Genetic therapies for cystic fibrosis lung disease

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The aim of gene therapy for cystic fibrosis (CF) lung disease is to efficiently and safely express the CF transmembrane conductance regulator (CFTR) in the appropriate pulmonary cell types. Although CF patients experience multi-organ disease, the chronic bacterial lung infections and associated inflammation are the primary cause of shortened life expectancy. Gene transfer-based therapeutic approaches are feasible, in part, because the airway epithelium is directly accessible by aerosol delivery or instillation. Improvements in standard delivery vectors and the development of novel vectors, as well as emerging technologies and new animal models, are propelling exciting new research forward. Here, we review recent developments that are advancing this field of investigation.

INTRODUCTION

Since the discovery of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene in 1989, CF has been in the sights of scientists hoping to prevent or delay the onset and progression of lung disease through the use of gene transfer. Although loss of CFTR function adversely affects multiple cells and tissues, progressive lung disease accounts for the majority of the morbidity and mortality. For this reason, most effort in the field has focused on gene transfer to the airways. CFTR is expressed in multiple epithelial cell types in the surface and submucosal glands of the conducting airways where its mRNA is expressed in low abundance. The gene product is an apical membrane anion channel that is regulated by nucleotides and phosphorylation (1–3). Loss of CFTR function likely alters the volume and composition of airway secretions, but key details of the molecular pathogenesis of CF lung disease remain the subject of intense study.

Complementation of this autosomal recessive disease by the delivery of a CFTR cDNA to the airway epithelium with a viral or non-viral vector holds appeal, as the envisioned target cells are accessible via direct instillation or aerosol delivery approaches. Furthermore, early studies indicated that complementation of as few as 6–10% of CF epithelia generated wild-type levels of chloride transport in vitro (4). However, since the completion of the first human gene therapy trial in 1993, the achievement of this goal has proved challenging.

There is controversy regarding which cells to target for CF gene therapy. Arguments can be made in support of correcting cells of the surface epithelium, the submucosal glands, or both (5–8). A heterogeneous population of cell types express CFTR in the airways, including ciliated cells within the surface epithelium and a subpopulation of cells in submucosal gland ducts and acini. There appear to be several epithelial cell types in the lung that provide progenitor functions, providing the possibility of long-term correction if such cells can be targeted with integrating vectors (9). These cells may represent a pluripotent population or serve as progenitors for a specific lineage. Experiments from several species and model systems identify potential progenitor populations, including: basal cells (10,11) and non-ciliated columnar cells of the airways (10,12,13), submucosal gland epithelia (14–16), Clara cells (17,18) and alveolar type II cells in the distal lung (19,20). Studies using integrating vectors [Moloney murine leukemia virus (MLV) and lentivirus based] suggest that if cells with progenitor capacity are targeted in vitro and in vivo, long-term expression can be attained (21–25).

RECENT VECTOR DEVELOPMENTS

Enveloped viral vectors

Current lentiviral vector technology has made considerable progress toward the aims of efficiently, safely, and persistently expressing CFTR in the appropriate pulmonary cell types. Studies are beginning to examine the consequences of repeated administration of lentiviral-based vectors in the airways. Sinn et al. (26) repeatedly administered (seven doses, one dose/week) a baculovirus envelope (GP64)-pseudotyped feline
immunodeficiency virus (FIV)-based lentiviral vector to the nasal epithelia of mice and observed dose-dependent increases in reporter gene expression that persisted for the 80-week duration of the experiment (Fig. 1). The observed innate or adaptive immune responses to the vector or vector-encoded transgenes were minimal and failed to curtail reporter or therapeutic gene expression. In contrast, Limberis et al. (27) reported that gene transfer with a VSV-G-pseudotyped HIV vector resulted in activation of transgene-specific T cells in mice. Transduction by VSV-G-pseudotyped HIV vectors can be further improved by formulations including magnetofectins (28), polyethyleneimine (29) or lysophosphatidylcholine (30,31). Such methods may prove vital for achieving transduction efficiencies sufficient to correct the chloride transport defect in vivo. Careful preclinical studies in large animal models will be needed to further assess the safety and efficacy of the different lentiviral vector platforms under investigation for pulmonary gene transfer.

Fetal and neonatal re-administration of a GP64-pseudotyped HIV vector was also investigated in the mouse lung (32). Buckley et al. (32) compared a single fetal intra-amniotic administration, one fetal and two neonatal administrations and two neonatal administrations. The levels of macrophage transduction increased with neonatal re-administration. The authors speculated that following the initial dose of lentiviral vector, macrophages are recruited to the pulmonary lumen and are subsequently transduced by the second and third doses. The authors further concluded that intra-amniotic administration of GP64-pseudotyped HIV was the most efficient mode of delivery for achieving airway epithelial cell transduction in the mouse model.

