We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,300

Open access books available

172,000

International authors and editors

190M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

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

Ashley L Cooney, Paul B McCray, Jr and
Patrick L. Sinn

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/60977>

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 $\Delta F508$ 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	$\Delta F508$	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”

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

HCO₃⁻ transport through CFTR might be more relevant to early disease pathogenesis than Cl⁻ secretion [46-49]. The relationships between HCO₃⁻ 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, vpr, 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].

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].

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 polyethylenimine (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-

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 $\Delta F508$ 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 $\Delta F508$ 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

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

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.

Acknowledgements

We acknowledge the support of the University of Iowa DNA Sequencing Core, In Vitro Models and Cell Culture Core, Viral Vector Core, and Cell Morphology Core. This work was supported by the National Institutes of Health R01 HL-105821 (PLS), P01 HL-51670 (PBM), and the Cystic Fibrosis Foundation SINN14G0 (PLS). Core facilities were partially supported by the National Institutes of Health: P01 HL-51670, P01 HL-091842 and the Center for Gene Therapy for Cystic Fibrosis P30 DK-54759.

Author details

Ashley L Cooney¹, Paul B McCray, Jr^{1,2} and Patrick L. Sinn²

*Address all correspondence to: patrick-sinn@uiowa.edu

1 Department of Microbiology, Carver College of Medicine, The University of Iowa, Iowa City, IA, USA

2 Department of Pediatrics, Carver College of Medicine, The University of Iowa, Iowa City, IA, USA

References

- [1] Rowe SM, Miller S, Sorscher EJ. Cystic fibrosis. *The New England journal of medicine*. 2005 May 12;352(19):1992–2001. PubMed PMID: 15888700.
- [2] Davis PB. Cystic fibrosis since 1938. *American journal of respiratory and critical care medicine*. 2006 Mar 1;173(5):475–82. PubMed PMID: 16126935.
- [3] Welsh MJ, Smith AE. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell*. 1993 Jul 2;73(7):1251–4. PubMed PMID: 7686820.
- [4] Clunes MT, Boucher RC. Front-runners for pharmacotherapeutic correction of the airway ion transport defect in cystic fibrosis. *Current opinion in pharmacology*. 2008 Jun;8(3):292–9. PubMed PMID: 18468487. PubMed Central PMCID: 2517236.
- [5] Van Goor F, Hadida S, Grootenhuis PD, Burton B, Stack JH, Straley KS, et al. Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. *Proceedings of the National Academy of Sciences of the United States of America*. 2011 Nov 15;108(46):18843–8. PubMed PMID: 21976485. PubMed Central PMCID: 3219147.
- [6] Accurso FJ, Rowe SM, Clancy JP, Boyle MP, Dunitz JM, Durie PR, et al. Effect of VX-770 in persons with cystic fibrosis and the G551D-CFTR mutation. *The New Eng-*

land journal of medicine. 2010 Nov 18;363(21):1991–2003. PubMed PMID: 21083385. PubMed Central PMCID: 3148255.

- [7] Clancy JP, Rowe SM, Accurso FJ, Aitken ML, Amin RS, Ashlock MA, et al. Results of a phase IIa study of VX-809, an investigational CFTR corrector compound, in subjects with cystic fibrosis homozygous for the F508del-CFTR mutation. *Thorax*. 2012 Jan; 67(1):12–8. PubMed PMID: 21825083. PubMed Central PMCID: 3746507.
- [8] Konstan MW, Byard PJ, Hoppel CL, Davis PB. Effect of high-dose ibuprofen in patients with cystic fibrosis. *The New England journal of medicine*. 1995 Mar 30;332(13):848–54. PubMed PMID: 7503838.
- [9] Saiman L, Marshall BC, Mayer-Hamblett N, Burns JL, Quittner AL, Cibene DA, et al. Azithromycin in patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa*: A randomized controlled trial. *JAMA: The journal of the American Medical Association*. 2003 Oct 1;290(13):1749–56. PubMed PMID: 14519709.
- [10] Gibson RL, Emerson J, McNamara S, Burns JL, Rosenfeld M, Yunker A, et al. Significant microbiological effect of inhaled tobramycin in young children with cystic fibrosis. *American journal of respiratory and critical care medicine*. 2003 Mar 15;167(6): 841–9. PubMed PMID: 12480612.
- [11] Ramsey BW, Banks-Schlegel S, Accurso FJ, Boucher RC, Cutting GR, Engelhardt JF, et al. Future directions in early cystic fibrosis lung disease research: An NHLBI workshop report. *American journal of respiratory and critical care medicine*. 2012 Apr 15;185(8):887–92. PubMed PMID: 22312017. PubMed Central PMCID: 3360572.
- [12] Pedemonte N, Lukacs GL, Du K, Caci E, Zegarra-Moran O, Galletta LJ, et al. Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. *The journal of clinical investigation*. 2005 Sep;115(9): 2564–71. PubMed PMID: 16127463. PubMed Central PMCID: 1190372.
- [13] Sondo E, Tomati V, Caci E, Esposito AI, Pfeffer U, Pedemonte N, et al. Rescue of the mutant CFTR chloride channel by pharmacological correctors and low temperature analyzed by gene expression profiling. *American journal of physiology Cell physiology*. 2011 Oct;301(4):C872–85. PubMed PMID: 21753184. PubMed Central PMCID: 3512166.
- [14] MacDonald KD, McKenzie KR, Zeitlin PL. Cystic fibrosis transmembrane regulator protein mutations: ‘class’ opportunity for novel drug innovation. *Paediatric drugs*. 2007;9(1):1–10. PubMed PMID: 17291132.
- [15] Rogan MP, Stoltz DA, Hornick DB. Cystic fibrosis transmembrane conductance regulator intracellular processing, trafficking, and opportunities for mutation-specific treatment. *Chest*. 2011 Jun;139(6):1480–90. PubMed PMID: 21652558.

