Chapter 14

Integrating Viral and Nonviral Vectors for Cystic Fibrosis Gene Therapy in the Airways

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Additional information is available at the end of the chapter

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Abstract

An important goal for cystic fibrosis (CF) gene therapy is to achieve long-term functional correction. While many vector options have been evaluated, integrating vectors have the greatest potential to maintain stable expression over time without a requirement for repeated administration. In this chapter, we discuss the importance of correcting the appropriate cell types, options for integrating vectors, animal models for CF gene therapy, and clinically relevant endpoint measurements. Lentiviral vectors are a promising option for CF gene therapy, as they integrate into the host genome and persistently express a transgene of interest. Airway cell tropism can be conferred by pseudotyping. Nonviral vectors such as DNA transposons can also integrate into the genome. Recent advances in hybrid viral/transposon vector technology improve the ability to deliver transposons to the airways *in vivo*. Integrating vector technology and new animal models have allowed considerable progress toward the goal of using gene therapy to correct life-long genetic diseases such as CF.

Keywords: lentivirus, transposons, animal models, progenitor cells, genotoxicity

1. Introduction

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, a cAMP-regulated anion channel. While our knowledge of CFTR function has advanced greatly since the discovery of the gene in 1989, CF remains fatal [1, 2].

While CF is a multi-organ system disease, most people with CF die of progressive lung disease that begins early in childhood and is characterized by chronic bacterial infection and inflammation [2]. Nearly 90% of CF patients have at least one copy of the ΔF508 mutation, but there are >2,000 disease causing mutations that result in a range of disease severities [2]. These mutations can be divided into six classes based on the type and consequence of the mutation (Table 1): class I, no synthesis; class II, defective processing; class III, defective regulation; class IV, altered conductance; class V, reduced synthesis; and class VI, accelerated turnover [3]. However, new mutations continue to be identified and one mutation may fit into more than one category by disrupting CFTR transcription, protein trafficking, or protein regulation in more than one way. Pharmacologic approaches aimed at activating alternative ion transport pathways [4-7], reducing inflammation [8, 9], and inhibiting or eliminating bacterial infection [10, 11] are active areas of therapeutic development. There is also intense interest in identifying interventions that might restore function to the mutant protein [5, 6, 12, 13]. The promise of restoring function to mutant protein was recently validated in a clinical trial for the CFTR conductance mutation G551D present in 2%–3% of CF patients [6]. However, unlike small molecule potentiators or correctors, a CFTR gene replacement approach would be efficacious regardless of the disease causing mutation and is potentially a single dose, life-long curative therapeutic strategy for a devastating disease.

Class	~Frequency	Mutation Type	Common Representative	CFTR Protein Outcome
I	10%	Nonsense, splice	G542X	No CFTR
II	70%	Missense	ΔF508	Defective Processing
III	2%–3%	Missense	G511D	Defective Regulation
IV	<2%	Missense	R117H	Altered Conductance
V	<1%	Missense, splice	3349+10KB	Reduced Synthesis
VI	<1%	Missense	N287Y	Accelerated Turnover

Table 1. Six classes of mutations in CFTR that result in CF disease [3, 14, 15].

There are a great number of vector options for *CFTR* gene delivery. Non-integrating viral vectors (i.e., adenovirus or adeno-associated virus) and non-integrating nonviral vectors (i.e., plasmid DNA or *in vitro* transcribed RNA) each have important attributes and have resulted in significant advances in the CF gene therapy field (reviewed in [16-18]). However, potential limitations to these episomal expression systems may include gradual decreases in transgene expression over time and limiting host immune responses following vector readministration. These pitfalls could be avoided if a therapeutic transgene is stably integrated into the genome of a progenitor cell population. Thus, in this chapter, we focus on the use of integrating vectors for gene delivery, although some of the topics covered will be relevant to multiple vector systems.

This chapter discusses the common and emerging options for integrating vectors and efforts to deliver integrating vectors to CF animal models. Defining "curing CF with gene therapy"

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is not nearly as simple as it appears at first glance. As such, we explore a variety of relevant outcome measures. In addition, there are challenges that need to be taken into consideration for pre-clinical and clinical *in vivo* studies, such as: delivery, efficiency, persistence, the potential for insertional mutagenesis, and cell types to transduce.

