REVIEW

Gene therapy for cystic fibrosis: an example for lung gene therapy

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Gene therapy is currently being evaluated for a wide range of acute and chronic lung diseases. The requirement of gene transfer into the individual cell types of the complex lung structure will very much depend on the target disease. Over the last decade, the gene therapy community has recognized that there is not even one vector that is good for all applications, but that the gene transfer agent has to be carefully chosen. Gene therapy is particularly attractive for diseases that currently do not have satisfactory treatment options and probably easier for monogenic disorders than for complex diseases. Cystic fibrosis (CF) fulfills these criteria and is therefore a good candidate for gene therapy-based treatment. This review will focus on CF as an example for lung gene therapy and discuss the progress made in this field over the last couple of years.

Keywords: cystic fibrosis; lung gene therapy; airway gene transfer

Introduction

Gene therapy is currently being evaluated for a wide range of acute and chronic lung diseases including acute respiratory distress syndrome (ARDS), cancer, asthma, emphysema and cystic fibrosis (CF), not least because of the comparatively easy noninvasive accessibility of the lungs through aerosols. The lung is a complex organ and can be roughly divided into two main regions: the airways, consisting of trachea, bronchi, large and small airways, which transport air to the peripheral lung, and the alveoli, where gas exchange takes place (Figure 1). The cell types facing the lumen vary greatly from pseudostratified, columnar ciliated and nonciliated epithelium in the larger airways, to single-layer cuboidal epithelium in the small airways and type I and II pneumocytes in the alveolar epithelium. The requirement of gene transfer into the individual cell types will very much depend on the target disease. In addition, tumour and perhaps inflammatory cells may also be important targets for gene transfer. Over the last decade, the gene therapy community has recognized that there is not even one vector that is good for all applications, but that the gene transfer agent (GTA) has to be carefully chosen depending on the cell type to be targeted, the number of treatments (one versus repeat administration) required, and the size and nature (secreted versus cellular product) of the gene to be delivered.

Gene therapy is particularly attractive for diseases that currently do not have satisfactory treatment options, and is probably easier for monogenic disorders than for complex diseases. CF fulfills these criteria and is therefore a good candidate for gene therapy-based treatment. This review will mainly focus on CF as an example for lung gene therapy.

CF is the most common lethal autosomal recessive disease in the Caucasian population and affects approximately 70,000 individuals worldwide. Although several organs are affected, severe lung disease is the cause of most of the morbidity and mortality in CF individuals. The CF gene, the cystic fibrosis transmembrane conductance regulator (CFTR), was cloned in 1989 and is a chloride channel located in the apical membrane of epithelial cells. Mutations in the CFTR gene lead to imbalanced ion and water movement across the airway epithelium, resulting in accumulation of sticky mucus, chronic bacterial infection and inflammation. Proof-of-principle for CFTR gene transfer was quickly established in vitro and in animal models. The first clinical trials in CF patients were carried out in 1993 and to date 29 trial protocols, most of which have been completed, are published (http://www.wiley.co.uk/genmed/clinica/). The initial hope was that CF gene therapy would progress rapidly, due to the ease of noninvasive access to the lungs, but delivery of the gene to the relevant cells remains a difficult task. Here, we will review the considerable progress that has been made in pre-clinical and clinical gene therapy studies for CF over the last couple of years.

Extra- and intracellular barriers to lung gene transfer

In non-CF individuals, CFTR is not expressed abundantly in the lungs, but high expression is seen in serous cells in the submucosal glands and isolated epithelial cells in the small airways. It is currently unclear which
of these cell types is the main target for CF gene therapy. However, given that CF, at least in the early stages, presents as a small airway disease, airway epithelial cells (AECs) are likely to be an important target. Topical delivery of GTA to the lung is currently the preferred method for airway gene transfer. However, before the GTA can reach the surface of the epithelial cells, a number of extracellular physical and immunological barriers have to be overcome (reviewed in Ferrari et al. and Weiss). Briefly, the airway epithelium in the lung is generally covered by a thin mucus layer (Figure 2), whose main role is to trap invading foreign particles. It has been shown that mucus significantly reduces the transfection efficiency of most viral and nonviral GTAs. However, transfection efficiency could be increased through pretreatment with mucolytics or the anticholinergic drug glycopyrrolate in vitro and in vivo. In CF patients, particularly at later stages in the disease, the airways are also filled with sticky sputum, consisting of inflammatory cells, cell debris, mucus and DNA. To avoid the confounding effect of sputum in vivo, gene transfer should ideally be carried out early in the course of lung disease, before the lungs become filled with secretions. The glycocalyx is also a barrier to gene transfer and pretreatment with neuraminidase, which removes sialic acid residues, enhances adenovirus (Ad) transfection of polarized cells in vitro. Although not formerly shown, it is likely that cilia also lead to steric hindrance of GTA to the apical surface of epithelial cells.