- [16] Pringle IA, Hyde SC, Gill DR. Non-viral vectors in cystic fibrosis gene therapy: Recent developments and future prospects. *Expert opinion on biological therapy*. 2009 Aug;9(8):991–1003. PubMed PMID: 19545217.
- [17] Sinn PL, Anthony RM, McCray PB, Jr. Genetic therapies for cystic fibrosis lung disease. *Human molecular genetics*. 2011 Apr 15;20(R1):R79–86. PubMed PMID: 21422098. PubMed Central PMCID: 3095059.
- [18] Griesenbach U, Alton EW. Moving forward: Cystic fibrosis gene therapy. *Human molecular genetics*. 2013 Oct 15;22(R1):R52–8. PubMed PMID: 23918661.
- [19] Kreda SM, Mall M, Mengos A, Rochelle L, Yankaskas J, Riordan JR, et al. Characterization of wild-type and deltaF508 cystic fibrosis transmembrane regulator in human respiratory epithelia. *Molecular biology of the cell*. 2005 May;16(5):2154–67. PubMed PMID: 15716351. PubMed Central PMCID: 1087225.
- [20] Curradi G, Walters MS, Ding BS, Rafii S, Hackett NR, Crystal RG. Airway basal cell vascular endothelial growth factor-mediated cross-talk regulates endothelial cell-dependent growth support of human airway basal cells. *Cellular and molecular life sciences: CMLS*. 2012 Jul;69(13):2217–31. PubMed PMID: 22382924. PubMed Central PMCID: 3633460.
- [21] Staudt MR, Buro-Auriemma LJ, Walters MS, Salit J, Vincent T, Shaykhiev R, et al. Airway Basal stem/progenitor cells have diminished capacity to regenerate airway epithelium in chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine*. 2014 Oct 15;190(8):955–8. PubMed PMID: 25317467. PubMed Central PMCID: 4299582.
- [22] Randell SH. Progenitor-progeny relationships in airway epithelium. *Chest*. 1992;101:11S–6S.
- [23] Ford JR, Terzaghi-Howe M. Basal cells are the progenitors of primary tracheal epithelial cell cultures. *Experimental cell research*. 1992;198:69–77.
- [24] Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *The American journal of pathology*. 2004 Feb;164(2):577–88. PubMed PMID: 14742263.
- [25] Engelhardt JF, Schlossberg H, Yankaskas JR, Dudus L. Progenitor cells of the adult human airway involved in submucosal gland development. *Development*. 1995;121:2031–46.
- [26] Borthwick DW, Shahbazian M, Krantz QT, Dorin JR, Randell SH. Evidence for stem-cell niches in the tracheal epithelium. *American journal of respiratory cell & molecular biology*. 2001;24(6):662–70.
- [27] Liu X, Engelhardt JF. The glandular stem/progenitor cell niche in airway development and repair. *Proceedings of the American Thoracic Society*. 2008 Aug 15;5(6):682–8. PubMed PMID: 18684717. PubMed Central PMCID: 2645260.

- [28] Evans MJ, Johnson LV, Stephens RJ, Freeman G. Renewal of the terminal bronchiolar epithelium in the rat following exposure to NO₂ or O₃. *Laboratory investigation*. 1976;35:246–57.
- [29] Hong KU, Reynolds SD, Giangreco A, Hurley CM, Stripp BR. Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. *American journal of respiratory cell & molecular biology*. 2001;24(6): 671–81.
- [30] Evans MJ, Cabral LJ, Stephens RJ, Freeman G. Transformation of alveolar type 2 cells to type 1 cells following exposure to NO₂. *Experimental & molecular pathology*. 1975;22:142–50.
- [31] Adamson IY, Bowden DH. The type 2 cell as progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen. *Laboratory investigation*. 1974;30:35–42.
- [32] Rawlins EL, Hogan BL. Epithelial stem cells of the lung: Privileged few or opportunities for many? *Development*. 2006 Jul;133(13):2455–65. PubMed PMID: 16735479.
- [33] Rock JR, Randell SH, Hogan BL. Airway basal stem cells: A perspective on their roles in epithelial homeostasis and remodeling. *Disease models & mechanisms*. 2010 Sep–Oct;3(9–10):545–56. PubMed PMID: 20699479. PubMed Central PMCID: 2931533.
- [34] Randell SH. Airway epithelial stem cells and the pathophysiology of chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society*. 2006 Nov; 3(8):718–25. PubMed PMID: 17065380. PubMed Central PMCID: 2647659.
- [35] Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, et al. Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proceedings of the National Academy of Sciences of the United States of America*. 2009 Aug 4;106(31):12771–5. PubMed PMID: 19625615. PubMed Central PMCID: 2714281.
- [36] Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. In vivo differentiation potential of tracheal basal cells: Evidence for multipotent and unipotent subpopulations. *American journal of physiology lung cellular and molecular physiology*. 2004 Apr; 286(4):L643–9. PubMed PMID: 12871857.
- [37] Delplanque A, Coraux C, Tirouvanziam R, Khazaal I, Puchelle E, Ambros P, et al. Epithelial stem cell-mediated development of the human respiratory mucosa in SCID mice. *Journal of cell science*. 2000 Mar;113 (Pt 5):767–78. PubMed PMID: 10671367.
- [38] Hackett TL, Shaheen F, Johnson A, Wadsworth S, Pechkovsky DV, Jacoby DB, et al. Characterization of side population cells from human airway epithelium. *Stem cells*. 2008 Oct;26(10):2576–85. PubMed PMID: 18653771. PubMed Central PMCID: 2849005.