2. Correcting the appropriate cells

Because pulmonary disease is generally the most life-limiting complication of CF, gene therapy strategies focus on lung delivery of CFTR. Regardless of the gene delivery tool, an important consideration for CF gene therapy is the target cell. In the proximal airways, CFTR is normally most abundant in surface epithelial cells including ciliated cells, surface columnar cells, and submucosal gland epithelia (SMGs) [19]; in distal airways only superficial epithelia express CFTR. With this information in mind, we face two important questions. 1) What cell types need to be transduced to attain lasting expression? 2) What percentage of cells needs to be transduced to correct CF lung disease? A goal of gene transfer to the pulmonary epithelium with integrating vectors is to correct the CFTR defect in a population of cells that could pass the corrected gene to their progeny, thus eliminating the need for vector readministration. There appear to be several epithelial cell types in the lung that provide these functions, which has led to controversy regarding which cells to target for CF gene therapy. Arguments can be made in support of the necessity to correct basal cells [20, 21] and non-ciliated columnar cells of the airways [22-24], SMGs [25-27], club cells [28, 29], and alveolar type II cells [30, 31] in the distal lung.

Compelling evidence from both *in vitro* and *in vivo* studies indicate that basal cells are multipotent proximal airway progenitor cells that repopulate pulmonary epithelia under normal conditions and during regeneration (reviewed in [32-34]). Cell-labeling experiments with transgenic mice show that basal cells give rise to labeled basal, ciliated, and club cells, thus fulfilling the definition of progenitor cells [35, 36]. Several studies suggest that basal cells from human trachea or bronchi will repopulate denuded tracheal xenografts or differentiated epithelial cells *in vitro* [37-40]. Hematopoietic stem cells are an example showing that a single stem cell type can reconstitute a whole organ; however, there is no convincing evidence that a multipotent airway stem cell is capable of replenishing all regions of the intrapulmonary epithelium. The current literature supports that tracheal, bronchiolar, and alveolar epithelia are maintained by regionally distinct progenitor cell lineages.

What percentage of cells needs to be transduced to functionally correct the CF phenotype *in vivo*? This is one of the most important questions in the field of CF gene therapy, but remains unanswered. At least five studies examined the relationship between percentage of cells expressing CFTR and transepithelial Cl^- secretion [41-45]. With relatively good agreement, they suggest that expressing CFTR in 5%–15% of cells restores Cl^- secretion to near wild-type levels. As such, the benchmark of correcting ~10% of the cells is often cited. However, there are many caveats to this number. Indeed one limitation is that many of these studies were performed using *in vitro* models. In addition, as discussed below, other studies suggest that defective

HCO_3^- transport through CFTR might be more relevant to early disease pathogenesis than Cl^- secretion [46-49]. The relationships between HCO_3^- secretion, airway surface liquid pH, bacterial killing, mucociliary clearance, and mucus viscosity may be as important as Cl^- secretion as metrics for disease correction. The short answer to the question posed above is "we do not know"; however, given current animal models and improved vector technologies, the experiments necessary to address the question are feasible. Indeed, as we discuss below, existing vector technologies are being optimized for lung gene transfer and novel integrating vectors are being engineered.

3. Options for integrating gene delivery

3.1. Lentivirus

Lentiviruses comprise a genus of the virus family *Retroviridae.* All retroviruses are defined by the ability to reverse transcribe their RNA genome and integrate proviral DNA into the genome of the host cell [50]. Several features of lentiviral vectors (LVs) make them attractive vehicles for delivering therapeutic genes, including their large coding capacity, efficient gene transfer, persistent expression, directed tropism via pseudotyping, and lack of virus-encoded proteins that could elicit undesirable immune responses [51-53]. Unlike gamma-retroviral vectors such as murine Moloney leukemia virus (MMLV)-based vectors, the pre-integration complex of lentiviruses can transverse the nuclear envelope and integrate its cargo into the genomes of non-dividing cells [54]. The first recombinant lentiviral vectors were based on human immunodeficiency virus type-1 (HIV-1) and remain the most widely used lentiviral vector for gene transfer applications [55]. The earliest recombinant HIV-1 viruses were created in the late 1980s and were used to study HIV-1 biology [56].

Beginning in the 1990s and continuing to this day, there have been significant strides in improving the safety and utility of HIV-based gene transfer vectors (reviewed in [50]). To reduce pathogenicity and render the vector replication incompetent, most modern HIV production systems are divided into four expression plasmids (Figure 1A): 1) a plasmid containing the transgene of interest flanked by the HIV long terminal repeats (LTRs); 2) a packaging plasmid expressing the necessary structural and enzymatic proteins; 3) a separate plasmid expressing HIV rev; and 4) an envelope glycoprotein expression plasmid. Multiple viral proteins, such as nef, vif, vpu, env, and vpr, have been deleted as well as much of the U3 region of the 3'LTR. This latter deletion removes promoter and enhancer activity from the LTR and has been termed a self-inactivating (SIN) modified vector [57]. The woodchuck hepatitis virus posttranscriptional regulatory element (wPRE) is a common addition to LV vectors and functions to increase RNA stability, resulting in higher levels of transgene expression [58, 59].

