid/DNA ratio and the size of the complex [11,12].

Early studies in CF transgenic mice demonstrated that liposomes were capable of short-term correction of the CFTR Cl^- ion transport defect [7,8]. Subsequent clinical studies in the nasal epithelium of CF patients demonstrated general safety and proof of principal, showing partial correction of the ion transport defect and measurable vector specific DNA and mRNA in nasal biopsies [13-15]. Generally, the correction was brief and (although statistically significant) inefficient with only a 20% restoration of chloride conductance, which was maximal at day 3 and absent at day 7 [13]. A further nasal study found no evidence of inflammation or immune response even after repeat dosage of plasmid DNA (pDNA)/liposomes to the human nose [16]. Improvements in early cationic liposomes were generated by a massive functional screen of cationic lipids for gene transfer to the lungs of mice. Genzyme Corporation identified a cationic lipid called GL-67 that was 100-times more effective in terms of reporter gene expression than preceding liposomes, albeit transient [11]. A GL-67 lipid formulation (with no transgene) was used in the first clinical trial of aerosolised (Pari LC Jet nebuliser) liposomes to the lungs of normal volunteers with no clinically detectable adverse effects [17]. However, when GL-67/CFTR cDNA was aerosolised to the lungs of CF patients they did exhibit inflammatory side effects such as transient fever, myalgia, arthralgia and elevated IL-6 levels, which, although mild [18,19], were associated with a significant fall in lung function (15% decline in forced expiratory volume for 1 s [FEV_1]) in one study [18].

The inflammatory responses encountered with cationic liposome/pDNA complexes are thought to be the result of unmethylated CpG nucleotides in the bacterially derived pDNA. In mammalian DNA the frequency of CpG dinucleotides is suppressed and they are largely methylated. Host recognition of these DNA differences stimulates an inflammatory response that includes the activation of B cells, monocytes, macrophages, dendritic cells and natural killer cells [20].

Particular weaknesses of nonviral gene transfer agents include low-level endocytosis from the apical membrane of differentiated airway epithelia, but perhaps more importantly the inefficient intracellular trafficking and very limited nuclear delivery in nondividing cells resulting in transient, low level expression [9,10,21,22].

3.3 Improvements in nonviral gene transfer

Efforts at improving nonviral gene delivery agents have focused on targeting the complexes to the appropriate cells using receptor ligands, modification of the complexes to improve endosomal escape and facilitate nuclear entry, reducing toxicity by changing the mix of lipids or adding protective molecules to polycations and modifying plasmid DNA by removing inflammatory CpG sequences and using more effective promoter sequences.

3.4 Cationic polymers

Novel synthetic vectors have been generated by complexing DNA with cationic polymers that induce DNA condensation and significant compaction of the polyplex. Various cationic polymers have been used to form polyplexes with DNA, such as polyethyleneimine (PEI), polylysine, polyamidoamine dendrimer, histidylated polylysine, chitosan and polyallylamine. The variable characteristics and potential advantageous properties of different cationic polyplexes over earlier lipoplexes have recently been the focus of specific reviews [22,23]. Briefly, cationic polymers such as PEIs and histidylated polymers possess amines that can be protonated, which causes endosomal swelling and membrane destabilisation facilitating release of the vector from the endosomal compartment into the cytoplasm.

Improved transgene expression has been achieved by the addition of a targeting ligand to the surface of the DNA delivery vehicle. For example, adding carbohydrate moieties (lactosyl residues) to polyplexes facilitates selective binding and internalisation via lectins on the apical surface of airway epithelia and possibly enhances nuclear localisation *in vitro* [24].

Specific cell receptor, targeted vector delivery is another approach. Ziady *et al.* have developed a system of receptor-mediated gene delivery by targeting the serpin-enzyme complex receptor (sec-R) on the apical membrane of CF mouse airway epithelium. The sec-R internalises complexes of serine proteases bound to their cognate inhibitor and delivers them to lysosomes. Sec-R directed complexes were generated by condensing pDNA with a covalent conjugate of a peptide receptor ligand and poly-L-lysine. Using these complexes with pDNA/CFTR, they demonstrated partial correction of the ion transport defect in the nasal epithelium of CF mice for $\leq$ 12 days [25].

This and other forms of receptor-targeted delivery could enhance nonviral vector efficiency and selectivity, but their potential immunogenicity and efficacy have yet to be assessed in CF patients. In addition to receptor targeting, the nanoparticle size of the complexes formed in the sec-R study (18 – 25 nm diameter of pCF1CFTR) may have contributed to the increased efficacy by facilitating passive diffusion through the nuclear pore complex.