- [39] Hajj R, Baranek T, Le Naour R, Lesimple P, Puchelle E, Coraux C. Basal cells of the human adult airway surface epithelium retain transit-amplifying cell properties. *Stem cells*. 2007 Jan;25(1):139–48. PubMed PMID: 17008423.
- [40] Li X, Rossen N, Sinn PL, Hornick AL, Steines BR, Karp PH, et al. Integrin $\alpha 6 \beta 4$ identifies human distal lung epithelial progenitor cells with potential as a cell-based therapy for cystic fibrosis lung disease. *PloS one*. 2013;8(12):e83624. PubMed PMID: 24349537. PubMed Central PMCID: 3861522.
- [41] Johnson LG, Olsen JC, Sarkadi B, Moore KL, Swanstrom R, Boucher RC. Efficiency of gene transfer for restoration of normal airway epithelia function in cystic fibrosis. *Nature genetics*. 1992;2:21–5.
- [42] Goldman MJ, Yang Y, Wilson JM. Gene therapy in a xenograft model of cystic fibrosis lung corrects chloride transport more effectively than the sodium defect. *Nature genetics*. 1995 Feb;9(2):126–31. PubMed PMID: 7719338.
- [43] Farmen SL, Karp PH, Ng P, Palmer DJ, Koehler DR, Hu J, et al. Gene transfer of CFTR to airway epithelia: Low levels of expression are sufficient to correct Cl-transport and overexpression can generate basolateral CFTR. *American journal of physiology Lung cellular and molecular physiology*. 2005 Dec;289(6):L1123–30. PubMed PMID: 16085675.
- [44] Zhang L, Button B, Gabriel SE, Burkett S, Yan Y, Skiadopoulos MH, et al. CFTR delivery to 25% of surface epithelial cells restores normal rates of mucus transport to human cystic fibrosis airway epithelium. *PLoS biology*. 2009 Jul;7(7):e1000155. PubMed PMID: 19621064. PubMed Central PMCID: 2705187.
- [45] Dannhoffer L, Blouquit-Laye S, Regnier A, Chinnet T. Functional properties of mixed cystic fibrosis and normal bronchial epithelial cell cultures. *American journal of respiratory cell and molecular biology*. 2009 Jun;40(6):717–23. PubMed PMID: 19011164.
- [46] Garcia MA, Yang N, Quinton PM. Normal mouse intestinal mucus release requires cystic fibrosis transmembrane regulator-dependent bicarbonate secretion. *The journal of clinical investigation*. 2009 Sep;119(9):2613–22. PubMed PMID: 19726884. PubMed Central PMCID: 2735925.
- [47] Muchekehr RW, Quinton PM. A new role for bicarbonate secretion in cervico-uterine mucus release. *The journal of physiology*. 2010 Jul 1;588(Pt 13):2329–42. PubMed PMID: 20478977. PubMed Central PMCID: 2915510.
- [48] Gustafsson JK, Ermund A, Ambort D, Johansson ME, Nilsson HE, Thorell K, et al. Bicarbonate and functional CFTR channel are required for proper mucin secretion and link cystic fibrosis with its mucus phenotype. *The journal of experimental medicine*. 2012 Jul 2;209(7):1263–72. PubMed PMID: 22711878. PubMed Central PMCID: 3405509.

- [49] Song Y, Salinas D, Nielson DW, Verkman AS. Hyperacidity of secreted fluid from submucosal glands in early cystic fibrosis. *American journal of physiology Cell physiology*. 2006 Mar;290(3):C741–9. PubMed PMID: 16207791.
- [50] Sakuma T, Barry MA, Ikeda Y. Lentiviral vectors: Basic to translational. *The biochemical journal*. 2012 May 1;443(3):603–18. PubMed PMID: 22507128.
- [51] McCormack JE, Martineau D, DePolo N, Maifert S, Akabarian L, Townsend K, et al. Anti-vector immunoglobulin induced by retroviral vectors. *Human gene therapy*. 1997;8:1263–73.
- [52] Sinn PL, Sauter SL, McCray PB, Jr. Gene therapy progress and prospects: Development of improved lentiviral and retroviral vectors – design, biosafety, and production. *Gene therapy*. 2005 Jul;12(14):1089–98. PubMed PMID: 16003340.
- [53] Stocker AG, Kremer KL, Koldej R, Miller DS, Anson DS, Parsons DW. Single-dose lentiviral gene transfer for lifetime airway gene expression. *The journal of gene medicine*. 2009 Oct;11(10):861–7. PubMed PMID: 19634193.
- [54] Brady T, Bushman FD. Nondividing cells: A safer bet for integrating vectors? *Molecular therapy: The journal of the American Society of gene therapy*. 2011 Apr;19(4):640–1. PubMed PMID: 21455211. PubMed Central PMCID: 3070107.
- [55] Franz K, Singh A, Weinberger LS. Lentiviral vectors to study stochastic noise in gene expression. *Methods in enzymology*. 2011;497:603–22. PubMed PMID: 21601105.
- [56] Terwilliger EF, Godin B, Sodroski JG, Haseltine WA. Construction and use of a replication-competent human immunodeficiency virus (HIV-1) that expresses the chloramphenicol acetyltransferase enzyme. *Proceedings of the National Academy of Sciences of the United States of America*. 1989 May;86(10):3857–61. PubMed PMID: 2726755. PubMed Central PMCID: 287240.
- [57] Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, et al. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *Journal of virology*. 1998;72(12):9873–80.
- [58] Sinn PL, Goreham-Voss JD, Arias AC, Hickey MA, Maury W, Chikkanna-Gowda CP, et al. Enhanced gene expression conferred by stepwise modification of a non-primate lentiviral vector. *Human Gene Therapy*. 2007;18:1244–52.
- [59] Zufferey R, Donello JE, Trono D, Hope TJ. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *Journal of virology*. 1999 Apr;73(4):2886–92. PubMed PMID: 10074136. PubMed Central PMCID: 104046.
- [60] Cmielewski P, Donnelley M, Parsons DW. Long-term therapeutic and reporter gene expression in lentiviral vector treated cystic fibrosis mice. *The journal of gene medicine*. 2014 Sep–Oct;16(9–10):291–9. PubMed PMID: 25130650.