Mitomo et al. (33) described a simian immunodeficiency virus (SIV)-based lentiviral vector pseudotyped with the Sendai virus envelope proteins, hemagglutinin-neuraminidase (HN) and fusion (F) protein. F/HN-pseudotyped SIV vector transduced nasal epithelial cells, resulting in sustained transgene expression for the duration of the experiment (8–12 months) in vivo. Similar to studies with GP64-pseudotyped FIV (26), re-administration was feasible with F/HN-pseudotyped SIV, where transgene expression remained stable after three vector doses. In addition, F/HN-pseudotyped SIV conferred functional CFTR expression in vitro as determined by iodide efflux assay.

Paramyxovirus family members with known airway tropism are currently being explored as potential CFTR delivery vehicles for the treatment of CF lung disease using reverse genetics systems. Kwilas et al. (34) recently demonstrated that a respiratory syncytial virus (RSV)-based vector could deliver CFTR and correct the chloride transport defect in primary cultures of human CF airway epithelia. In addition, a human parainfluenza virus (PIV)-based vector mediated detectable but transient expression of GFP and α-fetoprotein in rhesus macaques (35). Both the RSV- and PIV-based vectors are replication competent; however, these studies may lead to replication-attenuated vectors that are further engineered to reduce the expression of cytotoxic and/or immunogenic proteins. If such engineering is feasible, it could improve the duration of gene expression, address the obstacle of pre-existing immunity, allow for repeat administration and make these vectors suitable for clinical studies.

**Encapsidated viral vectors**

Helper-dependent adenoviral (HD-Ad) vectors do not express viral-coding sequences and elicit reduced cell-mediated immune responses, compared with earlier generations of Ad vectors. However, HD-Ad capsid proteins remain targets for neutralizing antibodies and may trigger cytokine responses from innate immune effector cells. Recently, Cao et al. (36) demonstrated that transient immunosuppression significantly enhanced the efficiency of transgene expression and facilitated re-administration of HD-Ad vectors to mouse lungs. In addition to immunosuppression, serotype switching is a proposed technique to allow for redosing of CFTR expressing HD-Ad vectors in vivo (37). Granio et al. (38) delivered an Ad vector expressing GFP-tagged CFTR to primary cultures of human CF airway epithelia. They observed that swapping Ad5 fiber with serotype 35 fiber conferred more effective apical transduction and correction of the Cl⁻ transport defect. These data suggest that Ad vectors such as Ad5 that use the coxackie and adenovirus receptor (CAR) are less effective at transducing the apical surface of airway epithelial cells than CAR-independent vector serotypes such as Ad35. Taken together, these studies outline strategies for using HD-Ad,
immunosuppression, serotype switching and optimal fiber selection to improve the safety and long-term efficacy of adenovirus for gene transfer to the airways.

Recombinant adeno-associated virus (AAV) has been used for pulmonary gene transfer in several preclinical and clinical trials. Flotte et al. (39) demonstrated that AAV1 offered advantages over AAV5 in the chimpanzee airways, in terms of both gene transfer efficiency and reduced immunogenicity. Importantly, this observation was validated by studies in well-differentiated human airway epithelia, suggesting that the dual reporter virus co-infection approach can help predict efficacy of AAV vectors in vivo. Progress has also been made in engineering minimal CFTR expression cassettes that can be accommodated by the AAV vector (40).

Additional strategies to improve the efficiency of AAV transduction to airway epithelia include using different capsid serotypes or capsid mutants with a greater affinity for airway epithelial cells (41). As discussed in what follows, other novel AAV capsid variants have resulted from directed evolution and sequence shuffling (42–44). Although standard triple transfection methodology remains an option, new developments in baculovirus-based methods are better suited to meet AAV production requirements (45–47). In conclusion, improvements in AAV engineering, capsid serotype design and production methods have made AAV an attractive vector choice for delivering CFTR to the airways. Studies of efficacy and vector re-administration in large animal models will help guide vector development.

Non-viral vectors

Considerable progress has been made toward developing non-viral vectors for gene transfer to the lung. Typically, non-viral vectors fall into two categories: (i) non-integrating, such as plasmids (48), nanoparticles (49) and mini-circles (50), or (ii) integrating, such as transposons (51) and phage phiC31 (52,53). Both integrating and non-integrating non-viral vectors face many of the same delivery and transduction obstacles in vivo. Optimizing the delivery efficiency of DNA-based vectors to the in vivo airways remains a focus of the field (54,55). Doxorubicin (56), carboxymethylcellulose (57) and chitosan (58) improve plasmid-based gene transfer and expression in the airways. As an alternative, some groups are investigating hybrid vector systems combining features of adenovirus (59) or lentivirus with transposon-based vectors to improve delivery (60).

