Human Molecular Genetics, 2013, Vol. 22, Review Issue 1 **R52–R58**
doi:10.1093/hmg/ddt372
Advance Access published on August 4, 2013

Moving forward: cystic fibrosis gene therapy

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Received July 23, 2013; Revised July 23, 2013; Accepted July 29, 2013

Since cloning of the *CFTR* gene more than 20 years ago a large number of pre-clinical and clinical CF gene therapy studies have been performed and a vast amount of information and know-how has been generated. Here, we will review key studies with a particular emphasis on clinical findings. We have learnt that the lung is a more difficult target than originally anticipated, and we describe the strength and weaknesses of the most commonly used airway gene transfer agents (GTAs). In our view, one of the most significant developments in recent years is the generation of lentiviral vectors, which efficiently transduce lung tissue. However, focused and co-ordinated efforts assessing lentiviral vector safety and scaling up of production will be required to move this vector into clinical lung gene therapy studies.

INTRODUCTION

Gene therapy has been defined as 'The introduction or alteration of genetic material within a cell or organism with the intention of curing or treating a disease' (1). The first gene therapy-based drug Gendicine, an adenovirus (Ad) carrying the p53 tumor suppressor gene for the treatment of head and neck cancer, was only approved in China (2). However, in early 2012 Glybera, an adeno-associated virus (AAV) carrying the lipoprotein lipase gene, obtained marketing authorization for the treatment of patients with severe lipoprotein lipase deficiency in Europe. Although CF gene therapy has been at the forefront of gene therapy research since cloning of the *CFTR* gene in 1989 (see Table 1 for key publications), the lung is a more difficult target organ than first anticipated and market authorization for a lung gene therapy product is not yet available. The successful development of Kalydeco, a recently approved drug, that potentiates CFTR function in patients with certain types of CFTR mutations (~4% of CF patients) has provided proof of concept that correction of the molecular defect can lead to clinical benefit, and thereby boosted confidence in the concept of gene therapy. Importantly, gene therapy should be suitable for all CF patients, independent of the type of mutation that they carry.

WHY IS THE LUNG SUCH A CHALLENGING TARGET?

The lung has evolved to fight invasion of foreign particles, and gene transfer agents (GTAs) have to overcome a number of extra- and intracellular barriers to achieve their objective (10). In general, viral vectors are more efficient than non-viral GTAs, which is in part related to their relative strengths and weaknesses in overcoming these barriers. However, sputum obstruction particularly in patients with more advanced disease will impair both viral and non-viral GTAs and gene therapy will, therefore, be most efficient in young CF patients before disease has established (preventative therapy). However, this patient group is not suitable for early phase clinical trials, and a careful selection of patient populations to establish proof-of-principle is a critical aspect of the trial design.

Although it is difficult to identify the most important extra- or intracellular barrier, it has become clear that induction of adaptive immune responses after administration of most viral vectors prevents efficient repeated administration and, therefore, renders these vectors ineffective for CF gene therapy. However, induction of immune responses is vector-specific and will be discussed in the relevant sections below. Importantly, non-viral vectors are less likely to induce immune responses and consistent efficacy after repeat administration has been demonstrated in pre-clinical (11) and clinical studies (12).

To date we do not know if the levels of lung gene transfer that can be achieved are sufficient for generating clinical benefit (i.e. improvement in lung function). However, a body of evidence suggests that even modest amounts of CFTR expression may suffice to improve lung disease, including:

(i) CF individuals with certain 'mild' mutations that retain as little as 10% of normal *CFTR* expression per cell do

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Table 1. Important milestone studies since cloning of CFTR in 1989

Only 1 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* (3).
Three years after the cloning of CFTR Rosenfeld *et al.* provided evidence of successful CFTR mRNA and protein expression after Ad-mediated CFTR cDNA transfer into cotton rats (4).
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 (5).
In the same year, Zabner *et al.* performed the first, albeit small and not placebo controlled, CF gene therapy trial in three 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 (6).
Five years after the 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 (5×10^9 plaque forming units (PFU)/patient) (7).
Six years after cloning of CFTR, Caplen *et al.* provided first evidence that a non-viral GTA (DC-Chol:DOPE) complexed with CFTR cDNA could partially correct cAMP-mediated chloride transport in nasal epithelium of CF patients (8).
Ten years after cloning of CFTR Alton *et al.* demonstrated that a non-viral GTA (GL67A) complexed with CFTR cDNA could partially correct cAMP-mediated chloride transport in the lungs of CF patients (9). This study remains the only study assessing CFTR function after gene transfer into lower airways.

generally not suffer from lung disease although other organs such as the vas deferense may be affected (13).

