Genetic therapies for cystic fibrosis lung disease

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Abstract

Gene therapy for cystic fibrosis (CF) has been the subject of intense research over the last twenty-five years or more, using both viral and liposomal delivery methods, but so far without the emergence of a clinical therapy. New approaches to CF gene therapy involving recent improvements to vector systems, both viral and non-viral, as well as new nucleic acid technologies have led to renewed interest in the field. The field of therapeutic gene editing is rapidly developing with the emergence of CRISPR/Cas9 as well as chemically modified mRNA therapeutics. These new types of nucleic acid therapies are also a good fit with delivery by non-viral delivery approaches which has led to a renewed interest in lipid-based and other nanoformulations.

Introduction

Cystic fibrosis (CF) is a recessively inherited disease caused by mutations in the gene for the cystic fibrosis transmembrane conductance regulator protein (CFTR) located on the long arm of chromosome 7 [1]. CFTR is a membrane protein located on the apical surface of epithelial cells and acts as a channel for anions including chloride and bicarbonate. In CF, the mutated protein results in an imbalance of salt and fluid transport as well as pH of the airway surface liquids that leads to the production of thick sticky mucus, bacterial infections and inflammatory damage to the lung [1]. Advances in understanding of CFTR mutations and the nature of the protein defect [2] have enabled the development of CFTR modulator drugs such as Ivacaftor, which are effective for patients with rare types of mutations, such as G551D, while other small molecule drugs are now in development for different classes of CFTR mutations [3]. However, the majority of the 281 disease-causing CFTR mutations (https://cftr2.org/mutations_history) remain untreatable with small molecule drugs and so new approaches to gene therapy are under investigation (Figure 1). Advances in nucleic acid technologies, such as CRISPR/Cas9 have opened up new opportunities for both mutation specific and universal gene therapies while new viral and non-viral vectors offer improved delivery. Each approach offers different advantages of persistence and repeatability of administration. (Table 1). This review describes some of the recent developments in genetic therapies of specific relevance for CF.

Viral Gene therapy

i. Adeno Associated Virus (AAV)

Previous clinical gene therapy studies for CF using AAV serotype 2 (AAV2) were unsuccessful due to insufficient levels of CFTR transgene expression [4*]. Recent research has focused on

approaches to expand AAV tropism [6-8], reduce immunogenicity and enhance CFTR expression levels and persistence in the lung (Table 1). These studies exemplify the challenge of developing efficient AAV vectors for evaluation in relevant CF animal models, including pigs [4*], sheep [7], ferrets [8] and mice [6], but also provide efficient transduction of human epithelia for clinical translation. For example, nine naturally occurring AAV serotypes tested in human cells and murine lungs identified AAV6 as the strongest candidate in both human and mouse epithelium [5]. However, other serotypes, including AAV2/5, i.e. the AAV2 genome packaged in AAV5 capsid proteins [6], were also effective in human cells *in vitro* and murine lungs *in vivo*, while AAV8 was identified as the optimal serotype for testing in sheep airways [7].

An alternative approach to expanding AAV tropism, which also overcomes the packaging constrains of AAV, reported using the capsid of human Bocavirus-Type-1 (HBoV1) to package an AAV2 genome [8]. HBoV1, like AAV, is a parvovirus that displays high tropism for the apical membrane of human airway epithelium and transduces human air-liquid interface cultures efficiently. Apart from its tropism, it also has a larger capsid than AAV with a packaging capacity of 5543 nt compared to the 4679 nt of AAV. CF ferrets recapitulate many of the physiological aspects of CF lung disease and are thus proposed to provide a suitable model for the development and testing of CF gene therapy strategies and so rAAV2/HBoV1 vector was evaluated in ferret epithelial models [8]. It was shown that ferret epithelium *in vitro* and *in vivo* is permissible to transduction with rAAV2/HBoV1 vectors encoding luciferase reporter genes and that repeated dosing *in vivo* was effective at maintaining transgene expression [8]. This work opens up the possibility of using CF ferrets to evaluate rAAV2/HBoV1 vectors for CF gene therapy.