RECENT DEVELOPMENT OF CLINICAL STUDIES FOR CF GENE THERAPY

Since 1993, approximately 25 phase I/II trials using either viral or non-viral vectors for CF have been conducted (61). A currently ongoing clinical trial in the field was initiated by the UK CF Gene Therapy Consortium, funded by the Cystic Fibrosis Trust, using an aerosolized non-viral gene transfer agent (62). CF patients are receiving a single dose of a plasmid carrying the CFTR cDNA that is complexed to the cationic lipid GL67A. The plasmid, termed pGM169, is devoid of putative pro-inflammatory sequences (CpG islands), and gene expression is regulated by a hybrid elongation factor-1a promoter. When complexed to the cationic lipid GL67A, pGM169 led to >4-week expression in mouse models upon single dosing (48). The initial single-dose clinical trial will assess safety and duration of expression in patients and will guide a planned (approximately 100 patients) multi-dose placebo-controlled trial. The planned trial, due to start in autumn 2011 (Uta Griesenbach, personal communication), will determine whether repeated non-viral CFTR gene transfer (12 doses over 12 months) improves CF lung disease (61).

NEW TECHNOLOGIES

Directed evolution of viral vectors

As vehicles for gene therapy applications, all viral vectors have potential weaknesses, such as immunogenicity, tropism, transient transgene expression and production to high titers. The success in exploiting viral vectors will depend on the ability to overcome these limitations. Directed evolution of viruses is a method for generating new or improved viral protein properties using selection-based approaches. AAV and retroviruses have been the subject of combinatorial engineering approaches in the past decade. AAV has been attractive due to its safety profile, low immunogenicity and ability to transduce both dividing and non-dividing cells. The AAV capsid determines infectivity and cell tropism (63) and is therefore the target of modification by directed evolution (42) or phage panning (64). The breadth of naturally occurring AAV serotypes suggests that the capsid is tolerant to changes (65). Directed evolution of the AAV capsid by PCR-based mutagenesis combined with high-throughput in vitro recombination generated a library of chimeric cap genes with components from two diverse serotypes. These serotypes, AAV2 and AAV5, use distinct receptors, heparan sulfate and sialic acid, respectively (42). Further selection of this library for improved transduction of human airway cells in culture identified a novel AAV variant, AAV2.5T, a chimera between AAV2 (aa1–128) and AAV5 (aa129–725) with a single-point mutation (A581T) that exhibited enhanced binding to the apical surface of airway epithelia and improved gene transfer (42). Furthermore, AAV2.5T could efficiently express the CFTR cDNA in human airway cells in culture and correct the Cl− transport defect in human CF epithelia (42).

Gamma retrovirus vectors are also efficient gene delivery tools but insertional mutagenesis and potential oncogenesis due to preferential integration at transcriptional start sites (TSS) are limitations for their clinical use (66–69). Lim et al. (70) recently showed that the random insertion of engineered zinc finger domains throughout the MLV Gag-Pol region and selection of viable variants resulted in a shifting of the integration preferences of these vectors. Furthermore, these modified integration patterns did not favor TSS. This approach could be extended to lentiviruses and may serve as a powerful method to improve the safety profile of retroviruses as gene transfer vectors for clinical use.
Gene repair and gene addition

A technique known as ‘genome editing’ enables efficient and precise modification of a target sequence in a genome by introduction of a double-strand break (DSB) followed by modification of the locus during subsequent DSB repair by homologous recombination. The DSB is induced by a zinc finger nuclease (ZFN) (71–74), a specifically engineered endonuclease designed to cleave a chosen target in the genome. The ZFN consists of two components: the zinc finger protein (ZFP) and the non-specific cleavage domain of Fok1 endonuclease. The ZFP binds to the target sequence and contains a tandem array of Cys2-His2 fingers (75) each recognizing 3 bp of DNA. The arrays generally contain three or four fingers that bind a 9 or 12 bp target, respectively. The Fok1 domains must dimerize to cleave the DNA (76), consequently the specificity of the recognition site is doubled from 9 to 18 bp for a three-finger ZFN.

Homology-based genome editing can be exploited for correction of mutated genes responsible for monogenic disorders (Fig. 2). ZFN-based genome editing requires delivery of a donor DNA repair template along with the target specific ZFN pair. Methods for generating ZFN pairs targeting specific genomic loci are becoming widely available and include modular design approaches (77–79) and the selection-based oligomerized pool engineering (OPEN) strategy (80). ZFNs designed using OPEN technology have been shown to bind the genomic DNA-encoding CFTR (78) and to create DSBs near the ΔF508 mutation in exon 10. Provision of a wild-type donor DNA template with a non-integrating vector, such as integrase-deficient lentiviral vector (81), can facilitate repair of this mutation by homologous recombination.

