GENE THERAPY STRATEGIES FOR TREATMENT OF MUCO-OBSTRUCTIVE LUNG DISEASES

by
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Abstract

Knowledge of genetic origins and associations of muco-obstructive lung diseases has made inhaled gene therapy an attractive alternative to the current standards of care that are limited to managing disease symptoms. However, despite over two decades of intensive research and development, gene therapy has yet to help patients with cystic fibrosis (CF) or any other muco-obstructive lung diseases. The slow progress is due in part to poor understanding of the biological barriers to inhaled gene therapy.

In this dissertation, I first introduce the pathobiology of representative muco-obstructive lung diseases and examine pitfalls of clinically investigated gene vectors of the past and of current options. I then review key components for successful execution of inhaled gene therapy, including gene delivery systems, physiological barriers and strategies to overcome them, and advances in preclinical models with which the most promising systems may be vetted for clinical trials.

Secondly, I demonstrate that adeno-associated vectors (AAV), which are more commonly used gene vectors for clinical settings, differ in their ability to diffuse through the CF sputum barrier and mediate various levels of transduction depending on the surface chemistry. Specifically, I compared three AAV vectors in their ability to i) diffuse in CF sputum, ii) provide transgene expression in ALI culture of primary human CF bronchial epithelial cells, and iii) provide transgene expression in a mouse model of muco-obstructive lung diseases.

Thirdly, I present the application of a synthetic biodegradable gene vector in *ex vivo*, *in vitro*, and *in vivo* models relevant to muco-obstructive diseases. This gene vector is composed of engineered poly(β-amino ester) polymers and nucleic acid that encodes

reporter or therapeutically relevant genes. I found that this gene vector, compared to conventional gene vector, is able to i) efficiently diffuse through CF sputum, ii) safely mediate higher magnitude of and widespread transgene expression in healthy and muco-obstructive lung mouse model, and iii) apically transfer reporter gene to mucus-covered air-liquid interface (ALI) culture of primary human CF bronchial epithelial cells harvested from CF patient lungs with F508del homozygous mutation, the most common form of mutation in the CF patient population.

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1. Introduction

Muco-obstructive lung diseases consist of a group of respiratory disorders characterized by airway obstruction in the lungs of affected patients. All included diseases entail severe respiratory morbidity that often results in pulmonary failure and disease-associated mortality. Current treatments generally improve patient quality of life. However, these options do not tackle the root of the disease [1], and patients are required to follow cumbersome therapeutic regimens throughout their lifetime [2]. Gene therapy has emerged as a promising alternative approach as a result of the growing number of identified genetic causes and modifiers of muco-obstructive lung diseases. With advances in nucleic acid engineering, it is now conceivable to achieve gene transfer to specific cell types, including lung parenchymal cells [3-5], without affecting non-target cells [6]. In addition, persistent transgene expression during the life span of transfected cells can be achieved [7, 8].

Therapeutic delivery via inhalation provides direct access to the target of gene therapy for muco-obstructive lung diseases, namely the cells lining the lung airways and airspace (i.e., alveoli), in a relatively non-invasive manner. The vast majority of preclinical and clinical studies of respiratory gene therapy have employed a pulmonary delivery strategy, including intratracheal/intranasal instillation and nebulization [9-11]. Nebulizers generate inhalable micron-sized liquid droplets that can carry hundreds of gene vectors per drop and reach virtually all areas of the lung [12]. However, despite encouraging safety and tolerability results, therapeutically effective inhaled gene therapy of muco-obstructive lung diseases has not yet been achieved. Lack of clinical success is due to the limited ability of gene delivery vectors to overcome difficult biological

barriers, which stems from a relatively poor understanding of the barriers. Improvement of this understanding may allow for more rational vector design strategies to tackle them. For example, accumulation of thickened airway mucus was recognized early on as a key pathological event of muco-obstructive lung diseases, but overcoming its barrier properties has typically not been addressed in the design of inhaled therapeutic delivery systems. In addition, most clinical trials involving airway gene therapy have been pursued as a result of positive outcomes observed in animal models that lack key features of human muco-obstructive lung diseases [13-16]. The disappointments in more than 25 prior gene therapy clinical trials for cystic fibrosis (CF) alone have refocused research efforts on deepening knowledge of key issues that have prevented success of inhaled gene therapy.

This dissertation is focused on these critical issues, including clinically-relevant gene delivery vectors, important biological barriers to successful gene transfer in the lungs, and rationally-designed approaches to overcome the barriers. This work is guided by the central hypotheses that i) pathological mucus barrier is problematic to inhaled gene vectors and ii) we can compare and identify promising viral or non-viral gene vectors by using appropriate tools and experiments. I first describe various muco-obstructive lung diseases and their current state in the arena of academic research and the clinic, and suggest future directions for improved clinical outcomes. Then the following chapters introduce viral and non-viral strategies to treat muco-obstructive lung diseases. The respective gene vectors are validated for treatment of muco-obstructive lung diseases by using appropriate *ex vivo*, *in vitro*, and *in vivo* models that closely recapitulate the pathophysiological conditions of muco-obstructed lungs.

2. Background

2.1. Muco-obstructive lung diseases for inhaled gene therapy

2.1.1. Cystic fibrosis (CF)

CF is the most common inherited genetic disorder in the US, and more than 70,000 people are affected worldwide. CF is generally caused by one of many different possible mutations to a single gene that encodes cystic fibrosis transmembrane conductance regulator (CFTR). Alteration of the CFTR ion channel protein causes abnormal ion transport between lung airway epithelial cells and the airway surface liquid (ASL), as well as similar ion transport defects in the gastrointestinal and genital tracts [17]. Pulmonary complications are the primary cause of CF-related morbidity and mortality [18]. Aberrant ion regulation in CF, including impaired chloride secretion and dysregulated sodium absorption, leads to dehydration of the ASL. The result of dehydration is a thickened mucus gel in the airways that can impair mucociliary clearance (MCC) [19]. Altered biophysical properties of airway mucus, which impair mucus detachment, may also play a role in impaired MCC in CF [20]. Reduced rates of MCC cause mucus accumulation in the airways and provide a permissive environment for chronic bacterial infection and associated inflammation, which together can cause airway obstruction, fibrosis and, eventually, pulmonary failure [19, 21]. Airway dehydration, infection and inflammation are more pronounced in females with CF, which may be due to airway-related modulatory effects of estrogen [22]. As a result, females with CF have higher mortality compared to males with CF [22].

Common inhalable treatments for CF, including recombinant human DNase (Pulmozyme®), hypertonic saline and antibiotics (TOBI®), alleviate disease symptoms, but do not address the underlying root of the problem, CFTR dysfunction. In 2012, the FDA approved the first drug that directly addresses CFTR dysfunction, Ivacaftor (VX-770; Kalydeco®) [23]. Ivacaftor demonstrated significant improvements in lung function of CF patients [24, 25]. However, only patients with a specific class of mutations respond to Ivacaftor treatment, representing only a small subset of the patient population (~5%). Another drug, lumacaftor (VX-809), showed promising *in vitro* correction of the most prevalent CFTR mutation, F508del, which represents ~70% of CF patients [26]. This finding prompted its clinical evaluation, in combination with Ivacaftor [27], and the result was published in 2015 [28]. Although the trial demonstrated only modest improvement in pulmonary function compared to Ivacaftor in G551D patients [29], the combined formulation was recently approved by the FDA under the brand name of Orkambi®.