In addition to the physical barriers, specific and nonspecific immune defences are important inhibitors of airway gene transfer. Pulmonary macrophages have been shown to ingest GTAs, and removal of these cells before transfection has increased reporter gene expression by >90% in animal models. However, it is unlikely that removal of macrophages is clinically feasible. In addition to the cellular immune response, humoral immune responses against GTA are an important problem, severely restricting the use of viral vectors for chronic diseases such as CF.

Viral airway gene transfer

Adenovirus

Despite encouraging results in nasal and pulmonary tissues of pre-clinical models and being well tolerated at low-to-intermediate doses in humans, adenovirus-mediated gene transfer in the absence of epithelial damage has been inefficient in CF patients. This is mainly due to the absence of the coxackie-adenovirus receptor (CAR) on the apical surface of the majority of human AECs, and highlights the important differences in receptor distribution of animal models and humans. In an attempt to increase the transfection efficiency of adenoviral vectors in vivo, Gregory et al. assessed the effects of sodium caprate (a tight junction opener) application to the luminal surface of AECs in mouse lung, with the rationale that CAR expression is higher on the basolateral surface of epithelial cells. Gene expression in total lung homogenate was increased 25-fold, which further increased to 45-fold when adenovirus was complexed with 2-(diethylamino)ethyl ether (DEAE) dextran. However, it is unclear if this increase in gene expression was attributable to increased epithelial cell transfection. A controversial issue is whether such tight junction openers can be used clinically, given the heavy bacterial colonization present in the CF lung and the attendant risk of systemic invasion.

In addition to problems with low transfection efficiency, the use of adenovirus for a chronic disease like CF is limited due to effective cellular and humoral immune responses against the virus. Harvey et al. delivered three doses of Ad-CFTR to the lung of CF patients 3 months apart and demonstrated that after the third administration vector-specific CFTR mRNA was no longer detect-
able. Similar results have been obtained in animal models.

Helper-dependent adenoviral vectors, which are depleted of all viral genes, are less immunostimulatory and have improved safety profiles compared to first- and second-generation viruses, which have only a subset of viral genes deleted. Recently, it was shown that helper-dependent adenovirus combined with the epithelial cell-specific cytokeratin 18 (K18) promoter leads to reduced inflammation and more prolonged expression in murine airways.19

The use of adenoviral vectors for CF gene therapy is currently limited by low transfection efficiency and inability of repeated administration, but it remains to be seen if future virus improvements resurrect its use.

**Adeno-associated virus (AAV)**

AAV vectors have attracted much interest due to their good safety profile, broad tissue tropism, long duration of expression, and suggestion of their superior escape from immune system surveillance compared with other viruses. Several clinical trials have been carried out in the nose, sinus and single lobes of CF patients, all using the AAV2-based vector TgAAV-CFTR (Targeted Genetics Corp.). This vector contains the complete human CFTR cDNA and uses AAV inverted terminal repeat (ITR)-based promoter elements. Phase I studies aerosolizing AAV2-CFTR into CF patients with mild-to-moderate lung disease have been conducted. There were no safety problems and the vector was detected in the proximal sinuses of CF patients.21 Although the good safety profile was confirmed, none of the primary end points, including the time to sinusitis relapse, histopathology and interleukin (IL)-8 measurements, changed significantly when compared to the contralateral control sinus. Most recently, results of the first repeat-administration lung trial (three doses of nebulized AAV2 1 month apart) were published. The treatment was well tolerated and showed some evidence of improved lung function and reduced IL-8 in induced sputum after the first administration. A follow-up trial sufficiently powered to detect pulmonary changes has recently started.22