Other repair strategies using homing endonucleases (82–84) or transcription activator-like effector nucleases (85) provide alternative mechanisms for creating DSBs in genomic DNA and allowing for gene repair by co-delivery of a homology repair template. A potential advantage of each of these gene repair approaches is that correction of CFTR in progenitor cell types could preserve the native regulatory elements and allow for correction in subsequent daughter cells. Reagent development and delivery to airway epithelia will be important for this field to advance.

An alternative to the gene repair approach is termed ‘targeted gene addition’ (Fig. 2). Here, ZFNs may be used to create DSBs at potential ‘safe harbor’ loci such as AAVS1 (86), CCR5 (87) or the mouse Rosa26 locus (88). In this approach, the entire therapeutic transgene with flanking homology arms would be inserted into the safe harbor loci by homologous recombination. Modification of such a locus is reasoned much less likely to perturb expression of neighboring genes or disrupt the function of other genetic elements (89).

Applications of RNA interference to treat CF

The recent explosion of knowledge in the field of small interfering RNAs has led to applications of direct relevance to CF. First, RNAi has been used as a tool to identify gene products that contribute to steps in wild-type and mutant CFTR biogenesis, including ER and Golgi trafficking, residence time in the cell membrane and its removal by proteasomal degradation (90–92). This has raised the possibility that RNAi-based strategies might be developed to increase the expression of ΔF508 CFTR, to rescue ΔF508 CFTR from proteasomal degradation.
or enhance its residence time in the cell membrane. Any of these approaches might provide sufficient residual CFTR function to be therapeutically relevant. Similarly, targeting other cellular pathways, such as those involved in inflammation, might offer a means to ameliorate disease symptoms and progression. A significant hurdle for translational studies in this area is identifying the methods to efficiently deliver RNAi to well-differentiated airway epithelia. Another area of investigation relevant to the field is the identification of the microRNA repertoire in airway epithelia and other CFTR expressing cells, as well as their respective target gene products. Knowledge in this area may identify new targets for therapeutic manipulation.

Lung tissue engineering
Lung transplantation is currently the only definitive treatment for end-stage CF lung disease. The supply of donor lungs is limited and transplantation achieves only a 10–20% survival at 10 years (93). Recently, two groups independently used similar tissue-engineering strategies to develop an autologous bioartificial lung that may begin to help overcome the limited availability of donor tissues (94,95). The bioartificial lungs were created by first generating a whole-lung scaffold by perfusion and decellularization of the adult rat lung, followed by reseeding of the endothelial and epithelial surfaces of the scaffold with new cells. Evidence for gas exchange within the resulting grafts was demonstrated. With the further development of this technology, one could envision the ex vivo correction of patient-derived cells, followed by lung tissue engineering and transplantation. Although these initial results are very exciting, several steps need to be further optimized before long-term tissue-engineered lung function can be translated to the clinic (96).

NEW ANIMAL MODELS OF CF DISEASE
A significant bottleneck in the development of new CF therapeutics has been the lack of animal models that recapitulate key features of lung and other organ disease pathogenesis. The mouse models with CFTR null alleles and specific disease mutations available since the early 1990s have contributed greatly to disease understanding but fail to develop spontaneous lung disease similar to humans with CF. Recently, two groups used somatic cell targeting of the CFTR gene with AAV vectors, followed by nuclear transfer and cloning to develop novel models in pigs (97,98) and ferrets (99,100). These new animal models recapitulate key features of CF disease (98,100). At birth, the lungs of CFTR null pigs are free of inflammation but manifest a bacterial host defense defect without the secondary consequences of infection (98,101). CF pigs spontaneously develop a lung disease phenotype mirroring key features of human CF lung disease in the first months of life, including infection with bacteria, airway remodeling and mucus hypersecretion (Fig. 3). CFTR null ferrets also develop multi-organ system disease, and neonatal animals manifest a pulmonary host defense defect in the airways associated with colonization by bacteria (100). There is also early evidence that adult CF ferrets develop a lung disease phenotype with similarities to human CF, including bacterial colonization (John Engelhardt, personal communication). These phenotypic features make these new models very attractive for gene therapy studies. They offer the unique opportunity to test gene therapy interventions prior to the onset of lung disease and monitor the outcomes for prevention-based treatment strategies.

CONCLUSIONS
This is an unprecedented time in the development of new therapies for CF. The near-universal availability of newborn screening for CF in developed nations has made early diagnosis commonplace, allowing the potential for treatment of healthy lungs before the onset of chronic lung disease. However, this opportunity comes at a time where there is a dearth of sensitive and specific markers of early disease that can be used to assess lung disease onset and monitor responses to therapy. Additional work is needed to develop new specific and sensitive measures of the early stages of lung disease suitable for monitoring the response to therapies for use in infants and young children. Parallel developments in improved gene transfer tools should further aid the field and lead to new clinical trials.
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Conflict of Interest statement. None declared.

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