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HUMAN GENE THERAPY CLINICAL DEVELOPMENT 26:38–49 (March 2015)
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DOI: 10.1089/humc.2014.154

Ferret and Pig Models of Cystic Fibrosis: Prospects and Promise for Gene Therapy

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Abstract

Large animal models of genetic diseases are rapidly becoming integral to biomedical research as technologies to manipulate the mammalian genome improve. The creation of cystic fibrosis (CF) ferrets and pigs is an example of such progress in animal modeling, with the disease phenotypes in the ferret and pig models more reflective of human CF disease than mouse models. The ferret and pig CF models also provide unique opportunities to develop and assess the effectiveness of gene and cell therapies to treat affected organs. In this review, we examine the organ disease phenotypes in these new CF models and the opportunities to test gene therapies at various stages of disease progression in affected organs. We then discuss the progress in developing recombinant replication-defective adenoviral, adeno-associated viral, and lentiviral vectors to target genes to the lung and pancreas in ferrets and pigs, the two most affected organs in CF. Through this review, we hope to convey the potential of these new animal models for developing CF gene and cell therapies.

Introduction

Cystic fibrosis (CF) is a common lethal autosomal-recessive disorder caused by mutations in a single gene encoding a protein, the cystic fibrosis transmembrane conductance regulator (CFTR).[1–3] CFTR is an anion channel, located in the apical membrane of epithelial cells, that conducts chloride and bicarbonate across the cell membrane.[4,5] CF affects at least 70,000 people worldwide and almost 2000 sequence variations have been identified in the *CFTR* gene.[6,7] The most common *CFTR* mutant is the deletion of a nucleotide triplet that results in the loss of a phenylalanine residue at position 508 of the CFTR protein (ΔF508CFTR). Approximately 70% of patients with CF carry two copies of the ΔF508 mutation, whereas 90% carry one.[8–10] *CFTR* gene mutations result in a wide range of organ-level dysfunction, including severe lung infections, pancreatic failure, intestinal obstruction, male infertility, and nutritional deficits.[11,12] A recurrent theme in CF organ disease is thick secretions and reduced pH caused by impaired bicarbonate transport.

Although CF affects multiple organs, lung failure due to chronic bacterial infections and inflammation is responsible for most morbidity and mortality.[13] Because CF is a monogenic fatal disorder, and the airway epithelium is an easily accessible target for gene therapy vectors, CF lung disease is an ideal genetic disorder for treatment by gene therapy.[14] Twenty-five clinical trials for CF lung disease have been implemented in approximately 450 patients with CF since the mid-1990s,[15] including those using recombinant adenovirus vector (rAD) targeting the nasal and bronchial epithelium[16–22]; recombinant adeno-associated virus (rAAV) with aerosolized administration to nose, sinuses, and lungs[23–27]; as well as cationic liposome or formulated DNA nanoparticles for nonviral *CFTR* gene transfer.[28–31] Despite the success of preclinical studies demonstrating efficacy of these recombinant vectors to correct CFTR channel defects, using *ex vivo* and *in vitro* airway model

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systems, all CF gene therapy trials to date have failed either to meet molecular end points or to improve lung function in patients with CF.[32–34] These failures are likely due to several issues, including (1) the lack of efficient gene transfer to cellular targets required to correct *in vivo* CFTR function,[35] (2) the animal models in which various preclinical vectors were tested,[36–39] and (3) previously unknown intracellular and extracellular barriers that limit viral transduction.[40–43]

Basic research on airway biology has found that gene delivery to airway epithelial cells *in vivo* must overcome a number of intracellular and extracellular barriers that physically or biologically hinder the delivery of DNA or viral vectors to the nucleus,[40,41,44,45] or target clearance of the vectors or infected cells through host immune surveillance.[46–51] Importantly, lung infection and inflammation in CF lung disease enhance these barriers. Challenges surrounding the physical barriers in the airway of a patient with CF, such as the thick layer of airway mucus secretion and the mechanisms of mucociliary clearance, were not completely recognized when the early CF lung gene therapy trials were conducted. Of note, the gene transfer agents used in these early trials were also not fully validated at that time[42,43] because of the lack of an animal model system that fully recapitulates the pathological condition of human CF lung disease.

Research on vector biology and virology has also revealed some inherent weaknesses that required solutions before applications in *CFTR* gene therapy. For example, in the initial rAAV2 clinical trials, the relative small package capacity (<5.0 kb)[52] of the AAV genome necessitated the use of a weak cryptic promoter in the AAV2 inverted terminal repeat (ITR) to enable packaging of the 4.44-kb *CFTR* genome.[24,53] It was also not known in early trials that rAAV2 has relatively high airway tropism in the preclinical rhesus monkey model[54] in contrast to human airway.[36,38] Subsequent studies demonstrated that rAAV2 has low tropism for transduction from the apical surface of the human airway epithelium[40] and has impaired proteasome-dependent intracellular processing and trafficking to the nucleus for productive transduction.[41,55,56] A major limitation of first-generation rAD vectors is the leaky expression of vector-encoded viral proteins that elicit strong humoral and cellular immune responses.[57,58] In addition, it is now known that the type 5 rAD receptor (coxsackievirus–adenovirus receptor) is not presented on the apical surface of human airway epithelium,[59,60] despite the fact that type 5 rAD highly transduced the murine airways used in preclinical studies.[50,61] In contrast, nonvirus-mediated approaches for *CFTR* delivery lack many of the limitations of viral vectors, including immunological barriers and tropism-specific features defined by species-specific receptors. However, liposome-plasmid based formulations are generally much less efficient at transfecting airway epithelium than viral vectors.[62,63]