- [61] Goldman MJ, Lee PS, Yang JS, Wilson JM. Lentiviral vectors for gene therapy of cystic fibrosis. *Human gene therapy*. 1997;8(18):2261–8.
- [62] Limberis M, Anson DS, Fuller M, Parsons DW. Recovery of airway cystic fibrosis transmembrane conductance regulator function in mice with cystic fibrosis after single-dose lentivirus-mediated gene transfer. *Human gene therapy*. 2002 Nov 1;13(16):1961–70. PubMed PMID: 12427306.
- [63] Sinn PL, Burnight ER, Hickey MA, Blissard GW, McCray PB, Jr. Persistent gene expression in mouse nasal epithelia following feline immunodeficiency virus-based vector gene transfer. *Journal of virology*. 2005 Oct;79(20):12818–27. PubMed PMID: 16188984. PubMed Central PMCID: 1235842.
- [64] Sinn PL, Hickey MA, Staber PD, Dylla DE, Jeffers SA, Davidson BL, et al. Lentivirus vectors pseudotyped with filoviral envelope glycoproteins transduce airway epithelia from the apical surface independently of folate receptor alpha. *Journal of virology*. 2003 May;77(10):5902–10. PubMed PMID: 12719583. PubMed Central PMCID: 154009.
- [65] Wang G, Slepushkin V, Zabner J, Keshavjee S, Johnston JC, Sauter SL, et al. Feline immunodeficiency virus vectors persistently transduce nondividing airway epithelia and correct the cystic fibrosis defect. *The journal of clinical investigation*. 1999 Dec;104(11):R55–62. PubMed PMID: 10587528. PubMed Central PMCID: 483477.
- [66] Sinn PL, Cooney AL, Oakland M, Dylla DE, Wallen TJ, Pezzulo AA, et al. Lentiviral vector gene transfer to porcine airways. *Molecular therapy Nucleic acids*. 2012;1:e56. PubMed PMID: 23187455. PubMed Central PMCID: 3511674.
- [67] Sinn PL, Arias AC, Brogden KA, McCray PB, Jr. Lentivirus vector can be readministered to nasal epithelia without blocking immune responses. *Journal of virology*. 2008 Nov;82(21):10684–92. PubMed PMID: 18768988. PubMed Central PMCID: 2573216.
- [68] Sinn PL, Shah A, Donovan M, McCray PB, Jr. Viscoelastic gel formulations enhance airway epithelial gene transfer with viral vectors. *American journal of respiratory cell and molecular biology*. 2005;32:404–10.
- [69] Griesenbach U, Meng C, Farley R, Wasowicz MY, Munkonge FM, Chan M, et al. The use of carboxymethylcellulose gel to increase non-viral gene transfer in mouse airways. *Biomaterials*. 2010 Mar;31(9):2665–72. PubMed PMID: 20022367. PubMed Central PMCID: 4148698.
- [70] Nakajima T, Nakamaru K, Ido E, Terao K, Hayami M, Hasegawa M. Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. *Human gene therapy*. 2000 Sep 1;11(13):1863–74. PubMed PMID: 10986559.
- [71] Griesenbach U, Inoue M, Meng C, Farley R, Chan M, Newman NK, et al. Assessment of F/HN-pseudotyped lentivirus as a clinically relevant vector for lung gene therapy.

American journal of respiratory and critical care medicine. 2012 Nov 1;186(9):846–56. PubMed PMID: 22955314. PubMed Central PMCID: 3530223.

- [72] Mitomo K, Griesenbach U, Inoue M, Somerton L, Meng C, Akiba E, et al. Toward gene therapy for cystic fibrosis using a lentivirus pseudotyped with Sendai virus envelopes. *Molecular therapy: The journal of the American Society of Gene Therapy*. 2010 Jun;18(6):1173–82. PubMed PMID: 20332767. PubMed Central PMCID: 2889732.
- [73] Valori CF, Ning K, Wyles M, Azzouz M. Development and applications of non-HIV-based lentiviral vectors in neurological disorders. *Current gene therapy*. 2008 Dec; 8(6):406–18. PubMed PMID: 19075624.
- [74] Patel M, Giddings AM, Sechelski J, Olsen JC. High efficiency gene transfer to airways of mice using influenza hemagglutinin pseudotyped lentiviral vectors. *The journal of gene medicine*. 2013 Jan;15(1):51–62. PubMed PMID: 23319179.
- [75] Yant SR, Meuse L, Chiu W, Ivics Z, Izsvak Z, Kay MA. Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nature genetics*. 2000 May;25(1):35–41. PubMed PMID: 10802653.
- [76] Meir YJ, Wu SC. Transposon-based vector systems for gene therapy clinical trials: Challenges and considerations. *Chang Gung medical journal*. 2011 Nov–Dec;34(6): 565–79. PubMed PMID: 22196059.
- [77] Huang X, Guo H, Tammana S, Jung YC, Mellgren E, Bassi P, et al. Gene transfer efficiency and genome-wide integration profiling of Sleeping Beauty, Tol2, and piggyBac transposons in human primary T cells. *Molecular therapy: The journal of the American Society of Gene Therapy*. 2010 Oct;18(10):1803–13. PubMed PMID: 20606646. PubMed Central PMCID: 2951558.
- [78] Belur LR, Podetz-Pedersen K, Frandsen J, McIvor RS. Lung-directed gene therapy in mice using the nonviral sleeping beauty transposon system. *Nature protocols*. 2007;2(12):3146–52. PubMed PMID: 18079714.
- [79] Ma K, Wang DD, Lin Y, Wang J, Petrenko V, Mao C. Synergetic targeted delivery of sleeping-beauty transposon system to mesenchymal stem cells using LPD nanoparticles modified with a phage-displayed targeting peptide. *Advanced functional materials*. 2013 Mar 6;23(9):1172–81. PubMed PMID: 23885226. PubMed Central PMCID: 3718568.
- [80] Yant SR, Ehrhardt A, Mikkelsen JG, Meuse L, Pham T, Kay MA. Transposition from a gutless adeno-transposon vector stabilizes transgene expression in vivo. *Nature biotechnology*. 2002 Oct;20(10):999–1005. PubMed PMID: 12244327.
- [81] Cooney AL, Singh BK, Sinn PL. Hybrid nonviral/viral vector systems for improved piggyBac DNA transposon in vivo delivery. *Molecular therapy: The journal of the American Society of Gene Therapy*. 2015 Apr;23(4):667–74. PubMed PMID: 25557623.
- [82] Zhang W, Solanki M, Muther N, Ebel M, Wang J, Sun C, et al. Hybrid adeno-associated viral vectors utilizing transposase-mediated somatic integration for stable trans-