In dividing cells, nuclear membrane disintegration during mitosis allows plasmid DNA to enter the nucleus prior to membrane reformation. In nondividing cells (most airway epithelia) passage through the nuclear membrane is a significant barrier for cationic liposome-mediated gene transfer [26]. Earlier cationic liposome complexes form relatively large particles (several hundred nanometers), which greatly exceeds the size of the nuclear pore complex (< 25 nm), leading to inefficient nuclear delivery.

DNA compacted with polycations into nanoparticles (< 25 nm diameter) has been shown to penetrate the nuclear membrane pore more efficiently than large lipid/DNA

complexes [27,28]. However, until recently problems of instability and toxicity have limited the application of nanoparticle complexes consisting of only polycations and DNA [28].

The incorporation of polyethylene glycol (PEG) into the design of poly-L-lysine/DNA nanoparticles has helped overcome these problems and produced a stabilised formulation, which significantly enhanced reporter gene expression over noncompacted DNA in postmitotic cells [28]. Mice dosed with similar nanoparticles demonstrated reporter gene expression 200-fold greater than animals dosed with naked pDNA and with no detectable inflammatory response [29]. Recently, compacted DNA nanoparticles have also been safely administered to the nasal epithelium of 12 CF patients. Vector-specific mRNA could not be detected in the patients, but partial correction of the chloride transport defect for ≤ 15 days post gene transfer was found in seven patients [30]. Further clinical trials are needed with these nanoparticle formulations specifically to test whether lung administration, nebulisation and repeat treatment are safe and effective.

In addition to reducing the size of the polyplex, improving nuclear importation by the addition of a nuclear localisation signal (NLS) has been tried. The incorporation of SV40 NLS peptides into DNA/lipid or polymer complexes has been achieved, but success so far has been limited, probably owing to limited cytosolic diffusion [31].

3.5 Plasmid modifications

Improvements in plasmid construction have highlighted the importance of this area in optimising nonviral gene transfer. Yew *et al.* have shown that transgene expression can be significantly prolonged and toxicity minimised by deleting 80% of the unmethylated CpG motifs within the plasmid [32].

The wide variation in efficiency of the promoter sequences used for gene transfer has also been demonstrated. Gill *et al.* have shown that promoters from the human polybiquitin C (UbC) and the elongation factor-1α (EF-1α) genes resulted in persistent gene expression in the mouse lung (> 16 weeks) as opposed to the transient expression mediated with viral promoters such as cytomegalovirus (CMV) (< 1 week) [33].

Others have developed a promoter based on the DNA control elements of the human cytokeratin 18 (*K18*) gene. The rationale being that endogenous expression pattern of K18 in humans closely mirrors that of CFTR; therefore therapeutic gene expression should be targeted to the relevant cells. Using the K18 promoter (K18-driven lacZ plasmid complexed with cationic liposomes) epithelial cell-specific transgene expression in the lung has been achieved following systemic delivery in mice [34].

Ideally, the improved properties of specific nonviral delivery systems outlined should be integrated in a way that combines the advantages of each.

In addition to the modification of nonviral vectors with specific advantageous properties the application of physical methods such as electroporation, ultrasound and magnetism are also being investigated as adjuncts to improve the efficacy of nonviral gene transfer to the lung [35].

4. Viral gene transfer

4.1 Recombinant adenovirus

Adenovirus, a double-stranded DNA virus, has been used extensively for gene transfer in the lung as it effectively transduces nondividing cells and shows tropism for airway epithelia [36-38]. However, although early preclinical animal studies demonstrated efficient CFTR gene transfer to the airways of rodents [39,40] comparative studies have shown that gene transfer to human airway cells is much less effective than in rodents [41,42].

Whereas results of some early Phase I clinical trials in CF patients suggested transient functional correction of CFTR in the nasal epithelium, as assessed by nasal PD [43,44], others have not and demonstrated problems with antibody responses and associated inflammation [45].

The disappointing results of clinical trials utilising adenoviral vectors in CF patients have probably been due to the deficit of coxsackie adenovirus receptor (CAR) on the apical surface of distal airway epithelial cells in the human lung. Whereas the CAR and the αVβ5 integrin, which facilitate the uptake of adenovirus, are directly accessible on the apical surface of rodent airway epithelial cells, in humans they are situated on the basolateral membrane [46].