There are more than 1900 identified CFTR mutations, many of which are not expected to be responsive to currently available CFTR drugs [27]. Inhaled CFTR gene therapy, as a means to treat the underlying cause of the disease in the lungs, could benefit CF patients regardless of their specific CFTR mutation. However, over 25 clinical trials testing viral or non-viral gene vectors have failed to show clinical benefits, largely due to inefficient gene transfer to target cells [9, 10], including serous cells in the submucosal glands and ciliated airway epithelial cells [30]. Some viral CF gene therapy trials have been discontinued due to the generation of host immune response that renders subsequent treatments ineffective [10, 31]. It should be noted that lifetime repeated treatment is likely required for CF, as therapeutic effects will eventually fade away due to the

transient nature of episomal transgene expression [32] and/or the natural lifespan of transfected cells [7]. The UK CF Gene Therapy Consortium has recently completed the only CF gene therapy clinical trial that has been active in the past decade [33]. In this study, Alton et al. demonstrated, using a non-viral gene vector, a significant, yet modest, benefit compared to placebo control. They concluded that a more potent gene delivery vector is required to make gene therapy a viable option for treating CF [33].

Clinical trials for CF gene therapy have shown evidence of CFTR transgene expression based on measurements of CFTR mRNA and changes in nasal potential difference (NPD), but no significant improvement in lung function parameters has been reported [34, 35]. This suggests that the levels of gene transfer achieved in clinical trials have been insufficient to mediate functional cure in the CF airways. Nevertheless, optimism remains as several studies have suggested that a modest level of functional CFTR protein may be sufficient to improve lung function of CF patients. An early *in vitro* study suggested that only ~5% of airway epithelial cells need to produce functional CFTR proteins to restore chloride ion balance in the CF lung [36]. More recently, Pickles et al. used an *in vitro* model of human CF ciliated airway epithelium and found that at least 25% of cells may be required to express functional CFTR proteins in order to achieve mucus transport rates comparable to those in non-CF airways [37]. Interestingly, CF patients with certain mutations, which retain ~10% of normal CFTR expression per cell, are generally not afflicted by CF lung diseases [38]. Based on these observations, modest levels of CFTR protein expression throughout the airway epithelium could normalize pulmonary function in CF lungs.

All CF gene therapy clinical trials to date have tested delivery of wild-type CFTR genes in order to provide functional proteins. However, approaches to rescue defective CFTR have been introduced in the literature, which involve miRNA [39], peptide nucleic acid [40], zinc-finger nuclease [41] and CRISPR/Cas9 [42] technologies. These studies demonstrated *in vitro* and/or *in vivo* restoration of the F508del CFTR function. Recently, the CF Modifier Consortium, which combines research efforts from groups in North America and France, completed a genome-wide association study to identify genetic loci relevant to CF pathophysiology [43]. In this study, samples from 6,365 CF patients with over 8 million genetic variants were analyzed and five genetic modifier loci associated with disease severity were discovered. This finding may provide additional genetic targets and enable individualized treatment of CF.

2.1.2. α-1 antitrypsin deficiency

α-1 antitrypsin deficiency (AATD) is another attractive target for gene therapy since it is also a monogenic disorder. AATD is caused by mutation in the gene encoding the serine protease inhibitor (α-1 antitrypsin; AAT). In normal conditions, AAT is synthesized predominantly in the liver, secreted directly into the bloodstream, and transported to the lungs where it protects alveolar interstitial elastin from degradation by neutrophil elastase [44]. However, reduced AAT secretion in AATD leads to protease/anti-protease imbalance and airway inflammation in the lungs. As a result, patients with AATD develop emphysema and chronic obstructive pulmonary disease (COPD; discussed in section 2.3), which is often triggered by environmental factors such as acute infection and cigarette smoking [9]. A small subset of AATD patients (<10%)

develops symptomatic liver disease. It is generally accepted that mutant AAT molecules polymerize and accumulate in the endoplasmic reticulum of hepatocytes, leading to the elevation of pro-inflammatory signaling [45].

Weekly intravenous infusion of AAT protein (i.e. augmentation therapy) is the only therapeutic option for AATD lung diseases that is currently approved by the FDA [44]. This protein-based augmentation therapy is well tolerated, effective in restoring AAT serum levels to the therapeutic threshold of 11 μM, and improves lung function [44, 46]. However, the therapy is expensive and requires frequent dosing in the clinic. Thus, a gene therapy product that allows sustained production of AAT over a longer period of time may offer a significant advantage over the current therapy.

There have been four clinical trials conducted for AATD gene therapy to date [47]. The first proof-of-concept clinical trial involved intranasal administration of non-viral gene vectors carrying the AAT gene [48]. In this study, one-third of the level after AAT protein therapy was achieved, but the effect was transient. In subsequent trials, viral gene vectors were intramuscularly dosed to AATD patients, since the site or type of cells that produce AAT is irrelevant [47]. However, in a recent Phase II clinical trial, viral gene vectors administered via this route mediated AAT production at only 3-5% of the therapeutic target, necessitating an improved gene transfer strategy [49].

Directly targeting the lungs via inhalation may provide a therapeutically-beneficial level of AAT production at the site of action. Although there is no active clinical trial evaluating inhaled AATD gene therapy, inhalable AAT protein-based augmentation therapy is in progress in AATD patients with emphysema [46]. Encouragingly, an inhaled AATD gene therapy trial demonstrated superior anti-

inflammatory effects in the lungs compared to intravenous protein therapy [48]. Gene therapy approaches for AATD liver diseases include the use of short hairpin RNA (shRNA) or miRNA for the knockdown of mutant AAT in hepatocytes; detailed information is available elsewhere [44, 46, 47].

2.1.3. Chronic obstructive pulmonary disease

COPD is an incurable disease that is expected to be the third largest cause of death in the world by 2020 [50]. Cigarette smoking is generally accepted as the major cause of the disease, but exposure to environmental and/or work-related pollutants is also reported to be a significant factor [51, 52]. Abnormal inflammation and oxidative stress mediated by excessive inhalation of particulate matter and certain gases cause destruction of the extracellular matrix, leading to disease progression [51]. COPD is characterized by a progressive and irreversible airway limitation. This results from chronic bronchitis characterized by fibrosis, obstruction and remodeling of small airways [53]. Emphysema may also occur characterized by enlargement of airspace and destruction of lung parenchyma [53]. COPD patients with frequent exacerbations experience increased airway inflammation, dynamic lung hyperinflation, elevated bacterial colonization in the lower airways and increased susceptibility to viral infection of the airways, rendering the lungs the major target for treatment [54].

Smoking cessation is an important part of COPD treatment, however, many COPD patients continue to suffer from the disease due to irreversible functional and anatomical alterations [55]. Current treatments for COPD reduce symptoms only, and do not arrest or reverse deterioration in lung function and architecture that accompanies

moderate to severe disease. Available pharmacological treatments include bronchodilators, such as β-agonists and muscarinic antagonists, and inhaled corticosteroids, each of which offer short-term management of disease symptoms [56]. Other medications include mucolytic agents [57] and antibiotics [58], both recommended for patients undergoing acute exacerbations.

COPD gene therapy research has been slow due to the highly variable disease etiology and lack of good animal models, each of which limit the pace of drug development [9]. While clinically-tested AATD gene therapies may also prove useful for treatment of COPD, mutations in the AAT gene are responsible for only ~1-3% of COPD cases [51]. Several genetic loci involved in protease/anti-protease, antioxidant, or anti-inflammatory activities have been identified to exhibit polymorphisms associated with COPD [51]. However, preclinical evaluation of genetic intervention has not been extensively pursued. For example, pro-inflammatory cytokines like IL-18 and IL-1β have been implicated in COPD pathogenesis, but their potential role as therapeutic targets remains to be explored [59].