Two larger animal models of cystic fibrosis in the ferret and pig have been generated by either disruption of the *CFTR* gene or introduction of the ΔF508 *CFTR* mutation.[64,65] Both CF ferrets and pigs spontaneously develop the lung disease phenotype, as well as pancreatic, gallbladder, and intestinal disease.[66,67] These models will be useful to test CF gene therapy in the context of disease that reproduces the human condition. In this review, we first briefly review the history of the development of these CF animal models and then describe their disease phenotypes at the organ level, with a focus on similarities and differences in organ phenotypes that will differentiate the gene therapy approaches that can be tested in the various affected organs. We then review the progress of using viral vectors to deliver foreign genes to the lung and pancreas in ferrets and pigs, which are two key target organs for future CF gene therapy efforts. We also discuss the prospects and practical issues of using the CF ferret and pig models for the development of CF gene therapies.

The Development of CF Animal Models

Mouse models of CF have been invaluable tools in the study of CFTR physiology in multiple organs for more than two decades.[68] More recently, the development of conditional CFTR knockout mice[69] has aided in dissecting novel CFTR functions in myeloid-derived cells and T cells.[70,71] However, major limitations of CF mouse models are the lack of spontaneous lung infections and pancreatic disease observed in patients with CF.[66,67] Several biologic reasons may account for the lack of pathology in these organs of CF mice. From an anatomical perspective, airway submucosal glands, which express abundant CFTR in human cartilaginous airways[72] and play important roles in lung innate immunity through the secretion of antimicrobials,[73,74] are present only in the proximal trachea of mice.[75] From an ion channel perspective, alternative non-CFTR, cAMP-activated, chloride channel activity appears to compensate for the lack of CFTR in the trachea[39,76] and pancreas in CF mice.[76–78]

In contrast to mice, ferrets and pigs share a high level of similarity in airway cytoarchitecture with humans[79–82] and have a similar composition of chloride channels in the airway.[83] The use of the ferret as an animal model for hypersecretory diseases such as CF and chronic bronchitis was first suggested in 1982, based on the properties of mucus, goblet cells, and submucosal glands throughout the tracheobronchial tree.[84] However, until somatic cell nuclear transfer (SCNT) cloning of mammals was first demonstrated in 1996 with the cloning of the sheep named Dolly,[85] this hypothesis could not be tested. The approach of combining SCNT with rAAV-mediated gene targeting in primary fibroblasts led to the successful generation of CF ferret[64] and pig models[65] in 2008.

Multiorgan Disease in CF Ferrets and Pigs Is Similar to the Human CF Phenotype

Although the lung is the primary organ that leads to mortality in CF, CF is a multiorgan disease for which disease in secondary organs such as the gut and pancreas can influence the health of the lung. For instance, CF-related diabetes and malnutrition are two examples by which pancreatic and intestinal health can negatively impact lung health in patients with CF. Thus, each of these organs is a potential target for gene therapy. Disease phenotypes in the CF pig and ferret have subtle differences in disease severity and time of onset. Hence, these two models present unique opportunities for testing gene therapies at various stages of CF organ disease.

Intestinal disease

Whereas only 15% of infants with CF suffer from meconium ileus (MI) at birth,[86,87] MI occurs in 100% of *CFTR* knockout piglets[88,89] and 75% of *CFTR* knockout ferret

kits.[90] The phenotype of MI in CF ferret kits and piglets is extremely similar to that which occurs in CF infants, including intestinal atresia, diverticulosis, and microcolon. Although intestinal surgery in newborn CF ferrets has not been possible due to size, it has been successfully used in CF pigs to rescue newborn animals. Malnutrition and distal intestinal obstruction syndrome in older CF ferrets and pigs is similar to that observed in patients with CF.[91,92] The creation of gut-corrected *CFTR* knockout ferrets and pigs harboring a wild-type *CFTR* transgene under the direction of the fatty acid-binding promoter will be useful models with reduced intestinal pathologies.[90,92] Furthermore, gut-corrected *CFTR* knockout models provide the opportunity to test gene therapies without the potential complication of developing cellular immunity against the *CFTR* transgene.