(ii) *In vitro* cell mixing experiments suggest that 6–10% of non-CF cells intermixed with CF cells restore CFTR-mediated chloride secretion to non-CF levels (14,15). However, it is important to note that CFTR has to be expressed in at least 25% of cells grown in monolayer *in vitro* to restore mucus transport (16) and that correction of sodium hyper-absorption requires substantially higher numbers (close to 100%) of non-CF cells (15,17).

(iii) Ad-mediated *CFTR* gene transfer into 20–30% of sinus epithelial cells extracted from CF knockout pigs corrects chloride transport to 50% of non-CF levels. Even low levels of gene transfer (~7%) produced detectable levels of correction (~6% of non-CF) (18,19).

It is also important to consider several additional questions:

(i) Are low levels (≤10%) of CFTR required in all cells (as in CF subjects with milder mutations), or are normal levels of CFTR expression required in ~10% of cells (maybe the more likely scenario after gene therapy)?

(ii) Which cells have to express functional CFTR? CFTR is expressed in various lung regions and cell types (20–24). In our view, airway epithelial cells are currently the most likely target cell for gene replacement.

DO VIRAL VECTORS HOLD PROMISE FOR CF GENE THERAPY?

A diverse range of viral vectors including Ad, AAV, Sendai virus (SeV) and lentiviruses have been assessed for airway gene transfer. Key studies and available clinical experience will be reviewed below.

Adenoviral vectors

Adenoviral vectors were the first viral vectors to be assessed for CF gene therapy. Various viral genes have been deleted over the years culminating in the generation of 'gut-less' viruses which are completely devoid of viral genome (25). Adenoviral vectors do not integrate into the host genome, but remain episomal. Due to a natural tropism for the target organ, adenoviral vectors were an obvious first choice for CF gene therapy and the first ever CF gene therapy trial was carried out with an adenoviral vector just a couple of years after cloning the *cystic fibrosis transmembrane conductance regulator* gene in 1989. Zabner *et al.* administered Ad-CFTR to the nasal epithelium of 3 CF patients (6). The nasal epithelium was initially chosen as a target organ, because the cell composition is similar to the lower airways and efficacy endpoints were easier to assess (26). Due to the low number of subjects treated by Zabner *et al.* ($n = 3$), conclusions about efficacy were difficult to draw, but the study opened the door for the additional nine Ad CF gene therapy trials that were carried out in nose and lungs of CF patients between 1993 and 2001 (27). Taken together these trials showed that (i) low level gene transfer can be achieved in some subjects based on the detection of vector-specific *CFTR* mRNA and protein, (ii) partial correction of the CF ion transport defect, specifically the chloride transport, can be achieved in nasal epithelium of some patient, (iii) Ad administration can cause side-effects (lung inflammation), but this effect is dose-dependent, (iv) efficacy in general was lower than originally predicted by the pre-clinical models, due to the absence of the relevant receptor on the apical surface of human airway epithelial cells, (v) administration of adenoviral vectors induced humoral and cellular immune responses, which (vi) importantly affect the efficacy after re-administration of the virus. In parallel to the clinical Ad studies described above, a large body of pre-clinical work has been conducted in an attempt to overcome the shortcomings of the vector for CF gene therapy (28). However, in our view, to date there is no strong evidence that adenoviral vectors will be useful for CF gene therapy, although the vector has been very useful for pre-clinical proof-of-concept studies. For example, Potash *et al.* have recently shown that *ex vivo* Ad-mediated CFTR transfer can correct the chloride transport defect in sinus epithelium of CF pigs (18).

ADV vectors

Adenoviral vectors were superceded by AAV vectors which are depleted of viral genes and remain largely episomal once inside the nucleus (29–31). A large number of serotypes have now been identified (32), and capsids from serotypes 1, 5, 6, 8 and 9 appear to be most efficient in transducing airway epithelial cells (33). AAV2 was the first, and is currently the most extensively studied, serotype. Between 1999 and 2007, six clinical studies were carried out mainly led by Targeted Genetics, Corp., in which the vector was administered to the nose, sinuses and lungs of CF patients (34). Initial single-dose Phase I trials demonstrated that virus administration to the CF airways was safe, but provided little opportunity to assess efficiency of vector-specific CFTR expression. A large repeat-administration study (100 subjects), sufficiently powered to detect significant changes in lung function, did not meet its primary efficacy end

point (improvement in lung function) (35). There may be several reasons for these disappointing results: (i) AAV2 is too inefficient in transducing airway epithelial cells via the apical membrane, (ii) the LTR promoter, which was used to drive expression of the 4.7 kb *CFTR* cDNA due to the limited packaging capacity (~5 kb) of the virus, is too weak and/or (iii) repeat administration of AAV2 to the lung is not possible due to the development of an anti-viral immune response. No additional AAV lung trials have been performed since 2005, but research aimed at addressing and improving these potential limitations of AAV has been actively pursued (extensively reviewed in (28)) and some progress, particularly related to improving transduction efficiency, has been made.