Recent genetic, biochemical and histological evidence suggests altered transforming growth factor beta (TGF-β) signaling is associated with COPD development and progression [60-62]. TGF-β levels are elevated in the conducting airway (i.e., bronchi and bronchioles) and airspace compartments of patients with COPD [63-65]. In addition, Podowski et al. have shown that angiotensin receptor blocker antagonizes TGF-β signaling and, as a result, attenuates smoke-induced lung injury and rescues lung airway and airspace architecture in a mouse model of COPD [66]. Knocking down TGF-β signaling in the airways and alveolar sacs via gene silencing technologies may provide

similar therapeutic outcomes. Interestingly, cigarette smoking has been shown to reduce CFTR mRNA levels, CFTR protein levels and CFTR function, which contributes to mucus clearance defects in patients with COPD [67]. Thus, CFTR is another potential therapeutic target for COPD therapy.

2.1.4. Asthma

Allergic asthma is a global health problem caused by unregulated production of cytokines secreted by allergen-specific, type 2 helper T-cells (Th2 cells) [68]. Similar to COPD, both genetic predisposition and exposure to environmental irritants contribute to the development of asthma. Allergens presented to naïve T cells lead to expression of various cytokines, including IL-4, IL-5, IL-9 and IL-13, via the Th2 pathway, resulting in immunoglobulin E (IgE) production and mast cell recruitment [69]. Exposure of sensitized individuals to allergens induces release of histamine, leukotrienes and prostaglandins by mast cells in the lung airways, which promote vascular permeability, smooth muscle contraction and mucus production [69]. Subsequently, chemokines released by mast cells attract macrophages, eosinophils, Th2 cells and basophils to the airways, triggering airway inflammation, tissue damage, and allergen hypersensitivity [69].

Anti-inflammatory medication, specifically inhaled corticosteroids, is the most frequently prescribed therapy for asthma patients [70]. β2-adrenergic receptor agonists are also frequently used to dampen the inflammatory response by relaxing the smooth muscle [71]. While effective for most of patients, corticosteroids and β2-adrenergic receptor agonists are not always effective in patients with severe disease experiencing

exacerbation [72], and do not modify the course of the disease [73]. In addition, prolonged use of either medication elicits several side effects [74]. Gene therapy has emerged as a potential alternative or supplementary therapeutic approach that has been investigated in preclinical settings to date; gene silencing approaches are more common, but gene overexpression approaches are also being studied [69].

Unlike CF and AATD, but similar to COPD, there are multiple genetic loci involved in asthma development and progression. This opens many opportunities for gene therapies aimed at a host of target cells, including lung epithelial cells, smooth muscle cells, and immune and inflammatory cells [9]. Using non-viral gene delivery platforms, a broad range of targeted proteins could be simultaneously activated with a single administration, which may be an advantage compared to interventions with small molecules, such as oligonucleotide (ONT)-based knockdown approaches [75]. Repeated dosing with ONT-based therapies failed to show sufficient therapeutic benefit over time [9, 74, 75]. In contrast, plasmid DNA-based strategies could provide long-term therapeutic effects with relatively infrequent administration.

Evidence suggests that airway remodelling results in the progressive loss of lung function in asthmatics [76]. Thus, in addition to the therapies for attenuating airway inflammation, intervention of asthma-associated structural changes in the lung, including smooth muscle hypertrophy, wall thickening and collagen deposition, could reduce the rate of loss of lung function. Recently, da Silva et al. reported a proof-of-concept study where they evaluated effect of the gene encoding an active form of thymulin peptide in a mouse model of asthma [77]. Thymulin peptide has been shown to mediate anti-inflammatory and anti-fibrotic effects in several disease models by modulating T cell

differentiation [78]. They found that a single intratracheal administration of a polymer-based non-viral gene vector carrying thymulin plasmid DNA effectively prevented both the inflammatory and remodelling processes in the airways, thereby providing improved airway repair and lung mechanics in a mouse model of allergic asthma. Likewise, a viral gene vector carrying antisense against a cytokine that activates the Th2 pathway, IL-4, reduced airway remodelling in a rat model despite being systemically administered [79]. Overall, multiple targets may need to be tackled to cover the broad spectrum of the diseased population, but promising preclinical studies underscore that gene therapy is an attractive strategy for patients affected by severe asthma.

2.2. **Gene delivery platforms**

2.2.1. Viral gene vectors

Viruses have evolved to infect and transfer genetic payloads to host cells, which makes them attractive candidates for gene therapy applications. The recent approval of adeno-associated virus (AAV) type 1 as a vector for gene therapy in patients with lipoprotein lipase deficiency in Europe (Glybera™) [80] has provided renewed optimism in virus-mediated gene therapy. Numerous clinical trials using adenovirus (AdV) or AAV as gene vectors to treat muco-obstructive lung diseases have been completed, most of which have focused on CF due in part to its monogenic nature [81]. However, gene therapy clinical trials for CF using AdV and AAV2, dosed either intranasally or intratracheally, have failed to result in clinical benefits, and inefficient gene transfer to target cells has been cited as the primary reason [9, 10]. In addition, host immune responses to these vectors were found to limit gene expression after repeated

administration of the vectors [10, 31]. However, it should be noted both AdV and AAV2 were well tolerated with limited adverse effects in these studies. Recent preclinical studies aim to more clearly define tissue tropism of the many available viral vectors and their alternative serotypes. In addition, sophisticated optimization of viral capsids and genomes (discussed in section 2.4.1) are further advancing the capabilities of next-generation viral gene vectors.

2.2.1.1. Adenovirus (AdV)

AdV was the first viral gene vector tested in inhaled gene therapy clinical trials. AdV is a non-enveloped, icosahedral capsid virion with diameter ranging from 70 - 100 nm. AdV has a genome capacity (36kb) that is much larger than typical viruses [82]. This allows full length CFTR, AATD, and other relatively large therapeutic nucleic acids to be packaged into AdV. AdV does not introduce its nucleic acid payload into the host genome, which results in transient transgene expression [83]. However, lack of DNA integration is a benefit in terms of safety since insertional mutagenesis has been observed with integrating viral vectors [84]. Importantly, the receptor that mediates AdV entry into airway epithelial cells, coxsakievirus-adenovirus receptor (CAR), resides on the basolateral side of the epithelium, which limits AdV's potential efficacy *in vivo* for inhaled gene therapy [85, 86]. The innate immune responses, including generation of neutralizing antibodies against AdV, must also be addressed, as repeated administration of AdV will be required for most inhaled gene therapy applications [87, 88]. To begin to address this limitation, helper-dependent or "gutless" AdV (HD-AdV) have been produced wherein all viral DNA has been removed. Preclinical studies with HD-AdV

have shown promise [89, 90]. Toeitta et al. showed that HD-AdV administration resulted in reduced inflammatory response and improved airway transduction in mice compared to AdV [91]. Importantly, Croyle et al. showed that gene transduction in mouse lungs with HD-AdV was maintained after a second administration conducted 28 days after the initial dosing [92]. Airway transduction with HD-AdV has also been demonstrated in larger animals, including the ferret [93] and pig [94], further supporting its potential for inhaled gene therapy applications.