Pancreatic disease

At birth, only 3% of patients with CF have severe lesions associated with exocrine pancreatic destruction (EPD), whereas 72–92% infants demonstrate mild lesions associated with exocrine acinar duct dilatation (ADD).[93,94] However, damage to the pancreas continues after birth and ultimately leads to complete destruction of pancreatic exocrine function in most patients with CF, with 82% of adult patients with CF suffering pancreatic insufficiency. Inflammatory damage to the pancreas of *CFTR* knockout pigs begins *in utero* on embryonic day 83 (pigs have a 114-day gestation) and 100% of CF pigs have EPD at birth.[95,96] By contrast, newborn CFTR knockout ferrets exhibit relatively mild histopathology of the exocrine pancreas characterized by ADD similar to that seen in CF infants.[90,97] Interestingly, a small subset of *CFTR* knockout ferrets (<1%) demonstrates pancreatic sufficiency throughout life with normal weight gain and only minor exocrine damage.[91] These findings suggest that pancreatic modifier genes exist in CF ferrets, as has also been suggested in patients with CF. However, the exocrine pancreas of most *CFTR* knockout ferrets undergoes rapid destruction over the first month of life, leading to extensive fibrosis, loss of exocrine pancreas, islet remodeling, and diabetes.[91,97] Both CF ferrets and pigs also show abnormalities in insulin secretion at birth,[97,98] suggesting that abnormal islet function initiates early in CF. Because CF ferret and pig models have differing degrees of exocrine disease severity at birth, they present opportunities to test CF gene therapies that target early and late disease processes, respectively. Furthermore, both models may have unique utilities for testing gene therapies for CF-related diabetes.

Gallbladder and hepatic disease

Biliary cirrhosis and gallbladder disease are observed in 15–30% of patients with CF.[11,99,100] Both CF pigs and ferrets develop moderate hepatic lesions, including biliary cirrhosis, ductal hyperplasia, steatosis, and fibrosis.[88,89,91] Newborn CF ferrets also have abnormally elevated plasma alanine aminotransferase and bilirubin levels,[90] similar to those observed in CF infants,[101] and suggestive of early liver disease. Like the pancreas, gallbladder disease in CF ferrets and pigs progresses at different rates, with disease in the CF pig beginning *in utero* and in the CF ferret beginning postnatally. Microgallbladder with thick mucus secretions is found in 100% of CF piglets at birth,[88,89] whereas the newborn CF ferret gallbladder is histologically normal despite the electrophysiologic absence of cAMP-mediated chloride currents.[90,102] However, with age, the majority of CF ferrets develop gallbladder disease characterized by cystic mucosal hyperplasia as seen in humans.[91] The liver represents a tractable target for gene therapy in both CF pigs and ferrets, whereas gallbladder-directed gene therapy would likely be limited to CF ferrets because of the extent of disease observed in CF pigs at birth.

Lung and airway diseases

The CF lung is the primary target for gene therapy, as it is the most severely affected organ in CF. Both CF pig and ferret models develop spontaneous lung infections similar to that in human patients with CF. Although CF pigs lack lung inflammation at birth, they fail to eradicate bacteria and eventually develop lung disease within the first few months of life characterized by airway inflammation, remodeling, mucus accumulation, and infection.[88,103] CF ferrets also have a lung bacterial eradication defect, but demonstrate an abnormally elevated inflammatory response at birth.[104] Proteomics analysis of bronchoalveolar fluid from sterile Caesarean-sectioned and natural-born CF and non-CF ferrets suggests that alterations to lung immunity may begin before birth in CF kits and prime the lung for hyperinflammation after the first bacterial exposure during birth.[104] The onset of lung infections in CF ferrets is rapid and if animals are not reared on antibiotics, they succumb to polymicrobial lung infections within the first week of life.[90] However, improved methods of rearing CF ferrets on multiple antibiotics from birth to 6 months of age have allowed for the study of a more slowly progressive lung disease that recapitulates human CF lung disease.[105] Both CF pig and ferret models represent unique opportunities to evaluate gene therapies to the CF lung. Gene therapy end points to the neonatal CF ferret lung may also benefit from methods of closely monitored weight gain, for which small decreases have been demonstrated to be indicative of the onset of a lung infection (Fig. 1).[105]

Progress in Viral Vector Development for *CFTR* Gene Transfer

Over the last two decades, a significant effort has been made to solve the challenges encountered in early CF gene therapy clinical trials. Areas of focus have included the identification of viral vectors with the appropriate tropisms to infect the apical membrane of polarized human airway epithelia, understanding the intracellular barriers that limit viral transduction, and improving vector design to both limit immune recognition and allow for packaging of the large *CFTR* cDNA. Here we review some of the most significant advances in viral vectors for CF gene therapy.

Recombinant adenoviral vectors

The major limitation of recombinant adenoviruses for gene therapy is the immune recognition and T cell-mediated responses that lead to clearance of virally infected cells. Next-generation rAD vectors, also called helper-dependent adenoviral (HD-AD) vectors, have been developed.[106] HD-AD vectors have all viral protein-coding sequences deleted, which significantly reduces the host immune response.[107,108] Thus,