- gene expression in human cells. *PloS one*. 2013;8(10):e76771. PubMed PMID: 24116154. PubMed Central PMCID: 3792901.
- [83] Vink CA, Gaspar HB, Gabriel R, Schmidt M, McIvor RS, Thrasher AJ, et al. Sleeping beauty transposition from nonintegrating lentivirus. *Molecular therapy: The journal of the American Society of Gene Therapy*. 2009 Jul;17(7):1197–204. PubMed PMID: 19417741. PubMed Central PMCID: 2835211.
- [84] Staunstrup NH, Moldt B, Mates L, Villesen P, Jakobsen M, Ivics Z, et al. Hybrid lentivirus-transposon vectors with a random integration profile in human cells. *Molecular therapy: The journal of the American Society of Gene Therapy*. 2009 Jul;17(7):1205–14. PubMed PMID: 19240688. PubMed Central PMCID: 2835219.
- [85] Hackett CS, Geurts AM, Hackett PB. Predicting preferential DNA vector insertion sites: Implications for functional genomics and gene therapy. *Genome biology*. 2007;8 Suppl 1:S12. PubMed PMID: 18047689. PubMed Central PMCID: 2106846.
- [86] Clarke LL, Grubb BR, Gabriel SE, Smithies O, Koller BH, Boucher RC. Defective epithelial chloride transport in a gene-targeted mouse model of cystic fibrosis. *Science*. 1992 Aug 21;257(5073):1125–8. PubMed PMID: 1380724.
- [87] O'Neal WK, Hasty P, McCray PB, Jr., Casey B, Rivera-Perez J, Welsh MJ, et al. A severe phenotype in mice with a duplication of exon 3 in the cystic fibrosis locus. *Human molecular genetics*. 1993 Oct;2(10):1561–9. PubMed PMID: 7505691.
- [88] Dorin JR, Dickinson P, Alton EW, Smith SN, Geddes DM, Stevenson BJ, et al. Cystic fibrosis in the mouse by targeted insertional mutagenesis. *Nature*. 1992 Sep 17;359(6392):211–5. PubMed PMID: 1382232.
- [89] Ratcliff R, Evans MJ, Cuthbert AW, MacVinish LJ, Foster D, Anderson JR, et al. Production of a severe cystic fibrosis mutation in mice by gene targeting. *Nature genetics*. 1993 May;4(1):35–41. PubMed PMID: 7685652.
- [90] Zeiher BG, Eichwald E, Zabner J, Smith JJ, Puga AP, McCray PB, Jr., et al. A mouse model for the delta F508 allele of cystic fibrosis. *The journal of clinical investigation*. 1995 Oct;96(4):2051–64. PubMed PMID: 7560099. PubMed Central PMCID: 185844.
- [91] Delaney SJ, Alton EW, Smith SN, Lunn DP, Farley R, Lovelock PK, et al. Cystic fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations. *The EMBO journal*. 1996 Mar 1;15(5):955–63. PubMed PMID: 8605891. PubMed Central PMCID: 449990.
- [92] Colledge WH, Abella BS, Southern KW, Ratcliff R, Jiang C, Cheng SH, et al. Generation and characterization of a delta F508 cystic fibrosis mouse model. *Nature genetics*. 1995 Aug;10(4):445–52. PubMed PMID: 7545494.
- [93] Hodges CA, Cotton CU, Palmert MR, Drumm ML. Generation of a conditional null allele for *Cftr* in mice. *Genesis*. 2008 Oct;46(10):546–52. PubMed PMID: 18802965. PubMed Central PMCID: 2711445.

- [94] Tuggle KL, Birket SE, Cui X, Hong J, Warren J, Reid L, et al. Characterization of defects in ion transport and tissue development in cystic fibrosis transmembrane conductance regulator (CFTR)-knockout rats. *PloS one*. 2014;9(3):e91253. PubMed PMID: 24608905. PubMed Central PMCID: 3946746.
- [95] Sun X, Sui H, Fisher JT, Yan Z, Liu X, Cho HJ, et al. Disease phenotype of a ferret CFTR-knockout model of cystic fibrosis. *The journal of clinical investigation*. 2010 Sep;120(9):3149–60. PubMed PMID: 20739752. PubMed Central PMCID: 2929732.
- [96] Grubb BR, Vick RN, Boucher RC. Hyperabsorption of Na⁺ and raised Ca(2⁺)-mediated Cl-secretion in nasal epithelia of CF mice. *The American journal of physiology*. 1994 May;266(5 Pt 1):C1478–83. PubMed PMID: 7515571.
- [97] Liu X, Yan Z, Luo M, Engelhardt JF. Species-specific differences in mouse and human airway epithelial biology of recombinant adeno-associated virus transduction. *American journal of respiratory cell and molecular biology*. 2006 Jan;34(1):56–64. PubMed PMID: 16195538. PubMed Central PMCID: 1752084.
- [98] Liu X, Luo M, Zhang L, Ding W, Yan Z, Engelhardt JF. Bioelectric properties of chloride channels in human, pig, ferret, and mouse airway epithelia. *American journal of respiratory cell and molecular biology*. 2007 Mar;36(3):313–23. PubMed PMID: 17008635. PubMed Central PMCID: 1894945.
- [99] Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y, et al. Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *The journal of clinical investigation*. 2008 Apr;118(4):1571–7. PubMed PMID: 18324337. PubMed Central PMCID: 2265103.
- [100] Fisher JT, Tyler SR, Zhang Y, Lee BJ, Liu X, Sun X, et al. Bioelectric characterization of epithelia from neonatal CFTR knockout ferrets. *American journal of respiratory cell and molecular biology*. 2013 Nov;49(5):837–44. PubMed PMID: 23782101. PubMed Central PMCID: 3931095.
- [101] Rogers CS, Abraham WM, Brogden KA, Engelhardt JF, Fisher JT, McCray PB, Jr., et al. The porcine lung as a potential model for cystic fibrosis. *American journal of physiology lung cellular and molecular physiology*. 2008 Aug;295(2):L240–63. PubMed PMID: 18487356. PubMed Central PMCID: 2519845.
- [102] Mercer RR, Russell ML, Roggli VL, Crapo JD. Cell number and distribution in human and rat airways. *American journal of respiratory cell and molecular biology*. 1994 Jun;10(6):613–24. PubMed PMID: 8003339.
- [103] Wang X, Zhang Y, Amberson A, Engelhardt JF. New models of the tracheal airway define the glandular contribution to airway surface fluid and electrolyte composition. *American journal of respiratory cell and molecular biology*. 2001 Feb;24(2):195–202. PubMed PMID: 11159054.