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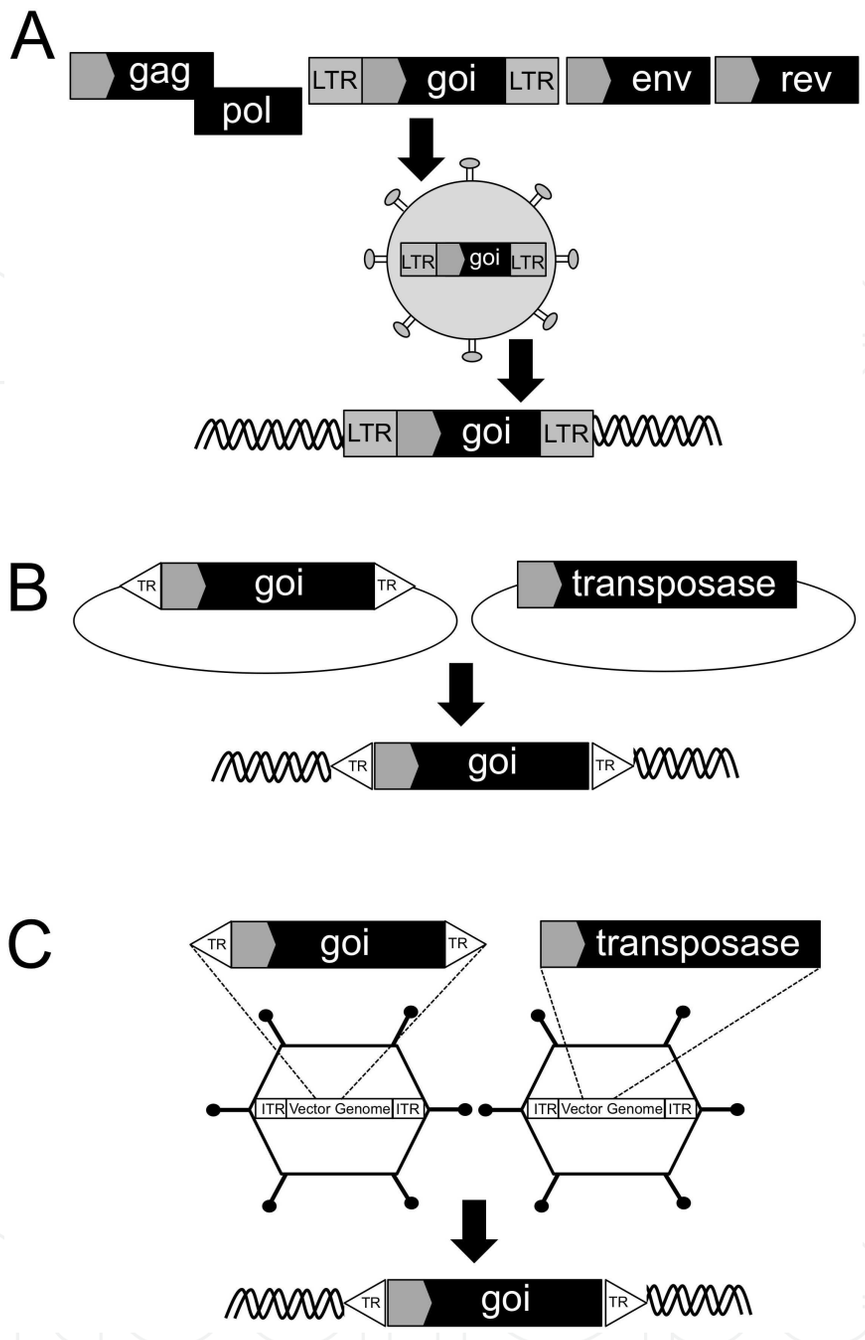

Figure 1. Schematics of integrating transgene delivery systems. A) HIV-based lentiviral vectors are produced by four plasmid transfection. The gag/pol plasmid supplies structural and enzymatic proteins; the env plasmid supplies the envelope glycoprotein (typically VSV-G); rev is delivered *in trans* via its own plasmid. The "gene of interest" (goi) is flanked by the long terminal repeats (LTRs) and driven by a heterologous promoter. Only the genetic material flanked by the LTRs is packaged and integrated into the host genome. B) Recombinant DNA transposons are typically delivered as a two-part plasmid system. Transposase catalyzes the transposition of the genetic material flanked by the appropriate terminal repeats (TRs) from the plasmid and into the host genome. C) DNA transposons can also be delivered by viral vectors to improve delivery efficiency. One viral vector carries the DNA transposon and the other carries the transposase. The goi is flanked by the transposon TRs, which in turn are flanked by the viral vector inverted terminal repeats (ITRs). Once inside the cell, transposition functions as described in B.