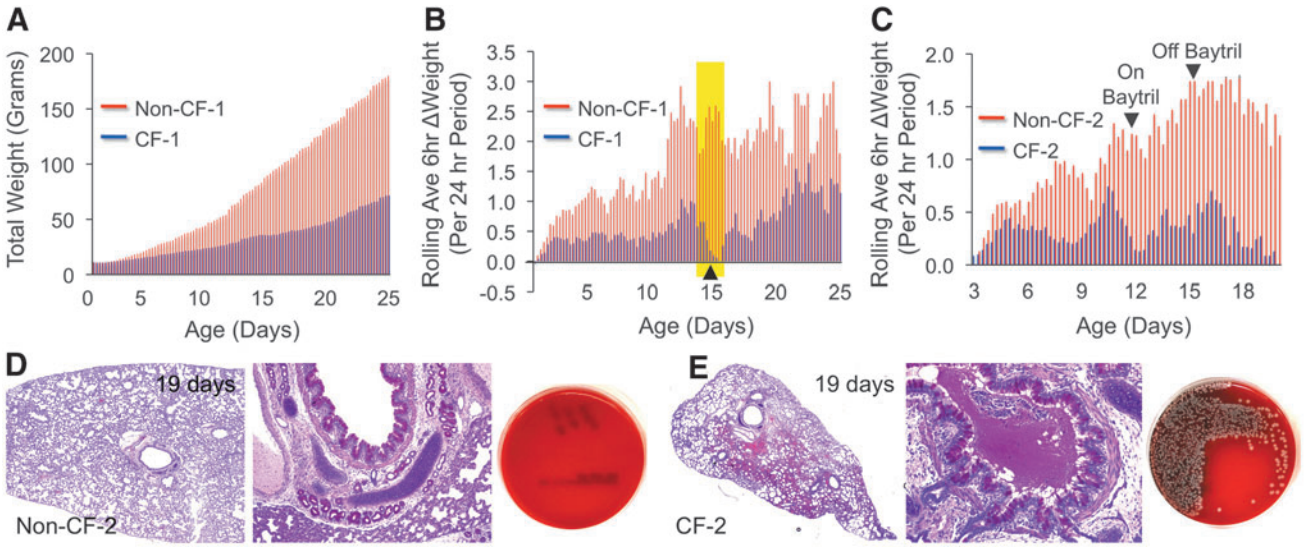

FIG. 1. Weight monitoring as a surrogate for lung infections in young cystic fibrosis (CF) ferrets. The weights of kits were measured every 6 hr and were compared between paired CF and non-CF kits born to the same jill. **(A)** Typical patterns of total daily weight gain for CF kits (*blue bars*) and non-CF kits (red bars). **(B)** The rolling average 6-hr delta weight gain over a 24-hr period (calculated as the average of five measurements over a 24-hr period) is plotted for a CF (*blue bars*)/non-CF (*red bars*) pair. A decline in this rolling average indicated early lung infection (*yellow shaded region*), and thus a second antibiotic (Baytril) was applied (*at arrowhead*). **(C)** A second set of CF and non-CF animals reared on Zosyn from birth, given Baytril on day 12 because of weight loss in the CF animal, and then removed from Baytril on day 15. The CF animal succumbed to lung infection at 19 days. **(D** and **E)** Lung histology and bacterial colony-forming units (CFU) in lung lysates from the 19-day-old **(D)** non-CF control and **(E)** CF animal shown in **(C)**. **(A)** and **(B)** are reproduced with permission from Sun *et al.*[105]

HD-AD confers longer term transgene expression in mouse lung and can be readministered through transient immunosuppression in mice.[109] Viral vector-mediated airway gene transfer has also been greatly improved by pharmacological interventions. Mucolytic agents have been used to break down the mucus layer and improve rAD transduction in mice.[110] A variety of pharmacological agents, such as sodium caprate,[111] ethylene glycol tetraacetic acid,[59] and lysophosphatidylcholines,[112] have also been used to transiently open the tight junction of airway epithelium, allowing access of rAD to the coxsackievirus–adenovirus receptor on the basolateral membrane of the airway epithelium.

Recombinant adeno-associated viral vectors

New rAAV vector serotypes, such as rAAV1[113,114] and rAAV6,[115] have demonstrated improved transduction efficiency after apical infection of polarized human airway epithelium compared with the rAAV2 vector used in initial clinical trials. Other rAAV capsid variants with enhanced tropism from the apical membrane of human airway epithelial cells were obtained by directed evolution in human airway cell cultures[116,117] or through genetic modification of the AAV6[115] and AAV2 capsid.[118] Other advances have focused on vector issues pertaining to the small packaging capacity of rAAV genomes. In this regard, shortened *CFTR* minigenes have allowed for the incorporation of stronger promoter/enhancer elements with rAAV vectors.[119] These have included deletion of 52 amino acid residues (156 bp) from the R-domain to create a CFTRΔR protein that retains ~80% Cl^- channel activity in comparison with the full-length CFTR.[120] In addition, a novel cross-genus hybrid parvoviral vector was developed that packages the rAAV2 genome into human bocavirus type 1 (HBoV1) capsids, a human respiratory virus that naturally infects human airway epithelium in infancy.[121] rAAV2/HBoV1 vectors have greater apical transduction of polarized human airway epithelium than rAAV1 vectors. Furthermore, rAAV2/HBoV1 vectors retain 20% greater packaging capacity than rAAV vectors because of the larger HBoV1 virion. The rAAV2/HBoV1 vector harboring a full-length CFTR expression cassette driven by the strong CBA promoter (a combination of the cytomegalovirus immediate-early [CMV IE] enhancer and chicken β-actin promoter) has been shown to efficiently correct CFTR-mediated chloride currents in CF human airway epithelium after apical infection.[121] Advances in our understanding of the intracellular barriers to rAAV transduction have also led to pharmacologic methods of enhancing transduction from the apical membrane of polarized airway epithelia. The use of proteasome inhibitors during or after infection can dramatically increase apical rAAV transduction of human airway epithelial by enhancing nuclear viral translocation.[41,122]