2.2.1.2. Adeno-associated virus (AAV)

Recent viral gene therapy trials have shifted to the use of AAV, as it overcomes many limitations of AdV. For example, AAV provides broad tissue tropism and more stable transgene expression with partial, site-specific integration into the host genome [10, 95, 96]. AAV is a non-enveloped, icosahedral, non-replicating capsid virus with a diameter of roughly 25 nm [97]. Infection of target cells with AAV is mediated by cell-surface associated glycans, such as sialic acids and/or heparan sulfate proteoglycans depending on the AAV serotype [98-100]. As AAV vectors have shown broad tissue tropism, debate remains over the best-suited AAV serotypes for pulmonary applications [10]. AAV2 was the first discovered serotype [95], and is the only serotype tested in clinical trials of inhaled gene therapy to date [101-103]. Although efficient gene transfer was evident in the nares of humans, lung function in CF patients was not improved [101, 102]. The disappointing outcomes with AAV2 mediated gene therapy In the lungs are likely partially due to the limited capacity of AAV2 to transduce airway epithelial cells via the apical membrane [10]. AAV2, similar to AdV, requires a receptor that is primarily

expressed on the basolateral side of the airway epithelium, specifically the heparan sulfate proteoglycan receptor, in order to introduce nucleic acid payloads into airway epithelial cells [99]. A Phase II clinical trial for CF utilizing AAV2 was dropped in 2005 due to inadequate efficacy following repeated administration [103]. To this end, identification and/or engineering of AAV variants with enhanced infection capabilities via the apical membrane, lower immunogenicity, and desired tropism is likely required, especially given the therapy-inactivating immunogenicity generated by repeated administration of AAV2. In Phase I and II clinical trials for AATD, AAV1 and AAV2 dosed intramuscularly did not reach their primary therapeutic endpoints, but rather showed transient and low production of wild-type AAT [47, 49]. Inhaled administration of next generation gene vectors, packaging AAT-encoding DNA, will allow production of AAT proteins at the target of therapy, which may provide a greater therapeutic benefit to patients afflicted with AATD.

Investigations into alternative AAV serotypes, including AAV1, AAV5, and AAV6, have shown promising results, motivating their development for inhaled gene therapy. AAV5 mediated 50-fold greater gene transfer efficiency than AAV2 in air-liquid interface (ALI) culture of primary human airway epithelium (HAE) *in vitro* in one study [104]. The inherent tropism of AAV5 for airway epithelium results from its interaction with α2,3 N-linked or O-linked sialic acid receptors present on the apical surface of the airway epithelium [98]. Recently, a pseudotyped (hybrid) AAV gene vector, bearing AAV2 rep and AAV5 cap expression cassette (AAV2/5), achieved persistent gene transfer lasting up to 15 months in the airways and alveoli of mice following intratracheal administration (Figs. 2.1. A, B) [105]. They also found that re-administration of AAV2/5

14 months after the initial administration did not significantly reduce the gene transfer efficacy, presumably due to the long dosing interval [105]. As a result of this dosing

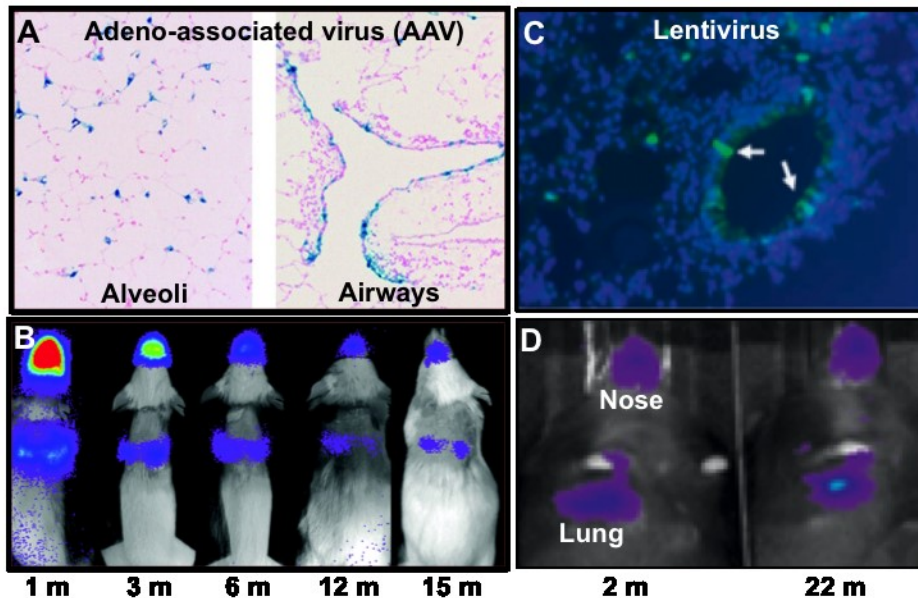

Figure 2.1. Viral vectors for inhaled gene therapy. (A, B) A recombinant AAV2/5 (AAV2 *rep* gene, AAV5 *cap* gene) delivered by intratracheal instillation demonstrates long-lasting gene expression (up to 15 months) in both conducting airways and alveoli of mice. (A) Immunohistological staining of β-galactosidase (β-gal) expression in alveoli and conducting airways 1 month post-administration. (B) Bioluminescence imaging of firefly luciferase expression in the lung and nose of mice at 1, 3, 6, 12, and 15 months post-administration. Reprinted from [105] with permission of Mary Ann Liebert, Inc. (C, D) A simian immuno-deficiency virus pseudotyped with the respiratory pathogen Sendai virus (F/HN-SIV) demonstrates sustained transgene expression in the nose and lungs of mice after intranasal administration, lasting 22 months post-administration. (C) Fluorescent microscopy images of GFP expression mediated by F/HN-SIV in mouse lungs. (D) Bioluminescence imaging of firefly luciferase expression in the mouse lungs and noses at 2 and 22 months post-administration. Reprinted from [131] with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society.

interval, neutralizing antibody levels were reduced by more than 50% compared to peak levels after the first administration [105]. The process and rationale of pseudotyping viral gene vectors is discussed in detail in section 5.1. Similar to AAV5, AAV1 and AAV6 require interactions with apically expressed receptors, α2,3 and/or α2,6 N-linked sialic acids [100], respectively, in order to transduce airway epithelial cells [100]. An *in vitro* study with ALI culture of primary HAE revealed that AAV1 exhibited orders of

magnitude greater transduction efficiency than AAV2 and AAV5 when the vectors were administered to the apical side of the airway epithelium [106]. AAV1 also exhibited gene transfer efficacy following intratracheal administration in the airways of chimpanzees that was 20-fold higher compared to that achieved with AAV5, presumably due to a weaker T-cell response to AAV1 [107]. More recently, AAV6 was found to transduce mouse and dog airway epithelium *in vivo* [108], and HAE *in vitro* [109], to a level surpassing that achieved with AAV1, AAV2 and AAV5. In addition to the identification of AAV serotypes with lung tropism, modification of AAV to overcome other key barriers and therapy-inactivating immunogenicity is needed prior to human testing of next generation AAV for inhaled applications.

A limitation of AAV is its relatively small packaging capacity for DNA payload (4.7 kb) [97]. All CF gene therapy trials to date have used the full-length, wild-type CFTR gene to produce functional CFTR protein. The relatively large size of CFTR cDNA (4.5 kb) has, as a result, greatly limited the selection of important regulatory elements in plasmid design, including promoter and enhancer components. [110]. Thus, clinical trials that have tested AAV-mediated CFTR gene transfer have utilized a weak promoter derived from inverted terminal repeat (ITR) [110]. ITR is a key element required for packaging therapeutic nucleic acid payloads into the AAV capsid [111]. Several approaches have been developed seeking to circumvent this issue. Yan et al. utilized human bocavirus virus-1 (HBoV1) capsids to create a hybrid AAV virus with a larger packaging capacity (5.5 kb) [112]. In order to incorporate stronger promoters into AAV, shorter versions of therapeutic genes have also been engineered with a specific focus on CF gene therapy [113]. Specifically, truncated CFTR genes that rescue defective

CFTR, rather than synthesize the protein *de novo,* have been developed [114, 115]. Cebotaru et al. demonstrated that a truncated CFTR, Δ27-264 CFTR, delivered via AAV2/5, provided F508del CFTR restoration *in vitro* in polarized HAE with chloride currents approaching that of wild-type CFTR [115]. In an earlier report, they also showed that expression of another truncated CFTR, Δ264 CFTR, led to an increased level of wild-type CFTR production in the lungs of monkeys [116]. Another strategy to overcome the packaging size limitation inherent to AAV is to split the genome between two AAV vectors. This is achieved by either packaging overlapping genomes, which reconstruct the full length gene through homologous recombination after viral entry, or by evenly splitting the genome between two vector genomes that then combine after infection through trans-splicing via heterodimerization [117]. Using the former approach, intranasal administration of dual AAV6 vectors, carrying overlapping fragments of a reporter gene, resulted in production of the encoded protein in the mouse lung to a level comparable to what achieved by a single AAV6 carrying the intact gene [118]. This finding suggests that the additional recombination step may not significantly impact the efficacy of transgene expression.