Another approach to expanding AAV vector tropism involves directed capsid selection

methods and this approach was used to identify a novel AAV serotype with specificity and high transduction efficiency for pig airway epithelium to enable testing of therapeutic correction in the gut-corrected CF pig [4*]. This virus packaged a truncated CF gene, CFTRΔR, and synthetic promoter/enhancer which rescued anion transport and restored bacterial killing after administration to the lungs of CFTR-null pigs. This AAV serotype, however, was specific for pigs so alternative serotypes will be required for man. The use of AAV2/5 encoding the same CFTRΔR minigene with a shortened CMV promoter was reported by other investigators to transduce CF epithelia and restore full channel activities, as measured by human intestinal organoid swelling after forskolin stimulation [6]. Moreover, treatment of a group of CF mice demonstrated, in most individuals, correction of ion transport as shown by the response of their nasal potential differences to low chloride and forskolin perfusion [6].

ii. Lentivirus

Lentiviral vectors are attractive vectors for CF gene therapy owing to their ability to integrate into the recipient genome, so producing long-term, stable gene expression (Table 1). They have been less well studied than AAV as gene therapy vectors in the lungs, and so less is known about their safety profile or immunogenicity on repeat dosing, while production of high-titre virus preparations is more challenging than for AAV. However, packaging of full length CFTR and strong promoters is not size-limited as with AAV and recent developments, described below, have indicated promising potential for their use in CF gene therapy. As with AAV, one of the major questions concerns viral tropism.

CFTR channel activity was corrected in the airways of CF pigs transduced with a feline immunodeficiency virus (FIV)–based lentiviral vector, pseudotyped with the GP64 envelope protein [9*]. Two weeks after aerosolized FIV-CFTR viral vector delivery to the nose and lung

of three new-born CF pigs, epithelial tissues revealed a significant increase in transepithelial cAMP-stimulated Cl^- current, indicative of functional CFTR. In addition, increases in tracheal airway surface liquid pH and bacterial killing in *CFTR* vector–treated animals were observed. Further studies are required to demonstrate the potential of this vector for gene therapy efficacy in treating or preventing lung disease and the ability to redeliver the treatment.

In another format, a simian immunodeficiency virus (SIV)-based lentiviral vector, pseudotyped with Sendai virus fusion protein (F) and Hemagglutinin/ Neuraminidase (HN) envelope proteins was reported to generate efficient transduction of cultured human bronchial epithelial cells and murine lung epithelium *in vivo* with no detectable immune responses, suggesting that these viral vectors possibly offer unique, non-immunogenic properties [10].

Non-viral Gene Therapy Formulations

Liposomal vectors for gene therapy offer advantages of safety, ease of scalable formulation and packaging capacity for large DNA molecules. Gene therapy trials for CF were performed with the pGM169/GL67A gene-liposome formulation in a Phase I/IIa clinical study [11], which confirmed safety of the formulation and procedure, and then in a repeat dosing trial in which patients received monthly repeated dosing for up to one year [12]. In terms of efficacy, a number of outcomes showed a trend favouring the treatment group as well as a small but statistically significant increase in lung function measurements at 12 months. The study also demonstrated the safety of repeated dosing, which will be required with most anticipated forms of CF gene therapy, but highlighted the need for improved nucleic acid delivery.