The small packaging capacity of AAV (<5 kb) precludes the use of this vector for transfer of larger genes. Although there is enough space for the CFTR cDNA, it is not possible to include potent promoter/enhancer elements. Thus, all clinical trials carried out with AAV2-CFTR have relied on the comparatively weak ITR regulatory elements, which may in part explain the disappointing efficacy data described above. Strategies to overcome the AAV packaging problem have therefore been developed, including approaches based on trans-splicing23 and homologous recombination.24 The basic principle of these techniques is to split the therapeutic cDNA and required regulatory elements, and package them into two viruses, which when transfecting the same cell may recombine and generate a full-length therapeutic gene. One would speculate that both of these strategies would lead to reduced transfection efficiency, when compared with the administration of one intact virus to the lung. However, surprisingly, Halbert et al25 have demonstrated that AAV2/6 (ITR from AAV2 and capsid from AAV6) recombination-dependent vectors transduced lung cells in mice almost as efficiently as intact vector, with 10% of AECs being positive.

Several different isoforms of human AAVs have been identified and further screening for new human and non-human primate isoforms is underway.25 It has already been documented that a virus with AAV5 or AAV6 capsid protein can enter AEC more efficiently than AAV2 viruses, but the overall transfection efficiency is still comparatively low.26 Recently, the atomic structure of AAV2 has been identified, which should enable rational engineering of vector capsids for specific cell targeting.27 Shi et al28 have already identified specific regions within the capsid protein that can tolerate the insertion of small exogenous peptides and have made an attempt at incorporating integrin-targeting peptides into this region.

It has been postulated that AAV may not infect antigen-presenting dendritic cells and thereby avoids activation of the host immune system. If this is true, AAVs, in contrast to other viruses, may be suitable for repeat administration. The results of repeat administration have been reported to vary greatly and may depend on the host, delivery route and AAV serotype tested.29-30 Aurichio et al30 have shown that AAV2/5 can be re-administered once to the mouse lung 5 months after the first delivery. Most recently, Fischer et al treated non-human primates with serial doses (three administrations) of aerosolated AAV2. This study goes some way towards demonstrating that repeat administration of AAV2 maybe possible, despite increasing titres of neutralizing antibodies.32 Importantly, repeat aerosolization of AAV2-CFTR into CF patients is safe and well tolerated22 and phase II efficacy trials are currently being carried out to determine if repeat administration in humans results in persistent gene expression.

**Negative-strand RNA viruses and lentivirus**

The murine parainfluenza virus type 1 (or Sendai virus (SeV)), the human respiratory syncytial virus (RSV) and the human parainfluenza virus type 3 (PIV3) have all been shown to efficiently transfect AECs via the apical membrane33,34 using sialic acid and cholesterol, which are abundantly expressed on the apical surface of AECs. These viruses have a negative-strand RNA genome and replicate in the cytoplasm. They do not go through a DNA intermediate and do not enter the nucleus. Only SeV has been assessed in animal models in vivo and is currently the most efficient virus for airway gene transfer. First-generation recombinant SeV carrying CFTR cDNA can produce functional CFTR chloride channels in vitro and after transfection of the nasal epithelium in CF knockout mice.35 Further improvements in the SeV vectors have been made by deleting the F-protein from the viral backbone (ΔF), which rendered the second-generation viruses transmission-incompetent. Inoue et al36 have further improved the ΔF/SeV vector by introducing mutations into the matrix (M) and hemagglutinin-neuraminidase (HN) proteins, which reduce the amount of virus-like particles that are produced after transfection, thereby further improving the safety profile. SeV-mediated gene expression is transient (lasting for about 7 days) and currently repeated administration
is inefficient. Several groups, including our own, are assessing a variety of immuno-modulatory strategies to improve the use of SeV for chronic lung diseases.

In contrast to retroviruses, lentiviruses transfect non-dividing cells and are, therefore, suitable for transfection of terminally differentiated AECs. The virus stably integrates into the genome of transfected cells and expression is therefore likely to last for the lifetime of the cell (approximately 100 days for AECs). However, when pseudotyped with the commonly used vesicular stomatitis virus G-glycoprotein (VSV-G), lentiviruses can only enter AEC via the basolateral membrane, using the inorganic phosphate receptor Pit2. Importantly, Pit2 is also expressed on the apical surface and binds ampiphotropic virus equally well on both membranes.36 Thus, other than unidentified factors contribute to the inefficient transfection of this virus via the apical membrane.