However, on balance we would argue that AAV vectors may face similar problems with repeat administrations as Ad vectors, but a clinical trial assessing repeat administration of an AAV vector, proven to transduce the human airway epithelium efficiently, is the only way to address this question reliably. Interestingly, Lui *et al.* have shown that AAV vectors may be able to transduce progenitor cells in the mouse lung (36), which if efficient may help to overcome repeat administration problem. Recently, Faust *et al.* have shown that depletion of CpG dinucleotides from the AAV vector, a strategy which reduced inflammation and prolonged gene expression of non-viral vectors, reduced adaptive immune responses and inflammation after intra-muscular injection of AAV vectors (37). It remains to be seen if CpG depleted AAV vectors offer any advantages for lung applications.

Cytoplasmic viruses

In addition to Ad and AAV, various cytoplasmic RNA viruses have been validated for airway gene transfer. The murine parainfluenza virus type 1 [or SeV], the human respiratory syncytial virus and the human parainfluenza virus have all been shown efficiently to transfect AECs via the apical membrane (38,39) using sialic acid and cholesterol, which are abundantly expressed on the apical surface of AECs. These viruses have a negative strand RNA genome and, in contrast to other viral vectors, replicate in the cytoplasm. Only SeV has been assessed in animal models *in vivo*, and is arguably the most efficient vector for transducing airway epithelial cells, but repeated administration was not feasible (extensively reviewed in (28)) and the vector did, therefore, not progress into clinical trials.

Lentiviral vectors

Most recently lentiviral vectors which integrate into the host genome have gained interest and for reasons described below, in our view, hold promise for CF gene therapy. Recombinant human immunodeficiency virus (HIV) is most commonly used, but in the context of lung gene transfer simian (SIV) (40,41), feline (FIV) (42) and equine (EIAV) (43) immunodeficiency viruses have also been studied. Lentiviral vectors are commonly pseudotyped with an envelope glycoprotein from the vesicular stomatitis virus G (VSV-G), allowing for a broad tissue tropism. However, VSVG-pseudotyped vectors only poorly transduce airway epithelial cells and require the addition of tight junction openers such as LPC to allow virus entry in airway cells (44,45). Several groups have replaced VSV-G with other envelope proteins to improve airway transduction (41–43,46,47). We, for example, used the SeV-derived F and HN envelope proteins and achieved efficient and persistent gene expression in mice (life-long expression after a single dose) and relevant human airway models (41) (Fig. 1). Stocker *et al.* have demonstrated that lentivirus-mediated CFTR expression in nasal epithelium of CF knockout mice allows partial correction of the chloride transport to persist for at least 12 months (48). In preparation for experiments in CF knockout pigs, Sinn *et al.* have recently shown that FIV pseudotyped with the baculovirus GP64 envelope protein can transduce porcine airways (49). In contrast to SeV, adeno and AAV vectors, we and others have shown that lentiviral vectors can be repeatedly administered to murine airways (41,42), which is a major requirement for the treatment of chronic diseases such as CF, although it remains to be assessed if repeat administration is feasible in man.

Before progression into clinical trials, two major points have to be addressed:

(i) Safety of genomic intergration of lentiviral vectors in the lung. Encouragingly, we have not observed any side-effects in a 2-year- mouse study (50).
(ii) Scale-up of vector production, because comparatively high virus titers will be required to treat a large organ such as the lung.

HOW SUCCESSFUL ARE NON-VIRAL VECTORS?

Non-viral gene transfer formulations have two components: (1) The nucleic acid ie the therapeutic cDNA and appropriate regulatory elements and (2) a carrier molecule which binds to the DNA.