Approaches to enhance transfection efficiency in the lung include equipping them to overcome better the epithelial barriers. For example, DNA nanoparticle formulations of the

biodegradable poly(β-amino esters) (PBAEs) polymers covered by a dense layer of polyethylene glycol (PEG) displayed increased penetration of mucus *in vitro*, which correlated with increased transfection efficiency of murine lungs *in vivo*. [13]. An interesting, alternative, non-viral approach to CF gene therapy reported the use of exosomes, naturally occurring extracellular vesicles formed by membrane budding from donor cells [14]. Exosomes from CFTR-expressing Calu3 epithelial cells, were incubated with CF15, an epithelial cell line expressing the most common CF mutation F508del-CFTR, and found to transfer both glycosylated exosomal membrane-bound CFTR and CFTR mRNA in the exosomal cytoplasm, restoring CFTR protein production and ion transport functionality to CF15 cells [14].

Improved Nucleic acids for Non-Viral Delivery

i. Minicircle DNA

Recent clinical trials of CF gene therapy have employed the plasmid pGM169, which is depleted of inflammatory CpG repeat elements, delivered by the GL67A liposome [11,12]. This plasmid previously achieved sustained gene expression in the mouse lung without causing inflammation [15]. However, the Phase I/IIa safety study revealed a dose-dependent, flu-like response to pGM169/GL67A while CFTR transgene mRNA levels in airway epithelial cells were very low [11] or undetectable [12] by quantitative, real-time PCR. The authors attributed the inflammatory response, particularly at higher doses, to the viscous, cationic formulation although the possibility of a DNA-specific response, despite the lack of CpG motifs, was not excluded [11]. This led to the repeat-dosing study being performed with the lowest dose of DNA together with anti-pyretic paracetamol [12]. Thus, for future studies, while improved lipids or other gene carriers would be beneficial, more potent gene expression vectors would enable more robust expression at lower doses, lowering the

amounts of cationic lipids required for delivery and so potentially reducing inflammatory responses.

Approaches to improving the activity of nucleic acid cargoes delivered by nanoparticles include minicircle DNA, circular pieces of DNA containing the transgene and regulatory elements but from which almost all vestiges of bacterial DNA have been removed, including the antibiotic selection marker. Studies using a lipid-peptide nanoparticle suggested that minicircles offer improved expression levels and duration of expression in human epithelial cells *in vitro* and in murine epithelia *in vivo*, along with reduced inflammatory responses [16*]. In another report, however, using polyethylenimine (PEI) as the carrier, murine lung transfections showed that the advantages of the minicircle in transgene expression levels over a plasmid were less evident if the transgene, in this case for luciferase, was additionally CpG-free [17]. Further studies will be required to evaluate whether minicircle DNA delivered by non-viral nanoparticles offers advantages for CF gene therapy over CpG-free plasmid DNA and viral vectors (Table 1).

ii. Oligonucleotides and siRNA

Approximately 13% of disease-causing CFTR mutations involve aberrant exon splicing [3] such as the c.2657+5G>A splicing mutation, which leads to exon 16 skipping or introduction of a premature stop codon [18*]. Single-stranded DNA oligonucleotides of 19 nucleotides containing 2’ O-methyl modified ribose and a phosphorothioate backbone were designed that hybridise to pre-mRNA to modify aberrant splicing and were found to restore correct splicing in HEK293 cells expressing the c.2657+5G>A mutant CFTR minigene [18*]. A similar approach could be taken with other such splice mutations as well as those that introduce cryptic splice sites such as the c.3849+10kb C>T mutation (Figure 1).

Small interfering RNA (siRNA) therapies in development include silencing of the epithelial sodium channel, ENaC. Sodium is the counterion to chloride, and ENaC is upregulated in CF, leading to dehydration of the airway surface liquid (ASL) [19]. Silencing of ENaC mediated by siRNA has proven challenging due to the lack of effective delivery formulations, but an efficient lipid-peptide nanocomplex was described recently that displayed efficient transfection both *in vitro* and *in vivo* that may enable siRNA therapy for CF [20].