HIV-based LV delivery of CFTR is a promising option for CF gene therapy. In proof of principle experiments using reporter genes, HIV conferred gene transfer to both ciliated and basal cells of the mouse, sheep, marmoset, and ferret airways [60]. Xenografts transduced with HIV expressing CFTR achieved functional correction as assessed by the measurement of transepithelial potential difference [61]. In a CF mouse model, delivery of CFTR by HIV resulted in sustainable transgene expression for 18 months in ciliated, non-ciliated, and basal cells [53] and partially recovered the anion channel defect [62]. These findings support HIV as a vector candidate for CF gene therapy. Further studies in a large animal CF model to restore anion channel defect to rescue pH and bacterial killing are important next steps in validating this vector for CF gene therapy.

For LVs, the native envelope glycoprotein is deleted and a heterologous envelope is supplied. This strategy, termed pseudotyping, modifies vector tropism. Glycoproteins from a wide variety of enveloped viruses can be used to package LVs and, as will be discussed, multiple groups have identified envelopes that confer lung gene transfer. The envelope glycoprotein from vesicular stomatitis virus (VSV-G) efficiently pseudotypes LVs, confers wide tropism, and is the most commonly used envelope glycoprotein. However, in well-differentiated airway epithelial cells, VSV-G pseudotyped LV (VSVG-LV) preferentially transduces the basolateral surface [63, 64]. By using pretreatments or vehicles that transiently disrupt epithelial tight junctions, VSVG-LV accesses the basolateral surface of airway cells following luminal delivery [65]. This strategy has also been shown to greatly improve VSVG-LV *in vivo* gene transfer efficiency in the lungs of mice [62].

Feline immunodeficiency virus (FIV) is a non-primate LV with a less complex genome than HIV. Unlike HIV, wild-type FIV naturally lacks tat, vpr, vpu, and nef. In addition, FIV-based vector has vif deleted. Additional modifications to the FIV vector, such as deleting the major splice donor, lead to a higher transduction efficiency and transgene expression [58]. Reporter gene studies demonstrate that FIV transduces cells in the conducting airways, bronchioles, and alveoli [66]. FIV-mediated delivery of CFTR corrects the anion channel defect in airway epithelia [65]. FIV pseudotyped with the baculovirus envelope glycoprotein (GP64) preferentially transduces polarized airway epithelia at the apical surface [63], results in persistent gene expression in mice [63], and supports gene transfer to airways of pigs [66]. Additionally, FIV vectors can be readministered to mouse airways without blocking immune responses [67]. To prolong virus exposure to the airways immediately following delivery, formulating vector with a viscoelastic gel increases transduction efficiency *in vivo* [68, 69].

Simian immunodeficiency virus (SIV)-based LV also successfully transduces airway epithelia. SIV carries little pathogenicity for its own host; therefore, modified strains of SIV could potentially be a safer alternative to HIV-based LVs [70]. In studies using Sendai virus envelope proteins (F and HN) pseudotyped SIV (F/HN-SIV), a single dose persisted for the lifetime in the nasal epithelia of a mouse and achieved a dose-dependent increase in reporter gene expression upon vector readministration [71]. *In vitro*, F/HN-SIV carrying CFTR can generate functional chloride channels [72]. Additionally, SIV transduction results in persistent transgene expression in both differentiated human airway cells and freshly excised human lung tissue [71].

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Equine Infectious Anemia Virus (EIAV) is another non-primate lentivirus that has been investigated as a gene transfer vector. It has been studied extensively for neurological disease applications, such as Parkinson's disease [73]. EIAV pseudotyped with the influenza HA envelope can transduce neonatal mouse airways, most notably, the nasal and lung epithelium. Readministration of HA-EIAV resulted in decreased gene transfer efficiency [74].

3.2. DNA transposons

Recombinant DNA transposons are integrating nonviral vectors that confer efficient and stable transgene expression in a variety of cell types. The DNA transposon *Sleeping Beauty* (SB) was resurrected from defective copies of a Tc1-like fish element. SB transposes its genetic cargo into host genomic loci using its catalytic transposase activity [75]. Recombinant DNA transposons used for gene transfer applications are comprised of a two-part system; one encodes terminal repeats (TRs) flanking a transgene of interest (transposon), and the other a catalytic protein responsible for transposition (transposase) (Figure 1B). Since the discovery of SB, other transposons such as *piggyBac* and Tol2 have also been used in gene transfer applications (reviewed in [76, 77]). DNA transposons are attractive tools for gene therapy because they have a large carrying capacity and integrate into the genome.