Agents such as EGTA or sodium caprate that disrupt epithelial tight junctions and expose the basolateral membrane have been used to augment adenoviral gene transfer efficiency [47,48]. This approach has demonstrated success in mouse models, but whether such agents can be used clinically in CF patients remains a matter of debate [49]. The clinical application of adenoviral gene transfer strategies have also been limited by the significant antibody and cytotoxic T-lymphocyte (CTL) response they induce on initial delivery [50] and importantly this response seems to be heightened in the setting of pseudomonas lung infection [51].

Helper-dependent adenoviral (HD-Av) vectors that are 'gutted' of all viral coding sequences can extend transgene expression and are less immunogenic compared with earlier generation recombinant viruses. Expression from HD-Avs given intravenously has been observed in mouse liver for 1 – 2 years [52-54] and > 1 year in baboons [55].

A HD-Av vector utilising the tissue-specific promoter K18 has been shown to generate more sustained expression of reporter genes in the murine airway than typically reported ≤ 28 days with β-gal and ≤ 15 weeks with human α-fetoprotein (AFP) in CF mouse airway, with minimal inflammation [56]. This vector has recently shown promise in ameliorating a CF mouse model of lung infection (discussed in Section 6). However, inadequate production capability is still a limiting factor for this type of vector for clinical testing.

In the absence of targeting airway stem cells (not covered in this review), correction of CF lung disease with gene

therapy will likely require both long-term CFTR expression and repeat treatment. However, adenoviral-mediated gene therapy in the human lung remains problematic due to transient, low level gene expression, acute inflammation and ineffectiveness of repeat administration. Common findings in all the studies published include a dose-dependent mild local inflammation and the progressive lack of expression following repeated administration.

4.2 Recombinant adeno-associated viruses

AAVs are safer, less immunogenic and in contrast to adenovirus have a simple 4700 nucleotide single-stranded DNA genome, making them less complex to engineer. However, the packaging capacity is limited (< 5 kb); therefore, the large size of CFTR cDNA has necessitated the use of compact weak endogenous promoter (ITR) elements, which may limit expression efficiency. Furthermore, although recombinant AAVs (rAAVs) are devoid of all viral genes, the capsid proteins can still induce neutralising antibodies that limit the possibility of repeat administration. Several serotypes (types 1 – 8 so far) of AAV exist with rAAV-2 being the most extensively studied. Preclinical studies have demonstrated prolonged transgene expression after single dose administration of rAAV-2 in the lungs of rhesus macaques (180 days) and New Zealand white rabbits [57-59]. AAV-2-CFTR vectors have also been used in Phase I/II clinical trials in CF patients and although there were no safety problems the level of *in vivo* CFTR expression was low [60-63].

Further animal studies with AAV-2 have demonstrated transduction in multiple cells types in the lung, but high particle numbers were required and expression of transgene was found to predominate in alveolar cells rather than airway epithelia. The low efficiency is in part due to the limited access of AAV-2 via the apical surface of human airway epithelia. As with adenovirus, rAAV efficiency may be hindered by a lack of specific receptors on the apical surface of epithelial cells. Heparin sulfate proteoglycan (HSPG) has been identified as the primary receptor for AAV-2 and (like CAR) is situated on the basolateral membrane resulting in similar access problems.

Which serotype of AAV is best suited for human lung gene transfer is unknown at present, but the indications from preclinical screening are that AAV-5 and -6 are superior to AAV-2.

After a single nasal administration of AAV-2/-5 (AAV-2 genome packaged in AAV-5 capsid) carrying an erythropoietin (Epo) transgene to mouse lungs, secretion of Epo was documented for 150 days. Furthermore, successful readministration of AAV-2/5 to the lung was achieved 5 months after the first delivery of the same vector [64].

Sirninger *et al.* have recently attempted to optimise rAAV-mediated gene transfer by characterising the gene transfer potential of a range of serotype capsids and larger promoters *in vitro* and combining the most efficient of each. They generated a rAAV-5-CB (CMV enhancer/β-actin) promoter-driven CFTR minigene vector, which prolonged transgene expression and ameliorated a pseudomonas-infected phenotype in the CF mouse lung [65].

This encouraging progress with rAAV would suggest that early phase clinical trials are warranted with these vectors.

Recently, the first clinical evaluation of repeated, nebulised AAV-2 delivery to the lungs of CF patients was completed [66]. The protocol involved three doses of nebulised (Pari LC Jet nebuliser) AAV-2/CFTR cDNA, 1 month apart in patients with mild lung disease (FEV_1 > 60%). The results were encouraging, demonstrating a good safety and tolerability profile even after repeat administration. There was also suggestion of clinical benefit, with a small but significant improvement in FEV_1 and reduction in sputum IL-8 levels ≤ 30 days. A further adequately powered study is under way to confirm these effects on pulmonary function and to address whether benefits beyond the first dose can be achieved.