iii. mRNA Therapy

Chemically-modified mRNA has been developed as an indirect means of protein replacement with CFTR delivery to the lungs as a therapeutic candidate (Table 1; Figure 1) [21] .Chemically modified mRNAs synthesised *in vitro* incorporate modified nucleosides that reduce their immunoinflammatory potential and enhance their stability and expression. Compared to DNA encoded gene delivery, mRNA offers higher levels of protein production with greater safety since there is no risk of chromosomal integration. Liposomal or polymeric non-viral vector formulations are essential for mRNA delivery across the epithelial barriers and such formulations often have different characteristics to those used for plasmid DNA delivery [22]. New approaches to developing more efficient nanocarriers for mRNA delivery include lipid library screening [23,24]. Because mRNAs have a short half-life *in vivo* in the lungs of about 7 hours [22], they would require regular, repeated delivery to maintain CFTR expression, which is usually a favourable property of liposomal systems (Table 1). However, mRNA can also be used to deliver Cas9 nuclease for therapeutic gene editing where transient expression is not a limiting factor, but a positive safety factor as described below.

Gene editing for CF

Gene editing offers an approach to repair specific mutations in CFTR with great precision, or to disrupt coding sequences in contributory genes, such as ENaC, or cryptic splice sites in CFTR (Figure 1; Table 1). Approaches include Zinc Finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs) and Clustered Regularly Interspersed Palindromic Repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9), all of which first introduce targeted, double strand breaks in the chromosome near to the mutation site, which are then repaired by the endogenous DNA repair pathways, either by Non-Homologous End-Joining (NHEJ) or, in the presence of a DNA donor, by Homology Directed Repair (HDR) [25,26]. ZFN and TALENs are protein targeted and therefore time consuming and expensive to use. By contrast, CRISPR/Cas9 is targeted to a specific chromosomal site by guide RNAs, while the simplicity and low cost of CRISPR has made gene editing a routine tool in most molecular biology labs [25,26].

ZFNs were reported to correct the F508del-CFTR mutation with a selectable plasmid donor in inducible pluripotential stem cells (iPSCs) generated from fibroblasts of a CF donor [27*]. Gene editing restored fully glycosylated CFTR protein production and correction of the ion transport defect. However, mutation specific therapies are problematic for CF as there are at least 281 disease-causing CFTR mutations (https://cftr2.org/mutations_history). An alternative approach to gene editing, termed “super-exon” therapy, has the potential to treat a far wider range of patients as a single therapeutic. ZFNs were used to insert a CFTR cDNA fragment covering exons 11-27, the super-exon, into exon 11, producing functional correction of ion transport characteristics in cell culture [28]. A potential limitation of this approach to

be determined is whether this may change the relative position of one or more known strong transcriptional enhancers, which may alter the expression of the CFTR gene [29].

Gene editing with targeted nucleases presents risks of off-target DNA cleavage introducing unwanted mutations with unknown consequences. Whole genome sequence may be the best way to detect nuclease-mediated mutations but this is not always practical and so sensitive and unbiased genome-wide methods have been developed to detect off-target effects including Digenome-seq [30], GUIDEseq [31] and CIRCLE-seq [32]. Efforts to minimise the potential for off-target effects include delivering Cas9 mRNA which has a shorter half-life *in vivo* than DNA, minimising opportunities for off-target cleavage. This approach was used for *in vivo* therapy of Surfactant Protein-B (Sp-B) deficiency in a murine model, with CRISPR/Cas9 system delivered by liposomes and the template DNA by AAV6 [33].

Nuclease-free approaches to gene editing would help to overcome these concerns such as the report of triplex-forming Peptide Nucleic Acids (PNAs) that were used to target and repair the F508del-CFTR mutation [34*]. PNAs were delivered with polymeric nanoparticles, achieving correction of CFTR ion transport of up to 25% *in vitro*. Another nuclease-free method for CFTR gene editing was described in which helper-dependent Adenoviral (HDAd) vectors, with donor DNAs with regions of homology to the *CFTR* locus of more than 20 kb, were used to transduce CF iPSCs, achieving levels of correction of more than 50% with positive selection [35]. The selectable marker was then excised by *piggybac* transposase leading to "footprintless" gene editing of *CFTR.* However, the high immunogenicity of adenovirus would limit its utility *in vivo*. RNA editing approaches are also under development using modified ADAR (Adenosine Deaminase that Acts on RNA) enzymes, in which the catalytic domain is linked to an antisense oligonucleotide [36]. ADAR converts an

A to a G in mRNA which might find particular use in correcting premature termination codons and offer fewer off-target effects in the genome compared to Cas9 [36].