2.2.1.3. Retro- and lentivirus

Retroviruses have also been investigated as viral vectors for use in inhaled gene therapy. Unlike AdV and AAV, retroviruses are capable of fully integrating nucleic acid payloads into the host genome via reverse transcription [119], thereby potentially providing longer and more stable transgene expression. However, insertion must be tightly controlled in order to avoid insertional mutagenesis that may result from random incorporation into

host chromosomal DNA [84]. In a prior clinical trial for X-linked severe combined immunodeficiency, 2 of 4 patients who were successfully treated with retroviral gene vectors carrying the γc gene developed treatment-related leukemia [120]. Another limitation of retroviral vectors is their inability to infect non-dividing cells [121], which limits their use for inhaled gene therapy applications given the slow turnover rate of airway epithelium [7].

Other integrating viral gene vectors of interest are lentiviral gene vectors, including recombinant human (HIV) and feline (FIV) immunodeficiency viruses. In contrast to retrovirus, lentiviruses are capable of transfecting post-mitotic cells [122, 123], including fully differentiated lung parenchymal cells. Lentiviral vectors have a packaging capacity large enough (8 kb) to accommodate full-length genes, such as CFTR [124]. However, lentiviruses have limited tissue tropism and, thus, capsid engineering is required to enable their use in gene therapy applications [124]. Initial preclinical studies have demonstrated efficient, persistent pseudotyped lentivirus-mediated gene expression in the lung [125-131]. For example, a recent study using pseudotyped lentivirus delivered intranasally showed gene expression in the lungs of mice that lasted up to 22 months after initial dosing without signs of toxicity or insertional mutagenesis (Figs. 2.1. C, D) [131]. The modifications used to introduce lung tropism to lentivirus-based gene vectors are discussed in detail in section 2.4.1. Further investigation into lentivirus-mediated lung gene therapy is warranted given the promising results thus far. However, whether lentiviruses can efficiently overcome physiological barriers unique to inhaled gene therapy of muco-obstructive lung diseases remains to be investigated.

2.2.2. Non-viral gene vectors

The majority of inhaled gene therapy clinical trials for muco-obstructive lung diseases to date have evaluated virus-based gene vectors. However, intrinsic limitations to their use for gene therapy over the lifetime of a patient, including therapy-inactivating immunogenicity and insufficient gene transfer in human airways to elicit clinical benefits, have spurred interests in development of synthetic systems [132, 133]. Synthetic systems, often referred to as non-viral vectors, are generally formed by multivalent electrostatic interactions between positively charged carrier materials and negatively charged nucleic acids. Unlike some viral vectors, non-viral vectors possess virtually unlimited nucleic acid packaging capacity [134], enabling the delivery of large, multiple and/or diverse nucleic acid payloads. Scale-up of non-viral vectors is relatively straight-forward compared to the complex procedures required for mass production of viral vectors [135]. Non-viral vectors can be altered to impart desired functionalities, such as the ability to penetrate through extracellular barriers [136], target specific cell types [137] and enhance intracellular delivery [138]. It is widely claimed that the primary disadvantage of non-viral vectors is that they provide lower gene transfer efficacy compared to viral vectors [139]. However, few studies have been conducted where advanced non-viral vectors have been directly compared with viruses *in vivo*, where physiological barriers such as the presence of thick mucus and the immune response may significantly contribute to their performance.

2.2.2.1. Lipid-based gene vectors

The earliest preclinical evaluation of inhaled non-viral vectors for gene therapy of the airways was conducted with lipid-based systems [140-142]. A seminal study reported by Stribling et al. reported results obtained from nebulized lipid-based gene vectors for inhaled gene therapy [142]. In their paper, aerosolization of a system based on N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) safely mediated transgene expression in the majority of airway epithelial cells and alveolar lining cells at least for 21 days without signs of toxicity [142]. The promise provided by this and other early work [13, 143-147] enabled the prompt translation of the approach to clinical evaluation for inhaled gene therapy, with a specific focus on monogenic disorders, including CF and AATD. There have been 11 clinical trials with non-viral gene vectors for inhaled gene therapy of muco-obstructive lung diseases to date, and all but one involved lipid-based formulations [148]. Based on promising observations in transgenic CF mouse models [13, 149], dimethylaminoethane-carbamoyl-cholesterol (DC-Chol)/DOPE was the first lipid-based system to be clinically evaluated [150]. These studies provided proof-of-concept for lipid-based inhaled gene transfer by showing partial NPD correction and a sign of CFTR transgene expression in several CF patients [11, 150]. However, similar to results with viral vectors tested in CF patients to date, the effects were modest and transient. Thus, the team tested whether repeated administration might provide more sustainable transgene expression [151]. Although clinical benefit was not achieved, the three doses of DC-Chol/DOPE carrying wild-type CFTR were well-tolerated by CF patients without any evidence of immunologic side effects [151]. In addition, gene transfer efficacy was not

attenuated by repeated administration [151], unlike viral vectors [10, 31]. Other lipid-based systems, including N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) [152] and p-ethyl-dimyristoylphosphatidyl choline cholesterol (EDMPC-Chol), have been tested in humans for inhaled gene therapy of CF. Unfortunately, the results were similar at best to the earlier single dose DC-Chol/DOPE studies [11, 150]. In the first and only clinical evaluation of the inhaled gene therapy of AATD using non-viral vectors, Brigham et al. compared nasally instilled DOTMA/DOPE carrying AAT-encoding plasmid DNA to the standard-of-care for AATD, weekly intravenous injection of AAT protein [48]. The concentration of AAT in nasal lavage fluids of patients who received the inhaled AAT gene therapy was about a third of the normal value that is achieved by AAT protein therapy. However, unlike the protein therapy, inhaled gene therapy reduced the levels of IL-8, a pro-inflammatory cytokine that is elevated in AATD patients [48].

The Genzyme lipid 67 (GL67) is by far the most extensively studied lipid-based gene vector for inhaled gene therapy of muco-obstructive lung diseases. GL67 provided 100-fold greater transgene expression compared to DC-Chol/DOPE [147], which was the first system tested clinically [150]. However, intranasal instillation of GL67, while achieving transgene expression on par with AdV, resulted in significant acute lung toxicity due to the need for high doses [147, 153]. Eastman et al. reported delivery of GL67 via aerosol significantly reduced the toxicity, but empirically determined that very high concentrations were required, which caused undesirable vector precipitation [142, 154, 155]. To address this, they modified the system with a fraction of dioleoulphosphatidylethanolamine covalently coupled with 5 kDa polyethylene glycol

(PEG) [155], yielding a formulation named GL67A. Surface stabilization with PEG was later confirmed by *in vitro* studies showing that exposure to physiological concentrations of CF mucus components, including albumin, mucin, and linear DNA, did not undermine the gene transfer efficacy of GL67A [156]. Clinical studies with GL67A showed signs of CFTR transgene expression, but flu-like symptoms were observed in treated patients [11, 157]. To potentially improve the safety profile, Hyde et al. investigated the effect of unmethylated CpG on GL67A-mediated *in vivo* gene transfer [158]. They demonstrated that while the presence of a single CpG in plasmid DNA was sufficient to elicit a pro-inflammatory response, CpG-free plasmid DNA delivered via GL67A provided sustained transgene expression at least for 56 days without causing lung inflammation. In the meantime, GL67A was compared for *in vivo* gene transfer efficacy with other non-viral systems, including 25 kDa polyethylenimine (PEI)-based systems and clinically-tested PEGylated poly-L-lysine (PLL) (see section 3.2.1; [159])-based systems [14]. Among these, GL67A exhibited the highest levels of CFTR transgene expression in sheep lungs (Fig. 2.2. A, B) [14]. However, it should be noted that the doses were not matched in this study. Recently, a clinical trial testing repeated administration of GL67A carrying CpG-free CFTR-encoding plasmid DNA was completed [160]. In this multi-dose trial, GL67A provided a significant, but moderate, improvement in the lung function of CF patients, suggesting that an improved gene delivery system is likely required to achieve therapeutically relevant outcomes [33]. Notably, monthly administration of the gene vectors to these patients was well tolerated over the course of the 12-month treatment without any detectable adverse effect [33].