- [104] Jacob S, Poddar S. Mucous cells of the tracheobronchial tree in the ferret. *Histochemistry*. 1982;73(4):599–605. PubMed PMID: 6175610.
- [105] Plopper CG, Mariassy AT, Wilson DW, Alley JL, Nishio SJ, Nettesheim P. Comparison of nonciliated tracheal epithelial cells in six mammalian species: Ultrastructure and population densities. *Experimental lung research*. 1983 Dec;5(4):281–94. PubMed PMID: 6662075.
- [106] Pack RJ, Al-Ugaily LH, Morris G, Widdicombe JG. The distribution and structure of cells in the tracheal epithelium of the mouse. *Cell and tissue research*. 1980;208(1):65–84. PubMed PMID: 6248229.
- [107] Widdicombe JH, Chen LL, Sporer H, Choi HK, Pecson IS, Bastacky SJ. Distribution of tracheal and laryngeal mucous glands in some rodents and the rabbit. *Journal of anatomy*. 2001 Feb;198(Pt 2):207–21. PubMed PMID: 11273045. PubMed Central PMCID: 1468211.
- [108] Dajani R, Zhang Y, Taft PJ, Travis SM, Starner TD, Olsen A, et al. Lysozyme secretion by submucosal glands protects the airway from bacterial infection. *American journal of respiratory cell and molecular biology*. 2005 Jun;32(6):548–52. PubMed PMID: 15746432. PubMed Central PMCID: 2715323.
- [109] Wine JJ, Joo NS. Submucosal glands and airway defense. *Proceedings of the American Thoracic Society*. 2004;1(1):47–53. PubMed PMID: 16113412.
- [110] Verkman AS, Song Y, Thiagarajah JR. Role of airway surface liquid and submucosal glands in cystic fibrosis lung disease. *American journal of physiology Cell physiology*. 2003 Jan;284(1):C2–15. PubMed PMID: 12475759.
- [111] Engelhardt JF, Yankaskas JR, Ernst SA, Yang Y, Marino CR, Boucher RC, et al. Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nature genetics*. 1992;2(3):240–8.
- [112] Choi HK, Finkbeiner WE, Widdicombe JH. A comparative study of mammalian tracheal mucous glands. *Journal of anatomy*. 2000 Oct;197 Pt 3:361–72. PubMed PMID: 11117623. PubMed Central PMCID: 1468138.
- [113] Ballard ST, Trout L, Bebok Z, Sorscher EJ, Crews A. CFTR involvement in chloride, bicarbonate, and liquid secretion by airway submucosal glands. *The American journal of physiology*. 1999 Oct;277(4 Pt 1):L694–9. PubMed PMID: 10516209.
- [114] Sehgal A, Presente A, Engelhardt JF. Developmental expression patterns of CFTR in ferret tracheal surface airway and submucosal gland epithelia. *American journal of respiratory cell and molecular biology*. 1996 Jul;15(1):122–31. PubMed PMID: 8679216.
- [115] Yan Z, Stewart ZA, Sinn PL, Olsen JC, Hu J, McCray PB, Jr., et al. Ferret and pig models of cystic fibrosis: Prospects and promise for gene therapy. *Human gene therapy clinical development*. 2015 Feb 12. PubMed PMID: 25675143.