4.3 Sendai virus and lentiviral vectors

Sendai virus (SeV) is an enveloped virus with a negative-strand RNA genome and is a member of the family *Paramyxoviridae.* Recombinant SeV-mediated gene transfer to differentiated airway epithelial cells has shown to be very efficient both in mice and ferrets [67,68]. The native virus is transmission competent and, therefore, unlikely to be suitable for use in clinical trials. However, deleting the envelope fusion (F) protein gene has generated a nontransmissible, replication-competent recombinant SeV (SeV/ΔF) that can still efficiently mediate gene transfer to the airway epithelium of mice *in vivo* [69]. The safety profile of SeV/ΔF has been further improved by introducing mutations to the matrix and haemagglutinin–neuraminidase proteins, which reduces the number of virus-like particles produced following transfection [70]. Nonetheless, the same concerns raised against other viral vectors apply to SeV, particularly how SeV can be administered repeatedly without invoking a significant immune response.

The development of lentiviral vectors (derived from the HIV-1 retrovirus) is another recent advance in the field of gene therapy. Lentiviral vectors can transfect nondividing cells and integrate into the genome of the transfected cell mediating persistent transgene expression. However, the efficiency of transfection is highly dependent on the initial interaction between the lentiviral envelope and receptors on the cell surface. Lentiviral vectors pseudotyped with the commonly used vesicular stomatitis virus (VSV)-G envelope protein are unable to enter airway cells via the apical membrane and require disruption of tight junctions to access receptors on the basolateral membrane for transfection [71]. The incorporation of envelope proteins derived from highly airway tropic viruses has the potential to expand the utility of lentiviral vectors. Novel lentiviral vectors have been generated by pseudotyping with apical membrane-binding envelope glycoproteins from various other viruses (including the filoviruses Marburg and Ebola, and also SeV), which can efficiently transfect polarised airway epithelial cells at the apical surface [71,72]. It should be noted, however, that all

randomly integrating vectors carry the risk of insertional mutagenesis and proto-oncogene activation.

Each of the GTAs has achieved limited success, with none obviously superior to others. Interestingly, despite nonviral vectors being arguably less efficient than viruses in animal models and laboratory studies, clinical trials have shown that this may be different in CF subjects. Consistently, the efficiency of *in vivo* gene transfer with currently available vectors has been modest. The vector improvements outlined above need to be tested in appropriate preclinical animal model systems and clinical trials to clearly define their full potential.

5. Alternatives to traditional gene therapy

In addition to the traditional gene therapy approaches (based on full-length CFTR DNA constructs), novel methods are being developed to improve the efficacy of gene transfer to airway epithelium. One example is gene repair in which only the faulty nucleotide is changed, leaving the rest of the CFTR sequence untouched, effectively rendering the endogenous mutant *CFTR* gene normal. Specific genomic sequences are targeted with small fragments of exogenous DNA (400 – 800 bp) that are homologous to the targeted endogenous DNA sequences except for the particular base pairs that encode the desired modification. The obvious advantage of this technique, if successful, is biologically normal expression of CFTR for the lifespan of the transfected cell. Studies using chimaeraplasts (chimeric RNA/DNA oligonucleotides) or small-fragment homologous recombination (SFHR) indicate the potential for genomic CFTR repair, although highly inefficient at present [73,74]. These techniques do promise significant advantages and the efficiency should improve with increased understanding of the mechanisms involved.

RNA-repair strategies have also been developed for use as therapeutic interventions by utilising the cell's endogenous splicing machinery as a strategy for modifying pre-mRNA. Spliceosome-mediated RNA *trans*-splicing (SMaRT) is a method of correcting endogenous CFTR mRNA transcripts wherein the mutated region responsible for the disease is excised and replaced with a normal coding sequence. The goal is to repair mutant pre-mRNA molecules and generate full-length repaired mRNA that is translated and processed into mature CFTR protein. Using recombinant adenovirus (Ad/CFTR-pretherapeutic molecule [PTM]) encoding a PTM designed to promote *trans*-splicing with endogenous CFTR mRNA, functional evidence has been demonstrated for repair of mutant (ΔF508) CFTR mRNA in human CF bronchial xenografts [75]. RNA repair methods offer the potential advantages of maintaining endogenous regulation and transgene expression in the appropriate cell types. However, at present, the efficiency and specificity of RNA repair appears to vary considerably [76].