Recombinant lentiviral vectors

Lentiviral vectors are able to transduce dividing and nondividing cells, conferring long-term expression through the integration of a transgene expression cassette into host chromosomal DNA.[123,124] The most commonly used lentiviral vector is derived from the human immunodeficiency virus (HIV),[125] but in the context of lung gene transfer feline (FIV)[126] and simian (SIV)[127] immunodeficiency viruses, and equine infectious anemia virus (EIAV),[128] have also been studied. These viruses do not have a natural tropism for the airway, and lentiviral vectors commonly pseudotyped

with an envelope glycoprotein from the vesicular stomatitis virus (VSV-G) are relatively inefficient at transducing polarized human airway epithelial cultures from the apical membrane.[129–131] However, promising new advances in developing retargeted lentiviral vectors for airway transduction have been made. Lentiviral vectors pseudotyped with GP64 glycoprotein from baculovirus of *Autographa californica*,[131] or M2 envelop protein and hemagglutinin (HA) from influenza virus,[128] demonstrated fairly high vector production yields and apical tropism to transduce polarized airway epithelial cultures *in vitro* and mouse airway epithelium *in vivo*. Similarly, SIV vector pseudotyped with Sendai virus hemagglutinin-neuraminidase (HN) and fusion (F) protein can efficiently transduce polarized human airway epithelia from the apical membrane and also efficiently transduces mouse nasal epithelial cells *in vivo*, resulting in transgene expression sustained for periods far beyond the proposed life span of differentiated airway epithelial cells.[132] Additional studies with lentiviruses demonstrate the feasibility of repeated administration to the respiratory tract without blocking antibody immune responses.[133,134]

Lung and Pancreatic Gene Therapy in Ferrets and Pigs

Before testing CFTR-mediated lung and pancreatic gene therapies in the CF ferret and pig models, it is necessary first to understand the optimal vector design for each species. Although efficacy studies in the CF models have yet to be completed, there has been a significant amount of research to aid in vector choice. Here we present both published and unpublished data that are being used to build a framework for future studies of *CFTR* gene delivery to important affected organs in these two animal CF models.

Gene transfer to ferret and pig airways, using replication-defective adenovirus

HD-AD vectors have been tested for their ability to deliver reporter genes to the airways of both normal pigs and ferrets. The HD-AD has been tested in 3- to 4-day-old newborn ferrets ($\sim$10–13 g body weight) by intratracheal injection of 3×10^{11} particles of HD-AD virus formulated with lysophosphatidylcholine and DEAE-dextran in a volume of 40 μl. The vector harbored a nuclear-targeted β-galactosidase reporter gene (nt-LacZ) driven by the human cytokeratin 18 (K18) promoter, which confers conducting airway epithelial cell-specific transgene expression. At 8 days postinfection, reporter LacZ expression was seen in the surface airway epithelial cells of intralobar conducting airways with little expression in alveolar regions of the lung (Fig. 2A–E). Similar HD-AD vectors carrying an K18 promoter driving nt-LacZ or human CFTR cDNA were delivered to 25- to 30-kg pigs as an aerosol under bronchoscopic guidance.[135]