Generally, transposon-based vectors are delivered as plasmids. This poses limitations for delivery to some somatic cell types. To improve delivery, several formulations have been investigated. Belur and colleagues described a protocol for complexing SB with polyethyleneimine (PEI) for delivery to the airways of mice [78]. The efficiency of gene transfer was approximated to be 1%–3% 2 months post delivery. The use of liposomes has also been investigated as an alternative transposon delivery strategy. Somatic delivery of liposome–protamine formulated SB resulted in a higher transduction efficiency than Lipofectamine 2000 while maintaining low toxicity and biocompatibility [79]. This non-viral vector approach overcomes biological barriers and allows for chromosomal integration; however, to date, this strategy has not been applied to delivering CFTR to the *in vivo* airways.

3.3. Hybrid DNA transposon/viral vectors

Delivery of plasmid-based transposon vectors to somatic cells *in vivo* is inefficient. To overcome this limitation, multiple groups have used viral vectors to deliver transposon components (Figure 1C). Recombinant DNA transposons delivered by non-integrating viral vectors, termed *hybrid vectors*, are intended to combine the advantage of efficient transduction of the viral vector with persistent expression of the transposon, creating an integrating vector. Adenovirus (Ad) [80, 81], adeno-associated virus (AAV) [82], and integrase-deficient lentivirus (ID-LV) [83, 84] have all been investigated as delivery tools for DNA transposons. An integration analysis of SB delivered by ID-HIV suggests a similar, near random, pattern as is observed for plasmid-delivered SB [83].

Yant et al. showed that SB delivered by a helper-dependent Ad vector can integrate into the host chromosome in a transposase-dependent mechanism [75, 80]. Interestingly, in this system, the initial transposition of SB out of the Ad genome required the use of Flp-recombinase to

successfully deliver the transposon transgene [80]. More recently, the generation of a hybrid *piggyBac*/Ad and *piggyBac*/AAV facilitated delivery of a nonviral transposon. However, unlike SB, neither *piggyBac*/Ad nor *piggyBac*/AAV required an extra recombinase step for transposition to occur [81]. In contrast to the hybrid SB, *piggyBac* has not exhibited overexpression inhibition or limitations on size of the genetic cargo [85]. *PiggyBac*/Ad hybrid vector successfully delivered a CFTR expression cassette to primary airway epithelial cultures *in vitro* that corrected the anion transport defect up to 4 months in culture. In reporter gene studies, transgene expression persisted for the 1-year duration of the experiment in mice [81].

4. Animal models of cystic fibrosis

Animal models serve an important testing ground for somatic cell gene transfer applications. Mice with null mutations [86-89], specific disease associated *CFTR* mutations [90-92], and conditional CFTR null alleles [93] have contributed to the understanding of molecular mechanisms of CF. However, mice do not recapitulate several aspects of CF lung disease pathogenesis. As discussed above, many studies evaluating integrating gene transfer vector delivery to the lungs of mice have been conducted. For these reasons, we now discuss efforts to deliver integrating vector to new animal models CF.

4.1. CF rat

Tuggle and colleagues used zinc finger nucleases to disrupt CFTR exon 3 in rats [94]. $CFTR^{-/-}$ rats recapitulate many aspects of human disease including intestinal obstruction, obstruction of the vas deferens, and abnormalities in nasal mucus production. It is currently not clear if CFTR null rats develop lung disease. To date, no gene correction studies have been reported in this novel model.

4.2. CF ferret

CFTR null ferrets were developed using AAV-mediated gene targeting in somatic cells, nuclear transfer, and cloning [95]. Unlike CF mice, CF ferrets develop early and reproducible lung infections that make it a promising platform for testing lung-directed CF therapies [95]. There are several potential reasons for these species differences. First, Ca^{++}-activated Cl^- channels in the mouse airway may compensate for cAMP-mediated CFTR Cl^- transport [96, 97], a pathway that appears to be less active in humans or ferrets [95, 98-100]. Second, in humans and ferrets, goblet cells are the predominant secretory cell type of the cartilaginous airways [101-104], whereas in mice the analogous secretory cell type is the club cell [105, 106]. Third, SMGs are virtually absent in murine cartilaginous airways, with only a handful in the most proximal regions of the trachea [106, 107]. SMGs are important for airway innate immunity in the ferret [108] and humans [109, 110], and a potentially valuable site for CFTR expression [111-114].