- [116] Cmielewski P, Farrow N, Donnelley M, McIntyre C, Penny-Dimri J, Kuchel T, et al. Transduction of ferret airway epithelia using a pre-treatment and lentiviral gene vector. *BMC pulmonary medicine*. 2014;14:183. PubMed PMID: 25413892. PubMed Central PMCID: 4258949.
- [117] Pabst R. The respiratory immune system of pigs. *Veterinary immunology and immunopathology*. 1996 Nov;54(1-4):191-5. PubMed PMID: 8988865.
- [118] Maina JN, van Gils P. Morphometric characterization of the airway and vascular systems of the lung of the domestic pig, *Sus scrofa*: Comparison of the airway, arterial and venous systems. *Comparative biochemistry and physiology Part A, Molecular & integrative physiology*. 2001 Nov;130(4):781-98. PubMed PMID: 11691614.
- [119] Baskerville A. Histological and ultrastructural observations on the development of the lung of the fetal pig. *Acta anatomica*. 1976;95(2):218-33. PubMed PMID: 961354.
- [120] Inglis SK, Corboz MR, Taylor AE, Ballard ST. In situ visualization of bronchial submucosal glands and their secretory response to acetylcholine. *The American journal of physiology*. 1997 Feb;272(2 Pt 1):L203-10. PubMed PMID: 9124370.
- [121] Inglis SK, Corboz MR, Ballard ST. Effect of anion secretion inhibitors on mucin content of airway submucosal gland ducts. *The American journal of physiology*. 1998 May;274(5 Pt 1):L762-6. PubMed PMID: 9612291.
- [122] Joo NS, Irokawa T, Wu JV, Robbins RC, Whyte RI, Wine JJ. Absent secretion to vasoactive intestinal peptide in cystic fibrosis airway glands. *The journal of biological chemistry*. 2002 Dec 27;277(52):50710-5. PubMed PMID: 12368280.
- [123] Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, et al. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science*. 2008 Sep 26;321(5897):1837-41. PubMed PMID: 18818360. PubMed Central PMCID: 2570747.
- [124] Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. *Genome research*. 1996 Oct;6(10):995-1001. PubMed PMID: 8908519.
- [125] Sausseureau EL, Roussel D, Diallo S, Debarbieux L, Edelman A, Sermet-Gaudelus I. Characterization of nasal potential difference in cftr knockout and F508del-CFTR mice. *PloS one*. 2013;8(3):e57317. PubMed PMID: 23505426. PubMed Central PMCID: 3591431.
- [126] Griesenbach U, Smith SN, Farley R, Singh C, Alton EW. Validation of nasal potential difference measurements in gut-corrected CF knockout mice. *American journal of respiratory cell and molecular biology*. 2008 Oct;39(4):490-6. PubMed PMID: 18458238.
- [127] Salinas DB, Pedemonte N, Muanprasat C, Finkbeiner WF, Nielson DW, Verkman AS. CFTR involvement in nasal potential differences in mice and pigs studied using a

- thiazolidinone CFTR inhibitor. *American journal of physiology Lung cellular and molecular physiology*. 2004 Nov;287(5):L936–43. PubMed PMID: 15246976.
- [128] Fasbender A, Marshall J, Moninger TO, Grunst T, Cheng S, Welsh MJ. Effect of co-lipids in enhancing cationic lipid-mediated gene transfer in vitro and in vivo. *Gene therapy*. 1997 Jul;4(7):716–25. PubMed PMID: 9282173.
- [129] Coakley RD, Grubb BR, Paradiso AM, Gatzky JT, Johnson LG, Kreda SM, et al. Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. *Proceedings of the National Academy of Sciences of the United States of America*. 2003 Dec 23;100(26):16083–8. PubMed PMID: 14668433. PubMed Central PMCID: 307696.
- [130] Pezzulo AA, Tang XX, Hoegger MJ, Alaiwa MH, Ramachandran S, Moninger TO, et al. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. *Nature*. 2012 Jul 5;487(7405):109–13. PubMed PMID: 22763554. PubMed Central PMCID: 3390761.
- [131] Abou Alaiwa MH, Beer AM, Pezzulo AA, Launspach JL, Horan RA, Stoltz DA, et al. Neonates with cystic fibrosis have a reduced nasal liquid pH; a small pilot study. *Journal of cystic fibrosis: Official journal of the European Cystic Fibrosis Society*. 2014 Jul;13(4):373–7. PubMed PMID: 24418186. PubMed Central PMCID: 4060428.
- [132] Hoegger MJ, Fischer AJ, McMenimen JD, Ostedgaard LS, Tucker AJ, Awadalla MA, et al. Cystic fibrosis. Impaired mucus detachment disrupts mucociliary transport in a piglet model of cystic fibrosis. *Science*. 2014 Aug 15;345(6198):818–22. PubMed PMID: 25124441.
- [133] Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. *Clinical microbiology reviews*. 2002 Apr;15(2):194–222. PubMed PMID: 11932230. PubMed Central PMCID: 118069.
- [134] Dodd JD, Lavelle LP, Fabre A, Brady D. Imaging in cystic fibrosis and non-cystic fibrosis bronchiectasis. *Seminars in respiratory and critical care medicine*. 2015 Apr;36(2):194–206. PubMed PMID: 25826587.
- [135] Kobinger GP, Weiner DJ, Yu QC, Wilson JM. Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. *Nature biotechnology*. 2001 Mar;19(3):225–30. PubMed PMID: 11231554.
- [136] Koehler DR, Martin B, Corey M, Palmer D, Ng P, Tanswell AK, et al. Readministration of helper-dependent adenovirus to mouse lung. *Gene therapy*. 2006 May;13(9):773–80. PubMed PMID: 16437131.
- [137] Koehler DR, Frndova H, Leung K, Louca E, Palmer D, Ng P, et al. Aerosol delivery of an enhanced helper-dependent adenovirus formulation to rabbit lung using an intratracheal catheter. *The journal of gene medicine*. 2005 Nov;7(11):1409–20. PubMed PMID: 15999396.