Non-viral vectors

A large number of carrier molecules have been developed, which broadly fall into either cationic lipids or cationic polymers. Both classes of molecules bind to negatively charged plasmid DNA through charge interaction and either encapsulate or condense the DNA to generate lipoplexes and polyplexes. The mechanism of non-viral gene transfer is poorly understood, but it is thought that lipoplexes and polyplexes bind to the cell membrane, are endocytosed and subsequently escape from endosomes by inducing rupture of the endosomal membrane (51). Non-viral vectors are generally less efficient than viral GTAs. This is due to a lack of specific components that would help with cell entry, endosomal escape, movement through the cytoplasm and nuclear uptake, all stages for which viruses have evolved efficient strategies and specific proteins (52). The simpler composition of non-viral vectors, however, may be an advantage. If free of 'non-human' protein components, successful re-administration may be easier to achieve compared with viral vectors (although a case-by-case assessment will be necessary to determine efficacy on repeat administration). Importantly, proof of principle for repeat administration of liposome-based gene transfer in human airways has been established in clinical studies. (12)

A flurry of activity in the 1990 s led to the development of a myriad of non-viral GTAs. However, it is our impression that progress in developing novel formulation for airway gene

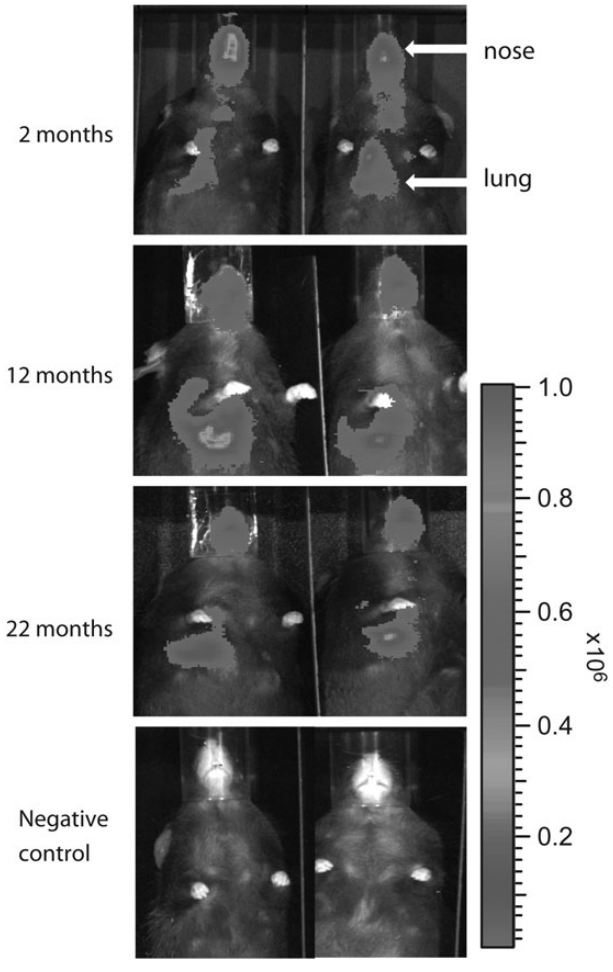

Figure 1. F/HN-SIV transduction leads to persistent gene expression in mouse airways. Mice were transduced with F/HN-SIV-Lux (5×10^8 TU/mouse) by nasal sniffing (or received PBS (negative controls). Luciferase expression was visualized using bioluminescence imaging. Bioluminescence *in vivo* imaging 2–22 months after transduction.

therapy over the last 5–10 years has been modest (see (52) for a more comprehensive review).

Nucleic acid components

In addition to the non-viral carrier, the nucleic acids component is an important factor in non-viral gene transfer, and improvements are at least as important as improving the lipid or polymer vector. Most pre-clinical and clinical studies have relied on the use of viral promoters such as the cytomegalovirus (CMV) promoter/enhancer to regulate gene expression. These generally lead to comparatively high level at early time-points (1–2 days), but very transient gene expression (9,53). Loss of transgene expression cannot simply be explained through plasmid degradation (54) Pringle *et al.* also assessed if plasmid silencing may be due to methylation of the DNA, which can cause silencing of integrating viral vectors (55), but did not find any evidence for *de novo* methylation after non-viral gene transfer (54). It has been shown, however, that vector-induced secretion of inflammatory cytokines silences the CMV promoter/enhancer (56). Importantly, problems related to transient lung expression after non-viral gene transfer have been overcome by the use of endogenous mammalian promoters (57,58) or, more recently, through the use of viral and mammalian hybrid regulatory elements (53). This study led to selection of a hybrid promoter consisting of a human CMV enhancer and the human elongation factor 1α promoter (hCEFI). This chimeric promoter/enhancer supports prolonged (months) gene expression in mice (53) and was selected for our ongoing clinical trial programme (see below). Unmethylated nucleotides (CpG motifs) in the plasmid can activate the innate immune system via toll-like receptor 9 (TLR9) and lead to production of pro-inflammatory cytokines. CpG-free plasmids not only reduce inflammation, but also lead to long-lasting gene expression when administered to the mouse lung (53).