Lentiviral gene transfer to the wild-type neonatal ferrets using EIAV- and FIV-based vectors expressing fluorescent reporter genes was recently reported [115]. The EIAV was pseudotyped with hemagglutinin (HA) from avian influenza A virus [74] and the FIV vector was pseudo-

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typed with GP64 [63]. A liquid bolus of the vector was delivered to newborn ferrets via a tracheal incision. Significant transgene infection was noted in respiratory epithelia of all lobes in both the conducting and small airways with both vectors. Cmielewski and colleagues delivered VSVG-HIV expressing the LacZ reporter to the lungs of 7—8-week-old ferrets [116]. Considerably less gene transfer was observed as compared to the HA or GP64 pseudotyped LV gene transfer in the neonatal ferrets. Currently, it is unclear if these differences are due to the vector pseudotype, age of the ferrets, or delivery protocol; however, these data suggest that ferrets may be useful pre-clinical models for lentiviral vector development.

4.3. CF pig

Pigs are an important model for many studies of human cardiovascular diseases, injury and repair, surfactants, inflammation, and pulmonary diseases (reviewed in [101]). Compared to rodents, the pig lung is anatomically and physiologically more similar to humans [117, 118] and has been studied extensively in xenotransplantation. The prenatal maturation of the pig lung is similar to humans and includes extensive alveolarization [119]. Pig airway branching and cell composition is much more akin to human airways than to those of mice. The cell types comprising the conducting airway epithelium in pigs and humans are similar, and notably lack the high percentage of club cells typical of mice. The pig bronchial epithelium is pseudostratified and contains ciliated, basal, and goblet cells, and abundant SMGs (reviewed in [101]). Importantly, the distribution of SMGs in the conducting airways and the CFTR-dependent and -independent secretion of liquid and macromolecules is similar to humans [112, 120-122].

Pigs with CFTR null and ΔF508 knock-in alleles were generated by AAV-mediated homologous recombination and somatic cell nuclear transfer [99]. Breeding heterozygous male and females generated homozygous *CFTR*$^{-/-}$ pigs, and their striking neonatal phenotype was described [99, 123]. Newborn CF pigs exhibit severe disease similar to humans including pancreatic insufficiency, meconium ileus with intestinal obstruction, absence of the vas deferens, and evidence of liver and gall bladder disease [123]. Importantly, CFTR null and ΔF508 pigs spontaneously develop lung disease with many features similar to humans with CF including bacterial infection, inflammation, abnormal mucociliary clearance, bronchiectasis, and remodeling.

In a recent study, we compared HIV- and FIV-based lentiviral vectors in well-differentiated human and pig airway epithelia [66]. FIV transduced pig airway epithelia with greater efficacy than HIV, but both FIV and HIV transduced human airway epithelia with equal efficacy [66]. We further screened a number of envelope glycoproteins and identified GP64 as one of the most efficient pseudotypes for transduction and persistent expression in both pig and human epithelial cells [66]. A mCherry marker virus was delivered to wild-type pigs 4 weeks of age. A bolus dose of GP64-FIV vector was delivered to the ethmoid sinuses or to the tracheal lobe through a catheter threaded through the suction channel of a pediatric bronchoscope. We estimated the range of transduction efficiencies in the pig airways to be from <1 to 7%. In future studies, we will deliver CFTR expressing vector to CF pigs to

determine the preferred gene transfer targets and the level of CFTR correction required to prevent or slow disease progression

5. Outcome measures

In pre-clinical studies of CF gene therapy, it is vital to define metrics of correction before the studies are initiated. It would be naïve to simply deliver vector to the airways and "look and see" if the airway disease is cured. CF is a complex disease with many phenotypic features and clearly defining the disease progression in an untreated CF animal model is vital. As experience is gained with new animal models, additional assays for correction will be established and refined. Importantly, these metrics may apply to multiple gene correction but may not be feasible in all animal models.

5.1. Quantitative real-time PCR and CFTR protein expression

Quantitative real-time RT-PCR is a sensitive assay for measuring vector expressed *CFTR* mRNA [123]. At progressive time-points post vector delivery, whole tissue or brushings of nasal or tracheobronchial epithelia can be obtained. Silent mutations can be engineered into the vector expressed *CFTR* cDNA so that transgene expression can be differentiated from endogenous *CFTR*. Using a similar strategy, vector genome copy number can be estimated. Genomic DNA from a portion of the same tissue or epithelial brushings can be purified and copy number estimated by normalizing to endogenous DNA and a standard curve. Appropriate controls would include wild type and untreated affected littermates. To identify cells expressing the CFTR protein, immunohistochemistry and immunofluorescence protocols have been reported [123]. Using these approaches, the percentage of cells expressing CFTR and the cell types expressing CFTR can be determined [124].