Most CF causing mutations prevent the biosynthetic maturation of the CFTR protein. As a result, most CFTR-processing mutants are degraded within the endoplasmic reticulum (ER) and never reach the cell surface. Using the technique of transcomplementation, the ΔF508-CFTR mutant can be rescued to form mature, functional chloride channels that reach the cell surface. The transcomplementation strategy essentially involves coexpressing other mutants or specific polypeptide fragments of the wild-type CFTR with the mutant CFTR (the effect required a specific match between the region flanking the disease-causing mutation and the complementing fragment). The transcomplementing fragments seem to promote CFTR maturation and normal export from the ER, although the exact mechanism is not clear. This opens up the exciting possibility of developing CF therapies (e.g., peptide therapy or mini-cDNA constructs for gene therapy) that are tailored to specific disease-causing mutants of CFTR [77].

6. Studies in animal models

Preclinical studies of CFTR gene replacement have been hindered by the lack of a pathophysiologically relevant animal model of CF lung disease. Unfortunately, although CFTR knockout mice display the characteristic airway ion transport defect they do not develop spontaneous lung disease as in the human condition, but rather, have severe intestinal defects [78]. Because of this anomaly, gene therapy approaches for CF have relied heavily on molecular end points for complementation such as electrophysiological correction and detection of vector specific mRNA, DNA and protein. CF mice have been of undoubted value in demonstrating the potential for functional correction of CFTR, but there are limitations when extrapolating from such studies to CF patients with complex lung pathology. Without a clear understanding of how CFTR/chloride transport is linked to disease progression, the use of such surrogate end points in human clinical trials may be misleading. In fact it is not clear whether increased CFTR expression or improved electrophysiology in airway epithelium constitutes successful gene therapy. Does correction of chloride transport equate to clearance of bacterial infection in the CF lung?

Mall *et al.* have recently generated a transgenic mouse that does demonstrate the characteristics of CF lung disease spontaneously. Interestingly, these were not traditional CFTR knockout models, but rather they overexpressed the β-subunit of the epithelial sodium channel (β-EnaC transgenics) causing increased sodium and water resorption in the airways [2,3]. This model should be useful for pathophysiological studies and testing alternative treatment strategies, but probably not for CFTR gene replacement.

Recently, two studies have advanced traditional CF mouse work by testing the therapeutic potential of CFTR gene transfer in bacterially infected lungs of CF knockout mice. It is known that lung infection (although not necessarily CF lung disease) can be induced in the CF mouse by exposure to high levels of bacteria, particularly Pseudomonas and *Burkholderia cepacia* (Bcc) [79-82]. For example, wild-type mice repeatedly instilled with Bcc clear the bacteria within 9 days,

whereas CF knockout mice retain Bcc and succumb to severe bronchopneumonia, exhibiting many of the histological signs of human CF lung disease including neutrophilia [82].

Van Heeckeren investigated whether CFTR delivery could improve the outcome in a CF mouse model of lung infection with a mucoid strain of *Pseudomonas aeruginosa* (PA M57-15). The CF mice treated with an adenoviral vector encoding CFTR (Ad2/CFTR-16) prior to inoculation with *P. aeruginosa* beads exhibited a significantly greater cumulative 10-day survival compared with sucrose-treated mice controls [48]. The survival improvement occurred without reduction of lung inflammation or bacterial burden. Interestingly, survival was similarly prolonged in a third group of mice treated with empty adenoviral vector alone (Ad2-EV) compared with control mice. This perplexing result suggested a protective effect related to adenoviral-induced inflammation alone and questioned the therapeutic activity of the CFTR transgene.

Koehler *et al.* demonstrated that nasal administration of a helper-dependent adenoviral K18CFTR vector (K18CFTR-HD-AD) to CF knockout mice protected against subsequent pulmonary infection by a clinical strain of Bcc complex (Bcc-ET-12), a particularly virulent pathogen of the human CF lung. CF knockout mice pretreated with K18CFTR vector were able to clear Bcc to basal levels indistinguishable from normal mouse controls [83]. The Bcc model may be more clinically relevant than the agar bead model of *P. aeruginosa*, as artificial immobilisation is unnecessary. This report represents the first demonstration that gene therapy using a tissue-specific promoter can ameliorate a CF-related bacterial phenotype in the CF mouse lung.