VSVG-pseudotyped HIV-derived lentivirus carrying the CFTR gene transiently and partially corrected the chloride defect in CF knockout mouse nose for up to 46 days.37 However, to achieve efficient transfection in the chloride defect in CF knockout mouse nose for up to 46 days, it is necessary to target the serpin-enzyme complex. It has previously been shown that lentivirus pseudotyped with envelope glycoproteins from the filoviruses Ebola or Marburg transfect AECs via the apical membrane, and that folate receptor alpha (FRα) is a cellular receptor for filoviruses. A recent report has shown that FRα is abundantly expressed on the apical surface of primary AECs, but interestingly does not appear to be absolutely required for filovirus uptake into the cells.38 In the presence of anti-FRα-blocking antibodies, virus entry was not affected. This indicates that cellular entry of lentivirus pseudotyped with filovirus envelope glycoproteins is likely more complex than via a single receptor.

As mentioned, above members of the paramyxovirus family, such as SeV and RSV, transfect AECs very efficiently. This is due to rapid interaction between the F and HN envelope glycoproteins with cholesterol and sialic acid residues on the cell surface, respectively. The F and HN proteins are therefore promising candidates for pseudotyping lentiviruses and Kobayashi et al.49 have recently demonstrated successful incorporation of F and HN envelope proteins into the capsid from simian immuno-deficiency virus (SIV). This vector was able to transduce polarized epithelial cells from both the apical and basolateral sides and we are currently evaluating this vector for airway transduction in animal models. Importantly, unless lentiviral vectors are able to hit airway stem cells efficiently, they will likely need to be re-administered and therefore will face the same immune-response problems as other viral vectors.

**Nonviral airway gene transfer**

Improving the efficiency of nonviral gene transfer to AECs has been a major focus with a variety of strategies being followed. Several groups are modifying polypelexes such as polylysine and polyethyleneimine (PEI) by adding sugars, based on the rationale that AECs express lectins, which selectively bind and internalize glycoconjugates. Although glycoconjugates containing lactose have been efficient in cell culture, their efficacy in vivo remains to be demonstrated.

Receptor-mediated gene delivery has been developed for AECs by targeting the serpin-enzyme complex receptor (Sec-R).43 This receptor is responsible for the uptake of serine proteases bound to their cognate inhibitors into cells. The receptor recognizes a conserved five-amino-acid-binding motif, but tolerates large variation in the attached cargo. Sec-R-directed complexes are prepared by condensing plasmid DNA with a covalent conjugate of a peptide receptor ligand (17 amino acids) and polylysine. Ziady et al.44 have recently demonstrated partial correction of the chloride transport defect in the nasal epithelium of CF knockout mice following administration of Sec-R ligand complexed to a CFTR plasmid. In nondividing cells, the nuclear membrane appears to be an important barrier to gene transfer and one reason why Sec-R ligand polylysine complexes transfect airway cells efficiently might be their small size. With a diameter of 18–25 nm, these nanoparticles may be able to enter the nucleus via passive diffusion through the nuclear pore complex, which has a cutoff size of about 25 nm. However, formulation and stability problems have so far prevented phase I clinical trials. Peptides resembling integrin-binding domains have also been linked to plasmid DNA and have been shown to transfect the airway epithelium of pigs when delivered at bronchoscopy.44 It remains to be established if antipeptide immune responses will interfere with using peptide-carrying nonviral formulations for chronic diseases, but the risk of immune responses against the peptide can be minimized by using conserved human peptide sequences. Importantly, traditionally used animal models may not be suitable to evaluate efficiency or repeat administration of human peptide formulation, if the chosen sequence is not conserved within the animal model.