A more detailed understanding of how plasmid DNA moves from the cytoplasm into the nucleus of non-dividing cells may help to improve gene transfer into differentiated airway epithelium. It has been hypothesized that DNA-binding proteins destined to move into the nucleus bind to plasmid DNA in the cytoplasm and support nuclear uptake of the DNA using the nuclear import machinery.

Published clinical trials

Nine clinical trials have evaluated non-viral gene transfer to nasal or lung epithelium (27). These studies were largely single-dose Phase I safety studies, but in most cases included some molecular or bioelectrical efficacy endpoints; detection of vector-specific CFTR mRNA or potential difference measurements to assess correction of the ion transport defect. However, detection of vector-specific mRNA proved difficult in most studies. This may be due to patchy gene transfer combined with comparatively small numbers of cells from a limited region being collected, although in some studies technical difficulties prevented extraction of mRNA. Interestingly, the majority of the studies have shown partial correction of the chloride transport defect in nasal epithelium. In addition, we were able to demonstrate ∼25% correction of the chloride transport defect in the lung (9). These findings provide proof of principle for non-viral CFTR gene transfer to the airways of CF patients and may argue that potential difference measurements may be more sensitive than RT-PCR.

Despite these promising results, it is currently unknown whether non-viral GTAs will be able to improve CF lung disease, because none of the trials were designed to assess clinical efficacy of gene therapy.

Ongoing clinical trials

A critical observer may question whether the field has benefited from the large number of non-viral Phase 1 proof-of-principle studies and may suggest a more coordinated and joined-up strategy. In an attempt to join-up CF gene therapy across Britain, we formed the UK CF Gene Therapy Consortium, (http://www.cfgenetherapy.org.uk) and we currently conducted the only active CF gene therapy clinical trial.

From the outset we understood that one-time administration of a GTA (single dose) to the lung will unlikely lead to significant improvements in CF lung disease severity and, therefore, planned our clinical trial programme to culminate in a multi-dose clinical trial sufficiently powered to support a clinically relevant primary endpoint (see below).

In an extensive pre-clinical research program, we determined that the cationic lipid formulation GL67A (Genzyme Corporation) a comparatively old lipid, which we had already used 15 years ago in a CF clinical trial (9), was still the most efficient non-viral vector currently available. We also showed that repeat aerosolization of GL67A/pDNA complexes was feasible without loss of efficacy (manuscript submitted). In addition, we further optimized the DNA component. Key modifications included: (1) Incorporation of a hybrid promoter consisting of a human CMV enhancer and the elongation factor 1α promoter (hCEFI) (2) CpG motif depletion to limit inflammatory responses in man (53). The final completely CpG-depleted plasmid carrying the CFTR cDNA under control of the hCEFI promoter is called pGM169.

We have completed a single-dose safety pilot trial ($n = 36$ subjects) and have succeeded in identifying a safe dose suitable for progression into a multi-dose trial (manuscript in preparation). The Phase 2B multi-dose trial is a double-blinded placebo-controlled study (59). Recruitment of trial participants has recently been completed. Trial participants are receiving 12 monthly doses of GL67A/pGM169 or placebo over a 1 year period. The primary endpoint is a change in $\%FEV_1$ from baseline. Secondary endpoints include lung clearance index, computed tomography and quality-of-life questionnaires.

CONCLUSIONS

Over the last two decades, our understanding of strength and weaknesses of both viral and non-viral vectors has significantly improved. CF remains a very promising target for gene therapy because it is a recessive condition, but the various extra- and intracellular barriers present a challenge. The transduction of bone marrow-derived hematopoietic stem cells *ex vivo*, an organ that present fewer barriers, led to the successful treatment of patients suffering from Wiskott–Aldrich syndrome and metachromatic leukodystrophy (60,61) for example. These studies provide proof of concept for successful implementation of gene therapy, but also highlight the need for overcoming the barriers presented by the lung through innovative approaches.

The question 'can gene therapy' improve CF lung disease remains unanswered, but the results of the first multi-dose non-viral trial designed to assess clinical efficacy are eagerly awaited. If successful, a Phase 3 study will rapidly follow. If unsuccessful, alternative strategies will have to be assessed. Although some progress has been made in improving non-viral gene transfer (recently reviewed in (28)), it is uncertain whether these will lead to a step change in non-viral vector efficiency. In our view, appropriately pseudotyped lentiviral vectors are more likely to provide a step change in efficacy of CF gene therapy and may also be suitable for treating other lung diseases. Specifically, using the lung as a factory to produce secreted proteins acting either locally (e.g. α1 anti-trypsin) or systemically (e.g. Factor VIII/IX) may be achievable using the potent lentiviral vectors.

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