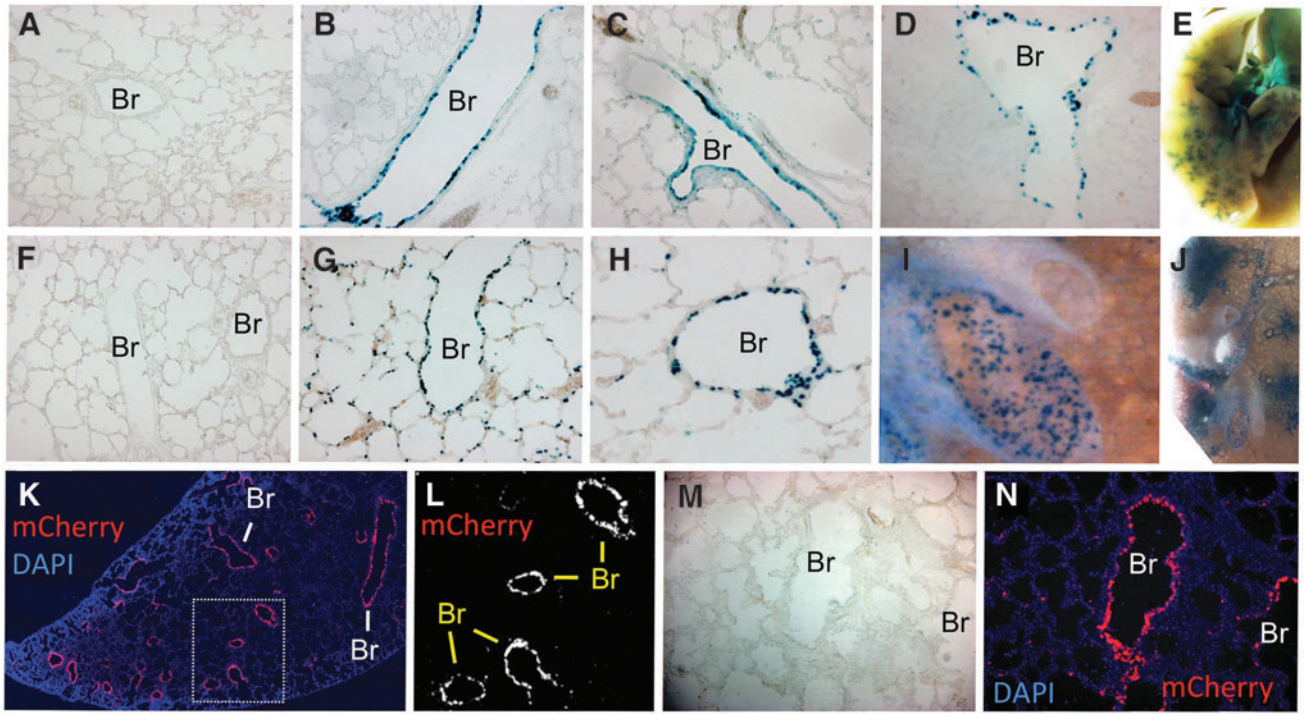

FIG. 2. Gene transfer in the airways of 3- to 6-day-old ferrets. **(A–E)** Airways after intratracheal delivery of **(A)** vehicle or **(B–E)** helper-dependent Ad5 (i.e., gutted adenovirus) with X-Gal staining 8 days postinfection. This vector expresses nt-LacZ under the control of the K18 promoter. **(A–D)** are sections and **(E)** is a whole-mount preparation. The transgene is expressed predominantly in the small airways. **(F–J)** Airways after intratracheal delivery of **(F)** vehicle or **(G–J)** equine infectious anemia virus (EIAV) pseudotyped with influenza A virus subtype H7 hemagglutinin (HA) with X-Gal staining 8 days postinfection. This EIAV vector expresses nt-LacZ under the control of the CBA promoter. **(F–H)** are sections and **(I** and **J)** are whole-mount preparations. The transgene is expressed in the large and small airways as well as in alveolar regions. **(K–N)** Newborn ferrets were infected with an RSVmCherry-encoding FIV-GP64 virus. mCherry expression (*red*) was observed almost exclusively in bronchioles (Br) at 7 days postinfection. **(L)** shows only the mCherry channel of the boxed region in **(K)**. **(M)** is the bright-field image of the fluorescence panel in **(N)**. **(K)** and **(N)** are counterstained with DAPI to mark nuclei.

Aerosol delivery of 5×10^{12} particles of HD-AD vector formulated with 0.01% lysophosphatidylcholine in 5 ml was directed to the left lung through the left mainstem bronchus. One week after infection, X-Gal staining revealed strong LacZ reporter expression in the surface epithelium of the segmental bronchus, bronchioles, and respiratory bronchioles from the infected left lung, whereas the right lung lacked reporter expression. Histological examination of tissue sections demonstrated that ~20% of the epithelial cells expressed LacZ transgene in the infected lobes. Importantly, LacZ expression was also found in the airway submucosal glands, which are considered an important target for CF lung gene therapy. HD-AD virus encoding the human CFTR gene also demonstrated expression throughout the infected lobes by real-time RT-PCR for the exogenous hCFTR mRNA and immunostaining using anti-hCFTR antibody for expression of the exogenous hCFTR protein. Although acute inflammatory cytokine and chemokine production was observed after HD-AD administration, as well as the infiltration of neutrophils into the pig airway epithelium 24 hr after vector delivery, there was no systemic toxicity observed after aerosol delivery of the HD-AD vectors, and no significant difference in inflammatory cell infiltration in the bronchi and alveolar regions before and after 1 week of vector delivery. The mRNA levels for cytokines and chemokines from the bronchoalveolar lavage cells and the lung tissue were also not significantly different on day 7 between the infected and noninfected animals.

Gene transfer to ferret and pig airways, using rAAV

Studies evaluating the use of rAAV for gene transfer to the ferret and pig lung have also demonstrated the feasibility of this vector for use in gene therapy in the CF animal models. Studies comparing rAAV1, rAAV2, and rAAV5 serotypes for their ability to transduce polarized human, pig, and ferret airway epithelial cultures suggest that these three species share a similar apical tropism for these serotypes, with rAAV2/1 being the most efficient.[37] Our studies have found that the newborn ferret airway is resistant to rAAV transduction by different serotypes including types 1, 2, 6, and 9 (our unpublished data). However, *in vivo* transduction with rAAV2/1 was significantly enhanced by the addition of 200 μM doxorubicin in the vector inoculum.[136] When the infections were conducted in 5- and 12-day-old ferrets, transgene expression was observed in the tracheobronchial epithelium, bronchioles, and scattered alveolar cells, whereas transgene expression was significantly lower in 18-day-old animals and undetectable in adult animals.[136] Interestingly, the resistance to rAAV2/1 transduction in older ferrets appears to be due, at least in part, to the increased abundance of a secreted inhibitory factor(s) in the ferret airway. The identity of the secreted inhibitory factor(s) remains unknown; however, resistance of the airway to rAAV1 infection appears to develop at 18 days after birth, a time point when submucosal glands are nearing a mature state in ferrets. Nevertheless, our studies demonstrated that rAAV2/1 may be a suitable viral vector to test gene therapy to the lung of neonatal CF ferrets. Notably, rAAV2/1 is also one of the most efficient natural serotypes of rAAV for transduction of polarized human airway epithelial cultures from the apical membrane.