5.2. Functional correction

Nasal potential difference (NPD) is an established assay for demonstrating *in vivo* correction of CFTR-dependent Cl^- transport [125-127]. For many of these studies, vector was delivered nasally and the NPD was used as evidence of CFTR complementation. Ideally, for integrating vectors, predelivery, early post-delivery, and late post-delivery timepoints in the intrapulmonary airways should be measured. As early as 1 week after gene transfer, the nasal voltage and its response to amiloride, low Cl^-, and cAMP agonists can be measured [90, 123, 128]. Animals could be followed with serial monthly nasal voltage measurements over a 12-month or longer period to document persistence of expression. Importantly, CFTR is an anion channel that conducts both Cl^- and HCO_3^-. As mentioned above, correcting ~10% of cells is often cited as a benchmark for restoring Cl^- transport and correcting the clinical phenotype. However, other studies also suggest that defective HCO_3^- transport might be relevant to disease. Multiple studies of CF mouse cervical mucus [46, 47], CF mouse small intestinal mucus [48], and human CF nasal SMGs [49] support the importance of CFTR-dependent HCO_3^- transport in CF pathogenesis. Loss of CFTR-dependent HCO_3^- transport acidifies liquid produced by surface

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epithelia [129, 130] and secretions from SMGs [49]. Thus, measurements of HCO_3^- transport may also be an important metric of functional correction.

As a result of abnormal CFTR-dependent HCO_3^- secretion, airway surface liquid pH is acidified. The ASL of primary cultures of CF pig airway epithelia [130], newborn CF pig airways [130], and the nasal pH of newborn babies with CF [131] is acidic. In CF pigs, the acidity has been shown to impair bacterial killing [130]. In addition, there are new techniques to measure mucus viscosity, mucociliary clearance (MCT), and lung function in large animal models [101]. Importantly, defects in airway MCT and SMG mucus detachment recently were identified in CF [132]. The bacterial killing defect is a quantifiable characteristic of CF airways. Bacterial killing is impaired as a result of reduced bicarbonate anion secretion and increasing ASL pH rescues bacterial killing [130].

5.3. Reduced infection and inflammation

A goal of gene therapy for CF is to prevent the onset or reduce the progression of lung disease. Signs of reduced infection and inflammation in treated animals can be visually inspected in the airways. Bronchoscopy can be used to detect signs of inflammation, such as mucosal inflammation and excessive purulent secretions. Total cell counts, cell differentials, and cytokine levels are obtained from bronchoalveolar lavage (BAL) as standard assay for infection and inflammation. In addition, standard quantitative microbiologic techniques are used to identify and quantify BAL bacteria [133]. Biopsies can also be obtained from larger animal models such as the CF pig. These samples can also be used for sequencing-based analyses. As we learn more about the disease progression in new animal models of CF, improved metrics of functional correction are being developed. High-resolution computerized tomography (HRCT) facilitates detailed structural analysis of the airways [101]. HRCT scans can discern anatomic changes in the airways over time in control and treated animals [134].

6. Challenges to pulmonary gene transfer with integrating vectors

6.1. Delivery

The lung is an attractive target for gene therapy because, unlike most other tissues, the vector can be topically delivered. Vector delivery to the airways of mice, rats, and newborn ferrets is most easily accomplished by nasal or intratracheal bolus delivery of vector resuspended in a liquid vehicle such as buffered saline [135], LPC [60, 62], or a viscoelastic gel [68, 69]. In small animals, bolus delivery using a relatively small volume of vector (25-50 μl) can achieve widespread gene expression throughout the airways. However, in large animal models such as pigs or sheep (and ultimately humans), aerosolization will likely be required to achieve a widespread pulmonary distribution. In general, devices for generating airborne vector fall into three categories: aerosolizing catheters, nebulizers, and atomizers. All of these devices convert liquids into particles small enough to be respired.

Aerosolizing catheters convert liquids into particles at the point of expulsion. Typically, an aerosolizing catheter is first passed into the trachea and then the vector is instilled. The Microsprayer® (PennCentury) and the AeroProbe® catheter (Trudell Medical International) are examples of delivery systems for this application. According to the manufacturer, it is possible to generate particles with aerodynamic diameters of 4–8 μm. The Trudell AeroProbe was previously used to aerosolize helper-dependent adenovirus vectors to rabbit airways [136, 137] and to deliver Sendai virus vectors to sheep [138]. Since Sendai virus and lentiviruses are both enveloped, it is likely that this approach is feasible with lentiviral vectors. In addition, aerosolized VSVG-LVs have been successfully delivered to the airways of mice [139].