- [138] Griesenbach U, McLachlan G, Owaki T, Somerton L, Shu T, Baker A, et al. Validation of recombinant Sendai virus in a non-natural host model. *Gene therapy*. 2011 Feb; 18(2):182–8. PubMed PMID: 20962870.
- [139] Hwang SK, Kwon JT, Park SJ, Chang SH, Lee ES, Chung YS, et al. Lentivirus-mediated carboxyl-terminal modulator protein gene transfection via aerosol in lungs of K-ras null mice. *Gene therapy*. 2007 Dec;14(24):1721–30. PubMed PMID: 17960162.
- [140] Koehler DR, Sajjan U, Chow YH, Martin B, Kent G, Tanswell AK, et al. Protection of Cftr knockout mice from acute lung infection by a helper-dependent adenoviral vector expressing Cftr in airway epithelia. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100(26):15364–9. Epub 2003 Dec 12.
- [141] Cao H, Machuca TN, Yeung JC, Wu J, Du K, Duan C, et al. Efficient gene delivery to pig airway epithelia and submucosal glands using helper-dependent adenoviral vectors. *Molecular therapy Nucleic acids*. 2013;2:e127. PubMed PMID: 24104599. PubMed Central PMCID: 3890457.
- [142] McLachlan G, Baker A, Tennant P, Gordon C, Vrettou C, Renwick L, et al. Optimizing aerosol gene delivery and expression in the ovine lung. *Molecular therapy: The journal of the American Society of Gene Therapy*. 2007 Feb;15(2):348–54. PubMed PMID: 17235313.
- [143] Davies LA, McLachlan G, Sumner-Jones SG, Ferguson D, Baker A, Tennant P, et al. Enhanced lung gene expression after aerosol delivery of concentrated pDNA/PEI complexes. *Molecular therapy: The journal of the American Society of Gene Therapy*. 2008 Jul;16(7):1283–90. PubMed PMID: 18500249.
- [144] Alton EW, Boyd AC, Cheng SH, Cunningham S, Davies JC, Gill DR, et al. A randomised, double-blind, placebo-controlled phase IIB clinical trial of repeated application of gene therapy in patients with cystic fibrosis. *Thorax*. 2013 Nov;68(11):1075–7. PubMed PMID: 23525080.
- [145] Xue FS, Yang QY, Liao X, Liu JH, Tong SY. Topical anesthesia of the airway using fiberoptic bronchoscope and the MADgic atomizer in patients with predicted difficult intubation. *Canadian journal of anaesthesia = Journal canadien d'anesthésie*. 2007 Nov;54(11):951–2. PubMed PMID: 17975244.
- [146] Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboultch P, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 2003;302(5644):415–9.
- [147] Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M, Kempinski H, et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *The journal of clinical investigation*. 2008 Sep;118(9):3143–50. PubMed PMID: 18688286. PubMed Central PMCID: 2496964.

- [148] Boztug K, Schmidt M, Schwarzer A, Banerjee PP, Diez IA, Dewey RA, et al. Stem-cell gene therapy for the Wiskott-Aldrich syndrome. *The New England journal of medicine*. 2010 Nov 11;363(20):1918–27. PubMed PMID: 21067383. PubMed Central PMCID: 3064520.
- [149] Stein S, Ott MG, Schultze-Strasser S, Jauch A, Burwinkel B, Kinner A, et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nature medicine*. 2010 Feb;16(2):198–204. PubMed PMID: 20098431.
- [150] Persons DA, Baum C. Solving the problem of gamma-retroviral vectors containing long terminal repeats. *Molecular therapy: The journal of the American Society of Gene Therapy*. 2011 Feb;19(2):229–31. PubMed PMID: 21289636. PubMed Central PMCID: 3034864.
- [151] Rothe M, Modlich U, Schambach A. Biosafety challenges for use of lentiviral vectors in gene therapy. *Current gene therapy*. 2013 Dec;13(6):453–68. PubMed PMID: 24195603.
- [152] Biasco L, Baricordi C, Aiuti A. Retroviral integrations in gene therapy trials. *Molecular therapy: The journal of the American Society of Gene Therapy*. 2012 Apr;20(4):709–16. PubMed PMID: 22252453. PubMed Central PMCID: 3321603.
- [153] Biffi A, Bartholomae CC, Cesana D, Cartier N, Aubourg P, Ranzani M, et al. Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. *Blood*. 2011 May 19;117(20):5332–9. PubMed PMID: 21403130.
- [154] Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, et al. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nature biotechnology*. 2006 Jun;24(6):687–96. PubMed PMID: 16732270.
- [155] McGarrity GJ, Hoyah G, Winemiller A, Andre K, Stein D, Blick G, et al. Patient monitoring and follow-up in lentiviral clinical trials. *The journal of gene medicine*. 2013 Feb;15(2):78–82. PubMed PMID: 23322669.
- [156] Aiuti A, Bacchetta R, Seger R, Villa A, Cavazzana-Calvo M. Gene therapy for primary immunodeficiencies: Part 2. *Current opinion in immunology*. 2012 Oct;24(5):585–91. PubMed PMID: 22909900.
- [157] Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, Baricordi C, et al. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science*. 2013 Aug 23;341(6148):1233151. PubMed PMID: 23845947.
- [158] Cartier N, Hacein-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, Kutschera I, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science*. 2009 Nov 6;326(5954):818–23. PubMed PMID: 19892975.

- [159] Biffi A, Montini E, Lorioli L, Cesani M, Fumagalli F, Plati T, et al. Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science*. 2013 Aug 23;341(6148):1233158. PubMed PMID: 23845948.
- [160] Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *The New England journal of medicine*. 2013 Apr 18;368(16):1509–18. PubMed PMID: 23527958.
- [161] Budde LE, Zhang MM, Shustov AR, Pagel JM, Gooley TA, Oliveira GR, et al. A phase I study of pulse high-dose vorinostat (V) plus rituximab (R), ifosphamide, carboplatin, and etoposide (ICE) in patients with relapsed lymphoma. *British journal of haematology*. 2013 Apr;161(2):183–91. PubMed PMID: 23356514. PubMed Central PMCID: 3618618.
- [162] Budde LE, Berger C, Lin Y, Wang J, Lin X, Frayo SE, et al. Combining a CD20 chimeric antigen receptor and an inducible caspase 9 suicide switch to improve the efficacy and safety of T cell adoptive immunotherapy for lymphoma. *PloS one*. 2013;8(12):e82742. PubMed PMID: 24358223. PubMed Central PMCID: 3866194.
- [163] Farinelli G, Capo V, Scaramuzza S, Aiuti A. Lentiviral vectors for the treatment of primary immunodeficiencies. *Journal of inherited metabolic disease*. 2014 Mar 12. PubMed PMID: 24619149.
- [164] Drumm ML, Pope HA, Cliff WH, Rommens JM, Marvin SA, Tsui LC, et al. Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. *Cell*. 1990 Sep 21;62(6):1227–33. PubMed PMID: 1698126.
- [165] Rich DP, Anderson MP, Gregory RJ, Cheng SH, Paul S, Jefferson DM, et al. Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature*. 1990 Sep 27;347(6291):358–63. PubMed PMID: 1699126.

IntechOpen