Capsid-directed evolution of rAAV in polarized human airway epithelial cultures has yielded some AAV variants with better apical tropism to the human airway.[116] This strategy was also used to direct the evolution of an AAV capsid library in pig airway *in vivo* and isolated a new AAV variant, AAV2H22.[137] The capsid sequence of this variant was identical to that of AAV2 except for five mutations of amino acid residues as E67A, S207G, Q598L, I648V, and V708I. A new rAAV vector generated by pseudotyping rAAV2 genome into the AAV2H22 capsid demonstrated its ability to selectively and efficiently transduce pig airway epithelium *in vitro* and *in vivo*. This vector will be useful in delivery of the porcine *CFTR* gene to test gene therapy for lung disease in the CF pigs.

Gene transfer to ferret and pig airways, using lentiviral vectors

Studies comparing HIV with FIV lentiviral vectors in well-differentiated human and pig airway epithelia screened a number of envelope glycoproteins and identified baculovirus protein GP64 as one of the most efficient pseudotypes for transduction from the apical membrane by both HIV and FIV vectors.[129] Furthermore, this study also demonstrated that FIV-GP64 recombinant virus was effective at transducing the airways of pigs *in vivo*. We tested the efficiency of two lentiviral vectors for gene transfer to the newborn ferret lung, including EIAV pseudotyped with hemagglutinin (HA) from avian influenza A virus[138] and FIV pseudotyped with GP64.[133] *In vivo* airway infection was conducted by intratracheal injection of 40 μl containing 7.5×10^6 infectious units (IU) of EIAV/HA-H7.CBAntLacZ into 3-day-old ferrets. Eight days after infection, tracheas and lungs were harvested and stained with X-Gal (Fig. 2F–J). Grossly, significant transgene expression was seen in all lobes and the large and small conducting airways of the lungs, but not in the trachea. Histologic analysis demonstrated that EIAV/HA-H7 virus efficiently transduced bronchi, bronchioles, and alveoli, ranging from ~10 to 70% of cells in these regions (Fig. 2F–J). Similarly, infection of 6-day-old ferrets with 1×10^8 IU (100 μl) of an RSVmCherry-encoding GP64-pseudotyped FIV vector efficiently transduced intralobar small airways at 7 days postinfection (Fig. 2K–N). These results suggested the HA-H7- and/or GP64-pseudotyped lentiviral vectors may be useful in testing lung gene therapies in the CF ferret and pig models.

Gene transfer to the pancreas of ferrets and pigs

The pancreas represents another important target organ for CF gene therapy. Several routes of viral gene delivery to the pancreas have been tested in animal models including direct pancreas injection, systemic delivery with temporary clamping of portal vein and hepatic artery, retrograde pancreaticobiliary duct delivery, intraperitoneal delivery, and intravenous delivery.[139–142]

A minimally invasive procedure to deliver rAAV to the pancreas via the celiac artery, the vessel that supplies major branches to the pancreas, was developed and tested in newborn pigs.[143] In this study, the celiac artery was used for vector delivery within 24 hr of birth and accessed via umbilical artery catheterization. One month after delivering 2×10^{12} particles of rAAV9-EGFP through the celiac artery,