Another nanoparticle formulation, consisting of a single plasmid molecule compacted with polyethylene glycol (PEG)-substituted polylysine (polymer of 30 lysines) has been developed. These DNA nanoparticles have a rod-like structure (12–15 nm diameter, 100–150 nm length). A single-dose escalation study to evaluate the safety of nasal administration into CF patients has recently been carried out in 12 subjects. In addition to assessing safety, secondary end points included assessment of electrical correction of the ion transport defect and molecular analysis for the presence of vector-specific DNA and mRNA. Administration of the nanoparticles was considered safe. In most patients, plasmid DNA could be detected in at least one nostril. There was no evidence of vector-specific mRNA in any patient, which may have been due to insufficient sensitivity of the assay. Partial correction of the chloride transport defect was demonstrated in seven out of 12 patients, which persisted for up to 15 days.45 Although these initial results are encouraging, further phase II trials will be necessary ultimately to determine the efficacy of these particles. In addition to improving nonviral DNA condensing agents, several groups are improving the plasmid vectors
for nonviral gene transfer. Yew et al. have demonstrated that reduction in CpG motifs in the pDNA reduces the immunostimulatory capacity of pDNA after systemic administration of liposome/pDNA complexes. Fewer changes in blood parameters of toxicity, reduced levels of inflammatory cytokines and decreased liver damage were observed after depletion of 80% of the CpG motifs. In addition, gene expression was prolonged in immunocompetent mice. Similar results were observed after topical administration of liposome/pDNA complexes to the lung (RK Scheule, personal communication).

Gill et al. have studied the effect of different promoters on persistence of lung gene expression by comparing the frequently used human immediate-early cytomegalovirus (CMV) promoter to the constitutive endogenous polyubiquitin C (UbC) and elongation factor 1α (EF1α) promoters. Although both eukaryotic endogenous promoters lead to about 10-fold less transgene expression at day 2, duration of gene expression was significantly improved when 'naked' pDNA was administered to the lung (CMV: <1 week, UbC: >16 weeks) and UbC-mediated gene expression reached CMV day 2 levels approximately 4 weeks after transfection. Similar results were reported by Yew et al. using the ubiquitin B (UbB) promoter. Promoter silencing is likely to contribute to these results and it has previously been demonstrated that the CMV promoter is silenced by TNFz and INFγ, which are both upregulated after gene transfer. However, it is currently unknown why the EF1α and UbC promoters are more resistant to gene silencing.

Despite the comparatively low transfection efficiency, nonviral GTAs offer important advantages over viral GTA for chronic disease. We and others are currently assessing a variety of physical delivery methods, including electroporation, magnetism, ultrasound and vibration, in an attempt to increase the transfection efficiency of nonviral formulations. Electroporation has been successfully used to enhance transfection in a variety of organs including muscle. Initial results for lung gene transfer are encouraging and demonstrate that the transfection efficiency of naked DNA can be enhanced in the presence of electrical fields (and Ian Pringle, personal communication). Clearly, important technical questions and safety considerations have to be resolved.

### Alternative delivery routes

Systemic delivery has long been postulated as a means for lung transfection and intravenous (i.v.) injection of many nonviral GTAs leads to lung transfection. It is important to note that for the vast majority of GTAs gene transfer is only achieved in alveolar endothelial cells and maybe pneumocytes, because the GTA gets trapped in the alveolar capillaries of the pulmonary circulation, the first capillary bed encountered after i.v. administration, but are found only rarely in the conducting airways, which are the targets for CF gene therapy. To be able to transfect the conducting airway epithelium, the GTA has to pass through the pulmonary circulation, reach the left side of the heart and travel from there to the bronchial circulation, which supplies the airways (Figure 3). Here, the GTA has to escape from the vessels and migrate through a dense layer of extracellular matrix to the basement membrane of the AECs. We have recently demonstrated that naked oligonucleotides are able to follow this route and transfect the cytoplasm of AECs efficiently. Koehler et al. have shown that the bronchial epithelium and submucosal glands can be transfected using plasmid DNA complexed to the cationic liposome DODAC:DOPE, although this was not reproducible with other lipids and appears to be a characteristic property of this particular liposome. A better understanding of the mechanisms involved in increasing organ and cell-type specific targeting will be important to minimize systemic gene transfer and toxicity.

### Alternative non-CFTR cDNA nucleotide-based therapies

Gene repair of the endogenous CFTR gene has two major advantages over traditional gene therapy. If successful, gene repair should ensure gene expression for the lifetime of the cells and appropriate control of gene expression is likely because the endogenous CFTR promoter is utilized. Our preliminary results indicated that the genomic CFTR locus could be modified in primary rat hepatocytes, but not primary AECs, using chimeraplasts (DNA/RNA hybrid oligonucleotides). Hepatocytes have previously been shown to be easily amenable for gene repair strategies, most likely due to efficient uptake of repair molecules into the nucleus. In addition, a similar approach using small-fragment homologous recombination (SFHR) was able to reintroduce the wild-type CFTR sequence into the lungs of CF knockout mice. Overall, the mechanisms involved in...
gene repair are not well understood and it is currently uncertain if the required ‘repair’ proteins are present in terminally differentiated AECs. In addition, uptake of repair oligonucleotides into the nucleus of AECs ex vivo and in vivo remains inefficient (Uta Griesenbach, unpublished observation) and is the first hurdle that needs to be overcome, before gene repair can be assessed.