However, a possible weakness of both studies was to deliver CFTR to CF mice before introducing the bacteria, as in reality the human CF lung is likely to have been chronically infected with bacteria by the time gene therapy can be contemplated. Furthermore, we know that pre-existing infection can significantly influence gene therapy in CF mice. Not only is transfection efficiency reduced by 50%, but antiadenoviral CTL activity is enhanced in the setting of pre-existing chronic *Pseudomonas*-induced lung inflammation [51,81]. These findings underscore the importance of considering the influence of the disease milieu when evaluating gene therapy protocols for CF in animal models. Preclinical gene therapy experiments testing the therapeutic efficacy of CFTR transgenes for CF should probably be tested in the already infected lung.

Nonetheless, demonstration that CFTR transgene expression can positively affect a clinically relevant end point of protection from infection is a promising addition to previous studies using electrophysiological and molecular end points. Further studies of this nature, using relevant clinical end points, may be more informative regarding the likely therapeutic potential of emerging vectors.

Experience dictates that previous successful gene augmentation studies in CF mice have not been predictive of the outcome of clinical trials. Differences in the scale, anatomy and physiology of the mouse and human lung may be pertinent. Mice lack bronchial submucosal glands and produce fewer airway secretions. Furthermore, CFTR mRNA levels are lower in the murine airway and CFTR is not the predominant chloride channel [84,85]. Moreover, by necessity, the mode of delivery and pulmonary deposition of vectors in mice and humans will differ significantly, possibly affecting transgene expression, host response and possibly even causing erroneous interpretation of data.

Recently, Emerson *et al.* described the use of the sheep as a novel large animal model system for respiratory gene transfer [86]. The size, anatomy and physiology of sheep and human lungs are similar, and human and sheep CFTR share significant similarities of sequence and developmental expression. They compared transfection efficiency and toxicity of DNA alone and DNA complexed with the cationic liposome, GL-67/dioleoyl-phosphatidyl-ethanolamine (DOPE) delivered bronchoscopically to specific lung segments. Levels of the reporter gene, chloramphenicol transacetylase (CAT), were assessed quantitatively at the levels of both protein and mRNA. Interestingly, compared with similar murine studies, CAT protein levels were, at best, 500-fold lower in the sheep tissue treated with the GL-67 liposome than were achieved in mice.

These results highlight not only potential differences in comparative animal biology, but also the problems inherent with extrapolating to human studies from a single, dissimilar animal model. However, the scale and similarity to the human lung perhaps allows more clinically relevant testing of the relative efficacy and toxicity of new vectors through clinically applicable approaches such as nebulised/aerosol delivery, repeated bronchoscopic assessment (and airway sampling) and may be helpful in guiding future gene delivery protocols in CF patients. At present, a CF transgenic sheep is unavailable, although attempts at generating both CF sheep and ferrets are ongoing [87].

7. Expert opinion and conclusion

Since the cloning of the CFTR gene in 1989 considerable progress has been made, particularly at the molecular and cellular level, but as yet this has been poorly reflected in clinical gene therapy trials.

Achieving expression of biologically relevant CFTR transgene in the correct cells at the correct level with the duration to reconstitute normal physiology and innate immunity in the CF lung will be difficult, but hopefully this degree of correction is not necessary to sustain clinical benefit.

A concerted and integrated approach to CF gene therapy has been adopted by UK Cystic Fibrosis Gene Therapy Consortium (UK CFGTC), where three major gene therapy centres in the UK have combined expertise and resources in developing preclinical studies and relevant end-point assays through to clinical trials. This cooperative strategy is logical given the effort that will be necessary for CF gene therapy to become a clinical reality.

Emerging vector strategies, both viral and nonviral, are showing promise towards this goal, but their safety and thrapeutic efficacy needs to be established in appropriate and informative preclinical studies and early clinical trials. The immunological hurdles, especially with regard to all viral vectors, remains problematic. The most successful improvements in nonviral delivery systems need to be integrated in a way that combines the advantages of each. Finally, with the advent of new transgenic technologies based on somatic cell nuclear cloning, the field is also now positioned to expand the use of larger and more relevant preclinical animal models for CF, which should facilitate a more seamless (and hopefully predictable) transition to clinical trials. The development and application of appropriate animal models in parallel with early clinical trials for testing new vector strategies is critical for informing basic research and the realisation of clinical gene therapy.

Potential conflict of interest

S Tate was formerly a member of the UK CFGTC (2000 – 2004) and S Elborn is currently Chair of the Scientific Advisery Committee.

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Websites

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