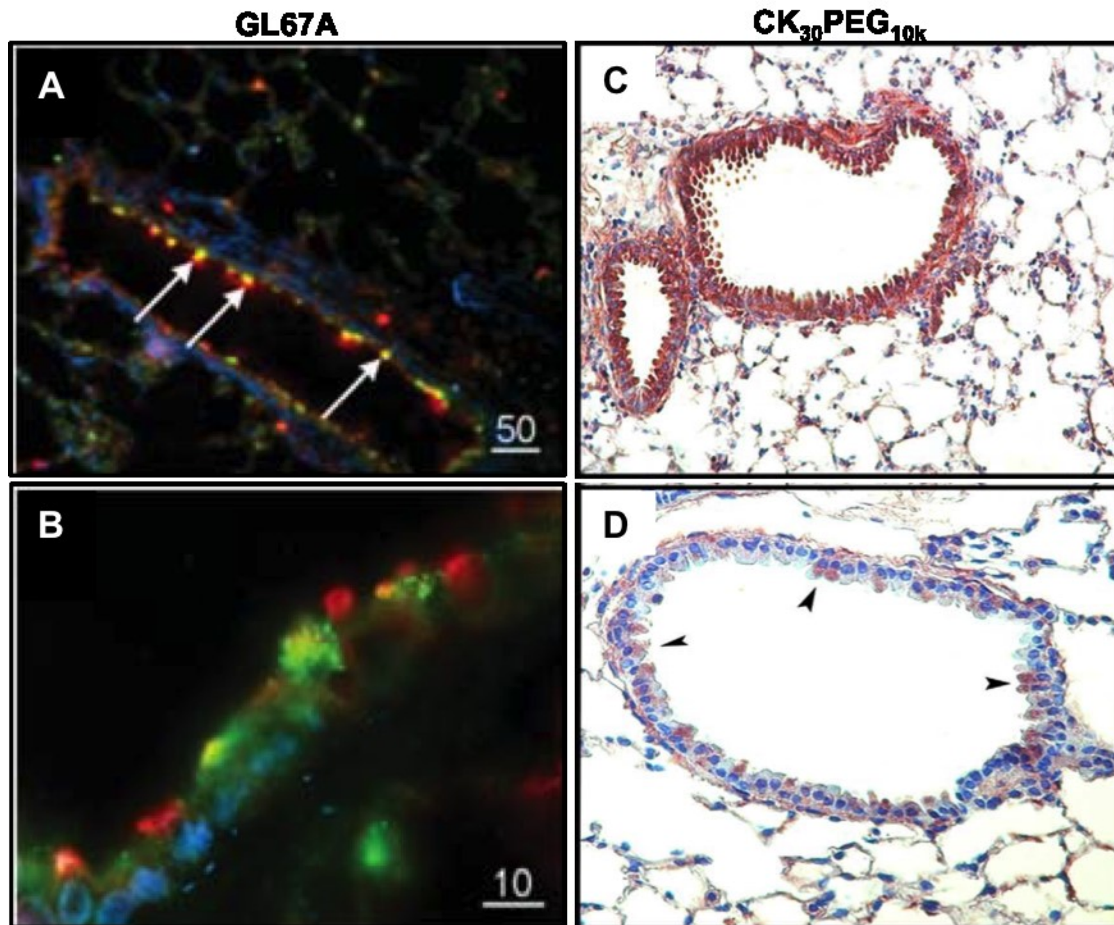

Figure 2.2. Non-viral gene vectors for inhaled gene therapy. (A, B) Localization of gene expression in sheep lungs treated with GL67A carrying human CFTR (hCFTR)-expressing plasmid DNA. (A) Dual labeling of two hCFTR epitopes (G449, Texas red; MATG1061, FITC) of lung sections. Arrows indicate epithelial cells that are positive with both antibodies. (B) Dual labeling of cytokeratin and G449 (anti-cytokeratin antibody, red; G449, FITC). Reprinted from [14] with permission of Macmillan Publishers Ltd. (C, D) Localization of gene expression in the (C) medium (20x maginification) and (D) small airways (40x magnification) in the mouse lungs. Lungs harvested from animals that received 100 μg of β-gal expressing plasmid DNA compacted with $CK_{30}PEG_{10k}$ were fixed, sectioned, and immunohistochemically stained for the bacterial β-galactosidase protein 2 days after intratracheal administration. Reprinted from [181] with permission of Macmillan Publishers Ltd.

2.2.2.2. Polymer-based gene vectors

Cationic polymers have also been explored as a means to produce non-viral gene vectors for gene therapy. Among polymer-based systems, PEI is the most extensively studied in the preclinical setting. Linear and branched PEI with molecular weights over

20 kDa have been used most frequently due to their high charge density that enables efficient complexation of nucleic acid payloads [161] and strong buffering capacity that may help the gene vectors escape acidic vesicles inside the cell [162]. Multiple groups have investigated PEI complexes with nucleic acids for inhaled gene therapy [14, 163-169]. Densmore et al. demonstrated that PEI-based gene vectors nebulized into mouse lungs produced a 10 – 100-fold greater pulmonary transgene expression compared to various lipid-based systems, including GL67/DOPE, DOTAP/DOPE and DC-Chol/DOPE [164]. Importantly, repeated administration at a dose interval of 56 days of 25 kDa branched PEI complexed with CpG-free plasmid DNA further boosted the transgene expression in the mouse lungs [163], suggesting that the effectiveness of repeated treatments was not limited by vector-induced immunogenicity. Nevertheless, clinical use of PEI alone as the condensing polymer in a non-viral vector system has been discouraged by some due to toxicity observed in animals caused by its high positive charge density and non-biodegradable nature [170, 171].

Interestingly, Boeckle et al. demonstrated that removal of residual free PEI polymers (polymers in a formulation that are not complexed to DNA) by size exclusion chromatography resulted in significantly reduced toxicity and increased transgene expression of gene vectors based on 22 kDa linear PEI [172]. Likewise, removing free PEI by ultracentrifugation resulted in significantly greater pulmonary transgene expression with negligible toxicity in mouse and sheep lungs following aerosolized administration of 25 kDa branched PEI-based gene vectors [169]. These findings suggest that the toxicity of PEI-based systems is largely attributed to uncomplexed polymers, and that complete removal of free PEI from formulations will be a necessary step in future

clinical evaluations. Furthermore, since low molecular weight PEI is less toxic than high molecular weight PEI, several groups have developed PEI derivatives capable of degrading into smaller subunits in physiological conditions, such as in aqueous, acidic and/or reducing environments [173-176]. Of note, albeit not for inhaled gene therapy, PEI has been tested in clinical trials for other gene therapy applications [177, 178].