Downregulation of gene expression through antisense molecules may be of therapeutic benefit in CF patients. Lambert et al. showed that antisense inhibition of the B-cell antigen receptor-associated protein (BAP) 31 increased expression of both wild-type CFTR and mutant CFTR and partially restored CFTR chloride channel function. The exact function of BAP31 is unclear, although the authors speculated that the protein may be involved in retaining mutant CFTR in the ER. Several other chaperone proteins, mucins or the epithelial sodium channel (ENaC), which is hyperactive in CF, may be suitable candidates for antisense strategies. We have recently assessed RNA interference-mediated gene silencing in the lungs in vivo, and although proof-of-principle could be demonstrated efficiency was low, likely due to low transfection efficiency.

Spliceosome-mediated trans-splicing (SMArT) has recently been introduced as a means to generate wild-type CFTR mRNA in CF xenograft models. Cells were transfected with very high titres of adenovirus that produced the so-called pre-therapeutic wild-type CFTR mRNA molecules (PTMs), which are designed to promote trans-splicing with the endogenous CFTR mRNA. Similar to gene repair, SMArT ensures cell-type-specific expression of wild-type CFTR mRNA; however, the technology requires further optimization with respect to efficiency and specificity.

Animal models

The choice of the correct animal model is a crucial factor in developing gene therapy for CF. Currently, the CF knockout mouse is the only CF animal model and although these mice do not develop the characteristic CF lung disease, they have the same ion transport defect as CF patients in their nasal epithelium. This, combined with the fact that the nasal epithelium can easily be exposed to GTAs, makes the CF mouse nose an ideal organ for assessing and optimizing gene transfer. In addition, non-CF primates, pigs and most recently sheep have been used to optimize airway gene transfer and allowed clinically relevant delivery methods such as nebulization to be assessed. More recently, first attempts have been made at generating CF ferret and sheep based on targeting of the CFTR locus in somatic cells coupled with nuclear transfer (and Jim McWhir, Roslin Institute, personal communication).

Assays

The success of pre-clinical and clinical CF gene therapy studies stands and falls with the assays used to evaluate gene transfer. The development of new CFTR-specific assays involving epithelial cell-specific detection of CFTR mRNA and protein, bacterial adherence to AECs, airway surface liquid height measurements and others are currently a major focus of the UK Cystic Fibrosis Gene Therapy Consortium (www.cfgenetherapy.org.uk). For clinical studies, the most relevant end points are a reduction in decline of lung function over time and of episodes of infection. However, these end points are not suitable for early phase II trials, because large patient numbers (>500) and long follow-up (>12 months) would be required. It is therefore crucial to identify clinical surrogate end points (such as bacterial burden, inflammatory markers and imaging) that can be assessed in smaller patient cohorts with shorter follow-up. It is unlikely that one-time administration of a short acting GTA will change these clinical surrogate end points, but will more likely require repeat administration and it is therefore important to design future gene therapy trials with these surrogate end points in mind. An extensive discussion about assays is outside the scope of this review, but has recently been reviewed.

Summary

Over the last decade, it became apparent that gene transfer to the AECs is difficult. This is perhaps unsurprising, given the lung has evolved to keep foreign particles out. The major obstacle for most viral GTAs is the effective immune surveillance mechanisms in the lung, which prohibit repeat administration. Many strategies to overcome this problem have already been explored, but have not yet been successful. In our view, this may be a difficult hurdle to overcome. Nonviral gene transfer has traditionally been inefficient, but recently developed nanoparticles and ligand-targeting appear to be overcoming this problem. Importantly, physical delivery methods to increase nonviral gene transfer are currently being assessed in the lung. Although gene therapy for CF is not yet a clinical reality, the many innovative strategies currently being assessed should lead to efficient and repeatable airway gene transfer within the next few years.

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