Successful vector aerosolization has been reported in mice [140], rabbits,[136, 137], pig [141] and sheep airways [142, 143]. By 2–3 weeks of age, wild type pigs are large enough to be sedated and have a pediatric bronchoscope passed into the trachea. The AeroProbe® catheter can be passed to the carina via the bronchoscope suction channel with the animal breathing spontaneously. In this way, an integrating vector can be aerosolized and targeted to specific bronchial segments.

In the case of nebulizers, the liquid is first converted into mist and then passively inhaled. Using this strategy, a plasmid-based vector was delivered to the airways of CF patients in a phase IIB gene therapy trial [144]. This approach could potentially be used to deliver DNA transposon or hybrid vectors. However, this strategy may not be feasible with enveloped viral vectors because this class of vectors may not be stable enough to withstand nebulization. In addition, nebulization requires a large volume of concentrated material; therefore, would be the least economic delivery strategy for LVs.

Atomizers are a subclass of aerosolizing catheters that deliver larger sized particles. An atomizer, such as the MADgic™ (LMA) atomizer, delivers large droplets (~30–90 μm in diameter), which may vary in size depending on the force applied to the syringe plunger. This type of device is often used to topically deliver medications to the airways [145]. Our group has observed that this type of atomizer is an effective delivery device for multiple viral vectors including FIV, particularly when formulated with a viscoelastic material such as methylcellulose.

6.2. Insertional mutagenesis

Since persistent gene expression from lentiviral vectors requires genomic integration, they show promise for treating life-long genetic diseases; however, there is inherent risk when introducing a transgene with integrating vectors. Insertional mutagenesis may disrupt normal cell functions by inactivating an essential host gene or inappropriately causing expression of an undesirable gene. The risk will vary depending on the vector used, the transgene cassette, and the cell type targeted. In many cases, enhancer effects pose the greatest danger. So far, malignant cell transformation after vector-mediated insertional mutagenesis has only been observed in three clinical entities (X-linked severe combined immunodeficiency (SCID-X1), chronic granulomatous disease (CGD), and Wiskott–Aldrich syndrome (WAS)), all of which occurred in conjunction with the use of first-generation gamma-retroviral vectors harboring LTRs with strong enhancer/promoter sequences [146-150]. These studies were conducted in

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immunocompromised patients where gene transfer conferred a selective advantage to corrected cells. The vector and disease settings likely influenced the risks for insertional mutagenesis and subsequent clonal expansion. Modern LVs are engineered to lack enhancer/promoter sequences within the LTRs and delivering *CFTR* to somatic cells has no known selective advantage. Considerable effort has been put toward mapping integration patterns and determining the functional consequences of LVs and retroviral vectors (reviewed in [151, 152]). LV integration analyses conducted on adrenoleukodystrophy clinical trial patients demonstrate that the genomic distribution maintain a polyclonal pattern [153]. Montini and colleagues demonstrated that LV integrations, even at high vector titer loads, did not accelerate tumorigenesis in tumor prone mice. In contrast, gamma-retroviral vector transduction triggered a dose-dependent acceleration of tumor onset [154].

The burden of proof has fallen on LV researchers to demonstrate that LVs do not cause cancer via insertional mutagenesis. It is unlikely that this can ever be demonstrated with absolute certainty; however, the evidence to date suggests that current LVs are considerably safer than the gamma-retroviral vectors that were first brought to clinical trials. In fact, results from human clinical trials using LVs are encouraging and the feasibility of gene therapy for monogenetic diseases is now firmly established [155]. Recent promising examples include Wiskott–Aldrich Syndrome [156, 157], metachromatic leukodystrophy [158, 159], acute lymphoid leukemia [160], lymphoma [161, 162], and multiple primary immuno-deficiencies [163].

7. Conclusions

Within a year of the discovery of CFTR, investigators validated the concept that gene replacement could reverse the ion transport defect *in vitro*, suggesting that gene therapy may be possible [164, 165]. We and others have demonstrated that CFTR delivery by integrating vectors can correct the CF anion defect *in vitro* and *in vivo*, and although further pre-clinical trials are warranted, there is great potential for translating this strategy to the clinic. As discussed, estimates of the percent of CF epithelia requiring correction vary and there is debate about which cell types must be corrected to achieve phenotypic correction; however, interest in CF gene therapy remains strong as barriers to gene transfer are identified, outcome measures are established, CF animal models with lung disease are developed, and better delivery systems are engineered. Demonstration of corrective gene transfer to pristine newborn lungs in CF animal models is a vital first step before looking ahead to correcting more diseased lungs. Newborn screening for CF is now established in all 50 states, allowing early disease detection. This offers an opportunity to introduce an integrating therapeutic gene transfer vector to the airway epithelium prior to the onset of chronic infection and inflammation. This strategy is a potentially life-long curative therapy regardless of the disease-causing mutation.

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