Poly-L-lysine (PLL) is another well-studied studied cationic polymer for gene delivery applications. While PLL alone has been associated with limited transfection efficiency and cytotoxicity [160, 179], a specific PLL-PEG based gene vector, namely $CK_{30}PEG_{10k}$, has been found safe when administered to human nares. $CK_{30}PEG_{10k}$ consists of a 30-mer PLL (30 lysine residues) covalently linked to 10 kDa PEG via a cysteine residue. $CK_{30}PEG_{10k}$ has been shown to transfect airway epithelial cells in the lungs of mice (Figs. 2.2. C, D) [180], perhaps owing to its ability to interact with nucleolin on the surface of airway epithelial cells [181], without eliciting significant toxicity [182]. In addition, $CK_{30}PEG_{10K}$ was shown to effectively complex plasmid DNA with sizes up to 20 kb [183] and to transfect post-mitotic cells [184]. The promise in the preclinical studies resulted in its evaluation in 12 CF patients who received $CK_{30}PEG_{10K}$ complexed with human CFTR-encoding plasmid DNA administered intranasally [159]. A majority of treated patients exhibited partial to complete NPD correction without any noted side effects [159]. The level of gene transfer to the upper airway achieved by $CK_{30}PEG_{10k}$ was comparable to the levels observed in trials of AAV2 at the highest titer [102, 159, 185].

Table 2.1. Gene vector platforms for muco-obstructive lung diseases

Gene vector type	Advantages	Disadvantages	Clinical Development Stage
Adenovirus (AdV)	Large nucleic acid packaging capacity (36 kb) compared to AAV [81] Non-integrating; no concerns over insertional mutagenesis [82]	Transient transgene expression [82] Requires basolaterally-expressed receptors for cell entry [84,85] Immune response limits efficacy upon re-administration [9]	Clinical trials for CF [9]
Adeno-associated virus (AAV)	Non-pathogenic [10] Multiple serotypes (e.g. AAV1, 5 and 6) able to enter airway epithelial cells via apical cell surface receptors [94] More stable gene expression compared to AdV [94,95]	Limited nucleic acid packaging capacity (4.7kb) [96] Immune response limits efficacy upon re-administration [10]	Clinical trials for CF [100-102] and AATD [46,48]
Lentivirus	Long-term, stable gene expression [118]; lower dosing frequencies are achievable [130] Capable of transfecting post-mitotic cells [121,122], unlike retrovirus [120]	Safety concerns over insertional mutagenesis [119] Requires vector engineering for lung applications [123]	Pre-clinical [124-130]
Lipid- and Polymer-based	Facile chemical modification [137] Minimal constraint in packaging capacity [151, 184] Large-scale production [186] Variety of FDA-approved drug delivery formulations [187] Controlled release is achievable [139]	Cytotoxicity [153, 173] No intrinsic tropism [139] (Lipid) Occasional stability issue [137]	(Lipid) Clinical trials for CF and AATD [148] (Polymer) Clinical trials for CF [35]

2.3. **Physiological barriers to inhaled gene therapy**

2.3.1. Barriers in conducting airways

2.3.1.1. Mucus gel layer

Initial efforts to improve lung gene transfer focused primarily on efficiently overcoming cellular barriers to DNA delivery [188-190]. More recently, mucus covering the airway epithelium has been recognized as one of the greatest obstacles to overcome [191-195]. Airway mucus is primarily composed of a dense mesh of gel-forming mucin fibers, large macromolecules containing a high density of negatively charged glycans interspersed with periodic hydrophobic regions [196]. Thus, inhaled foreign materials, including gene vectors, are most often immobilized in the mucus blanket via multivalent adhesive interactions (e.g. electrostatic interactions, hydrophobic forces and hydrogen bonding) and/or steric obstruction. Gene vectors trapped in the mucus gel are cleared from the lung via mucociliary clearance (MCC) (3.6 mm/min [197]), which precludes them from efficiently reaching underlying target cells. In the lungs of people with muco-obstructive lung diseases, including CF, COPD and asthma, mucus metaplasia and hypersecretion leads to mucus accumulation and impaired MCC, providing a permissible environment for chronic infection and inflammation [19, 21, 198-200]. Particularly in the CF airways, elevated levels of endogenous DNA and actin filaments released from necrotic neutrophils further contribute to the dense mesh structure of the airway secretions [191, 201].

The average pore size of CF mucus is 140 ± 50 nm (range: 60 – 300 nm) [202], which is markedly smaller than the average pore size of human cervicovaginal mucus secretions (340 ± 70 nm) [203]. Recently, Fahy et al. reported that elevation of oxidative

stress in the lungs of CF patients increases disulfide cross-links between mucin fibers that increases mucus elasticity [204]. The increase in mucin crosslinking density also most likely causes the mucus mesh spacing to tighten further, thereby reinforcing airway mucus as a steric barrier. It is conceivable that other muco-obstructive lung diseases characterized by elevated oxidative stress, including COPD and asthma [205], may share this feature. The viscous drag on gene delivery vectors in the pores alone is not likely to pose a significant diffusion barrier, since the viscosity of the fluid in that fills these pores in normal airway mucus [206] or CF mucus [207] is only moderately higher than that of water. Of note, Coakley et al. have reported that estrogen reduces airway surface liquid height on the CF airway epithelium, which is restored by estrogen antagonist, tamoxifen [208]. Thus, mucus barrier properties may be more pronounced in females. Cigarette smoke coupled with progesterone exposure has also been reported to significantly elevate mucus cell metaplasia and accumulation of eosinophils in an asthma model, while progesterone alone does not [209]. MCC is likely impaired in both cases, which contributes to infection and inflammation in the airway, thereby increasing mucus barrier properties.

Gene vectors that have been used in CF clinical trials recently have been shown to be incapable of efficiently penetrating CF mucus, including AdV [210], various serotypes of AAV including AAV1, 2 and 5 [210, 211], and $CK_{30}PEG_{10K}$ [212, 213]. Likewise, non-viral gene vectors based on the most widely explored cationic polymers, including PEI [214] and polyamidoamine (PAMAM) dendrimers [215], are unable to penetrate CF mucus, most likely due to the their positively charged surfaces that readily interact with negatively charged mucus constituents. It has also been shown that *in vitro* gene transfer

mediated by lipid- and polymer-based gene vectors and AdV was significantly reduced by CF mucus, underscoring its barrier property [216, 217]. Braeckmans et al. have demonstrated that negatively charged, hydrophobic polystyrene nanoparticles strongly adhere to the CF mucus mesh network (Fig. 3A) [207]. Thus, the limited success with gene delivery systems in clinical trials to date may be at least partly attributed to their inability to overcome the mucus barrier. Given the similar pathophysiological events in the airways of patients with other muco-obstructive lung diseases, the mucus blanket is likely to pose a similarly critical barrier to inhaled gene transfer. Indeed, diffusion of nanoparticles in COPD mucus has been shown to be hindered to a similar extent as in CF mucus [201].