Conclusion

Cystic fibrosis provides one of the finest examples of how a deeper understanding of disease genetics can lead to the development of effective personalised medicines including drugs such as Ivacaftor and Orkambi [37]. Although gene therapy for CF has had a long, and largely disappointing record, deeper understanding of epithelial biology, a renewed effort to develop improved viral and nanoparticle vectors and the development of novel nucleic acid therapeutics, including mRNA and gene editing with CRISPR/Cas9, are opening up new prospects for CF gene therapy. Model systems including CF ferrets and pigs are enabling the development of disease-relevant functional metrics in animal models while improved *in vitro* human cell models including air-liquid interface cultures and epithelial organoids are now more widely available which are essential for the development of personalised genetic medicines and assessment of their ability to correct the physiological defects of the CF airway epithelium.

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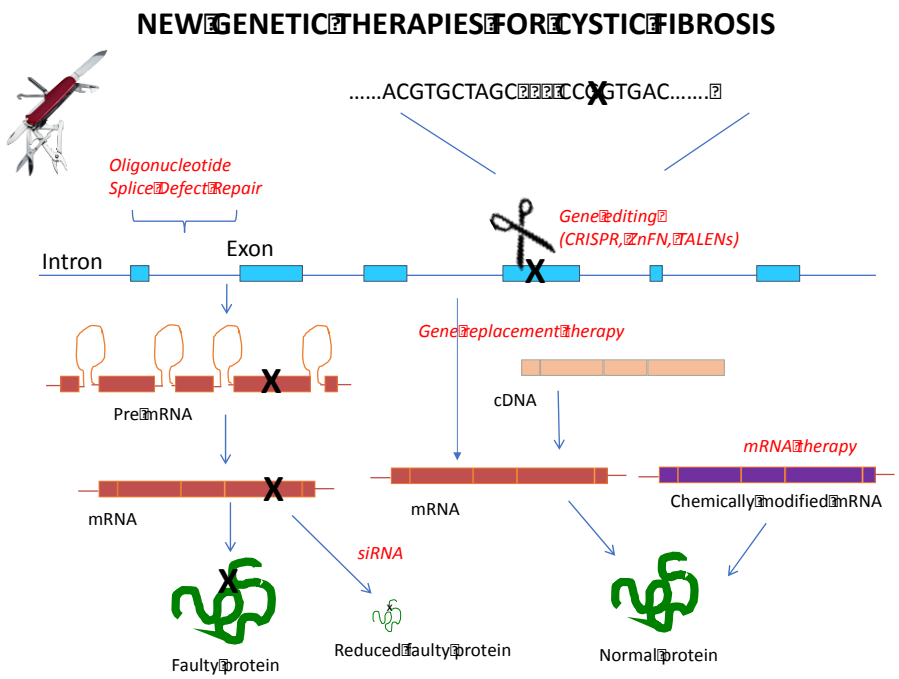

Figure 1

The figure shows the structure of a stylised gene with exons and introns and a disease-causing mutation in one of the exons. The left side of the figure shows that the mutation is transcribed to pre-mRNA and mRNA then translated to a defective protein. The right side of the figure illustrates different molecular approaches to correcting the mutation to either reduce expression, as achieved with siRNA, to replace the defective gene by delivery of a corrected cDNA, by oligonucleotides to correct a splice mutation and finally by gene editing to correct the mutation in the chromosome.