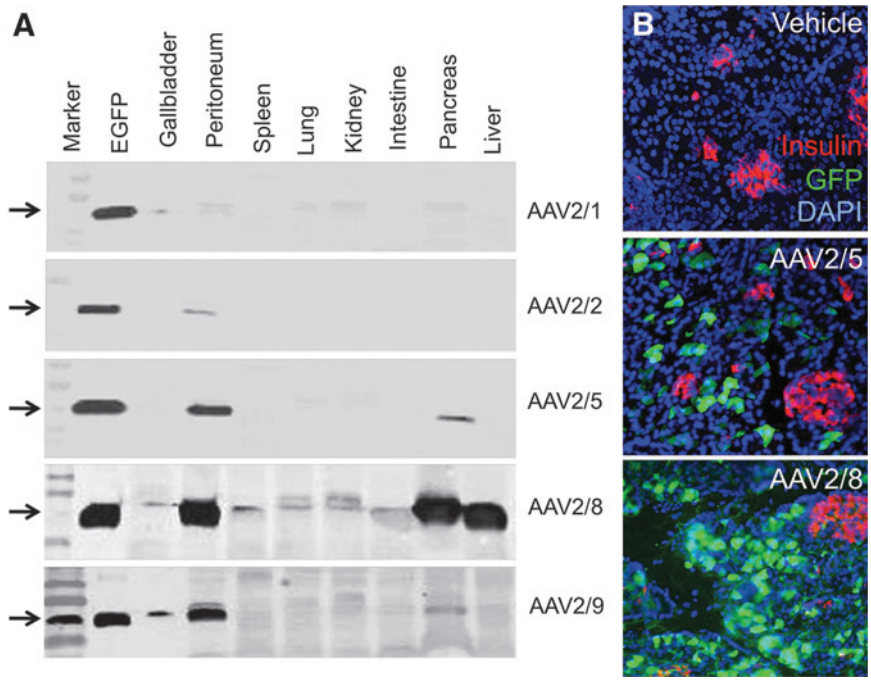

FIG. 3. rAAV transduction of the pancreas in 3-day-old ferrets. Three-day-old ferrets were injected intraperitoneally with 200 μl of PBS (vehicle) or the indicated serotype rAAV vector encoding EGFP (2×10^{11} particles/20 g body weight in 200 μl of PBS). Ferrets were sacrificed 2 weeks after infection. **(A)** Tissue homogenates of the various organs were used for Western blot detection of EGFP. The lane marked ''EGFP'' contained the same amount of EGFP protein (*marked by arrows*) on each blot. **(B)** Pancreatic tissue sections from animals injected with vehicle, rAAV2/5-EGFP, or rAAV2/8-EGFP were immunofluorescently stained for insulin (*red*) and EGFP (*green*) followed by mounting in the presence of DAPI (*blue*) to mark nuclei.

reporter expression was found in pancreatic ducts, including the intercalated and intralobular ducts; these ducts express the highest levels of CFTR in pig and human pancreatic tissue. rAAV2/9 also transduced pancreatic polypeptide (PP) cells of the islets, but not α, β, or δ cells. Celiac artery delivery of rAAV2/9 also transduced a number of other organs, as indexed by enhanced green fluorescent protein (EGFP) mRNA, including the liver, gallbladder, heart, spleen, salivary glands, trachea, and lung, but not stomach or duodenum. Notably, systemic venous delivery of rAAV2/9 did not transduce the pancreas in newborn pigs.[143]

We have also tested rAAV transduction of the pancreas in newborn ferrets. Because newborn ferrets are much smaller than piglets, celiac artery or umbilical artery cannulation is not feasible. However, it was previously reported that intraperitoneal injection of rAAV2/6 and rAAV2/8 can effectively transduce the pancreatic acinar cells and islets in mice.[142] We adopted this method and delivered 2×10^{11} particles of various serotypes of rAAV vectors (rAAV1, 2, 5, 8, and 9) to 3-day-old newborn ferrets by intraperitoneal injection. Western blot analysis of EGFP expression in various organs demonstrated that rAAV2/8 effectively delivered EGFP to the pancreas and liver (Fig. 3A). rAAV2/5 was the next most effective serotype at pancreatic gene delivery and demonstrated no hepatic gene transfer. Immunofluorescence staining for EGFP and insulin demonstrated that neither rAAV2/5 nor rAAV2/8 effectively transduced β cells of the islets (Fig. 3B). These findings suggest that the AAV8 serotype vectors may be most suitable for gene therapy to the ferret exocrine pancreas.

Conclusions and Perspective

The creation of CF pig and ferret models presents a unique opportunity to evaluate the ability of gene therapies to slow the progression of disease in multiple target organs. Differences in the rate of disease progression in certain organs of these CF models (e.g., pancreatic and gallbladder disease) also provide opportunities to understand at what stages of disease gene therapy can be effective. Both models appear to develop disease at an accelerated rate compared with humans, facilitating a variety of studies. These models may also be useful in testing stem cell-based gene therapies, either using induced pluripotent stem cells or adult somatic stem cells. Gene-editing technologies using engineered zinc finger nucleases,[144] transcription activator-like effector nucleases,[145] and clustered regularly interspaced short palindromic repeats/Cas9 nucleases[146] have begun to demonstrate the potential to correct endogenous mutations at the *CFTR* locus by homologous recombination.[147,148] Notably, the ΔF508-CFTR pig model exists[149] and new CFTR mutant ferret models are in the pipeline. Such models will further expand the usefulness of CF pigs and ferrets to test innovative gene and cell therapy strategies.

Acknowledgments

This work was supported by NIH grants HL108902 and DK096518 (to J.F.E.), DK092284 (to Z.A.S.), P01 HL051670 (to P.B.M.), P01 HL091842 (to P.B.M.), the Cystic Fibrosis Foundation RDP (to M.J.W.), the University of Iowa Center for Gene Therapy (DK054759), and the National Ferret Research and Resource Center on Lung Disease (HL123482), the Roy J. Carver Chair in Pulmonary Research (to P.B.M.), the Canadian Institute of Human Research (to J.H.), and the Roy J. Carver Chair in Molecular Medicine (to J.F.E.).

Author Disclosure Statement

The authors declare no financial conflict of interest.

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Received for publication December 18, 2014;
accepted after revision December 26, 2014.

Published online: December 30, 2014.