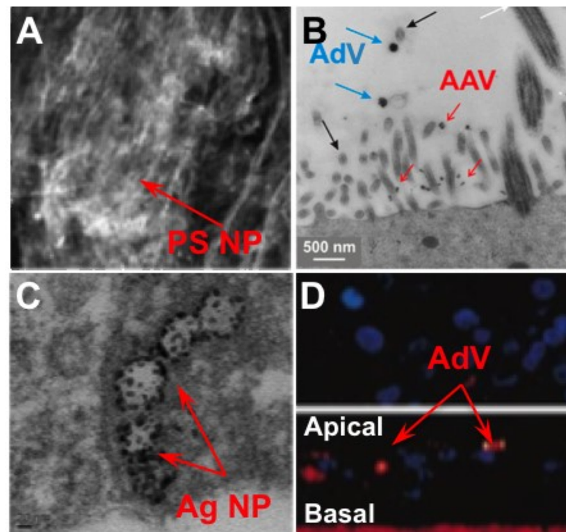

Figure 2.3. Primary physiological barriers to inhaled gene therapy. (A) Mucus: A confocal image showing 89-nm polystyrene nanoparticles (PS NP) trapped via adhesive interactions within CF mucus. Reprinted from [207] with permission from Elsevier. (B) The periciliary layer (PCL): Adenovirus (AdV, blue arrows) is excluded from the PCL while adeno-associated virus (AAV, red arrows) penetrates into PCL and reaches underlying epithelium. Reprinted from [240] by permission from Macmillan Publishers Ltd. (C) Alveolar macrophages: an SEM image showing aerosolized 110 nm silver nanoparticles (Ag NP) accumulating in macrophages collected from broncheoalveolar lavage fluid (BALF) after being administered to rats. Reprinted from [260] by permission from Oxford University Press. (D) Epithelial cell tight junctions: A confocal image (top: xy view, bottom: xz view) showing AdV restricted to the apical side of human airway epithelium due to the presence of tight junctions which prevents the access to receptors required

for cell entry. Reprinted from [272] with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society.

Antibody trapping and/or neutralization can also contribute to inefficient gene transfer, specifically for viral gene vectors. Although antibodies diffuse in human mucus relatively unimpeded due to their small molecular size [218], the Fc region of antibodies make low affinity adhesive interactions with mucus [219]. As antibodies accumulate on the surface of pathogen, multivalent antibody interactions with the mucus mesh can trap the pathogen, thereby preventing their penetration to the underlying tissue. For example, Wang et al. recently reported that HSV serotype 1 (HSV-1) readily penetrated fresh neutralized human cervicovaginal mucus, but was trapped therein in presence of anti-HSV-1 immunoglobulin G (IgG), which protected mice against vaginal infection [220]. A significant fraction of CF patients harbor active antibodies against AAV2 (32%) and AdV (55%) [221], which may further enhance adhesive entrapment in CF mucus and/or inactivate their capacity to transduce target cells. Further, neutralizing antibodies against AAV1, 2, 5, 6, 7, and 8 are often found in the airways of healthy people and people with CF [222, 223]. Neutralizing antibodies are also produced in response to administration of viral vectors, and their levels are elevated upon repeat dosing [10]. Thus, it is critical to determine if the presence of antibodies affects the mucus penetration rates of AAV in these patient populations.

In addition to its role as a barrier that prevents penetration of gene vectors, mucus may impair the colloidal stability of gene vectors due to the presence of various soluble macromolecules, proteins, lipids, surfactants and ions in the airways. For example, non-viral gene vectors based on cationic polymers and lipids readily interact with negatively charged mucus constituents, resulting in large aggregates. Cationic gene vectors have

been shown to aggregate in presence of albumin (the most abundant protein in airway secretions [224]), DNA or mucin [156, 225, 226]. Viruses that possess positively charged surfaces, including HSV [227] and HIV [228], may share similar fates in the mucus gel. The aggregation of gene vectors may reduce gene transfer efficacy by further hindering diffusion in mucus, especially if the aggregates become larger than mucus mesh spacings [229]. Alton et al. showed that even mucus diluted 100-fold markedly reduced gene transfer by AdV and lipid-based gene vectors [230], which is partly attributed to the altered physicochemical properties of gene vectors mediated by soluble mucus constituents [195]. Negatively charged soluble materials in mucus may also compromise non-viral gene vectors by de-stabilizing complexation of DNA with cationic carrier materials. DNA molecules released from their vector are susceptible to degradation by endogenous nucleases present in the airways [231, 232].

2.3.1.2. Periciliary layer (PCL)

Gene vectors trapped in the mucus gel layer of the airways are cleared by MCC or cough-driven clearance [233, 234]. In contrast, gene vectors that rapidly penetrates through the gel layer and into the periciliary layer (PCL) may be retained significantly longer in the lung, as the PCL is believed to be nearly stationary [235-237]. Button et al. found that the PCL presents a significant steric barrier to vector penetration [238]. Using ALI culture of primary human bronchial epithelial cells, they showed that fluorescent dextran probes larger than 40 nm were excluded from the PCL, while smaller probes partitioned into the PCL and penetrated further toward the epithelium as size decreased [238]. These studies demonstrated that the PCL has a fine mesh structure, as opposed to being just a watery

layer as was previously suggested [239], and that nano-sized objects must pay a free energy price to penetrate the PCL [238]. Similar to the mucus gel layer, the PCL may also serve as an adhesive barrier since the meshwork is primarily composed of cell-tethered mucins [238]. Kesimer et al. showed that the PCL excluded AdV (~100 nm in diameter), but not AAV (~30 nm in diameter; serotype not specified; Fig. 3B) [240]. In CF lungs, dehydration of the PCL mediated by dysregulation of epithelial sodium channels (ENaC) on the airway epithelium can cause an osmotically-driven collapse of the PCL [238, 241]. Collapse of the PCL likely further increases the barrier function to inhaled gene vectors by making the PCL mesh tighter. Despite being relatively less explored, airway dehydration appears to be a common concern for other muco-obstructive lung diseases, including COPD [242] and asthma [243]. Overall, gene vectors capable of penetrating the mucus gel layer but not the PCL would most likely be cleared via MCC [214] or by macrophages [244].

2.3.2. Barriers in airspace

2.3.2.1. Pulmonary surfactant

Mucus is not present in the alveolar sacs of the lungs. However, inhaled gene vectors that make it to the alveoli must retain their stability and function in presence of surfactants that are abundant in the airspace. Pulmonary surfactant is a surface-active lipoprotein complex, synthesized by type II alveolar epithelial cells, that is composed of various phospholipids, cholesterols and surfactant proteins, including SP-A, B, C and D. To date, studies exploring the effect of pulmonary surfactants on lung gene transfer have primarily involved non-viral gene vectors [170, 245-251]. Gene transfer mediated by cationic lipid-

based gene vectors was significantly reduced by pulmonary surfactants [248-251]. The stability of lipid-based gene vectors in the presence of various surfactants, including Alveofact (an extract from bovine lung lavage) and Exosurf (a synthetic surfactant) and individual components of pulmonary surfactant was investigated. Negatively charged phospholipids [248-251] and/or SP-B or C [250] were found to facilitate aggregation of, or DNA release from, lipid-based gene vectors, indicative of impaired colloidal stability or disruption of DNA-lipid complexation, respectively. While to lesser extents, Alveofact has been shown to reduce gene transfer efficacy of gene vectors formulated with cationic polymers, including PEI and PAMAM dendrimer [170, 248].

Studies have also demonstrated surfactant-mediated aggregation of polymeric gene vectors [245, 246], however, DNA complexation was maintained with cationic polymer-based gene vectors, unlike lipid-based vectors, in the presence of pulmonary surfactants [248]. This carrier material-dependent effect is likely due to the difference in the nature of complexation by cationic lipids and polymers that influences vector stability in pulmonary surfactants. In addition to the disruption of complexation by negatively charged surfactants, hydrophobic lipid-surfactant interactions may render lipid-based vectors more susceptible to destabilization. Interestingly, incubation of polymeric gene vectors with pulmonary surfactants has been shown to enhance gene delivery efficiency *in vitro* due to improved cellular uptake and/or increased cell membrane permeability [246, 247]. Similarly, surfactants have been found to enhance AdV-mediated gene transfer to peripheral lung cells *in vitro* and *in vivo* [252, 253]; lipid recycling in pulmonary epithelial cells was suggested as a potential mechanism [253]. These findings

suggest that the effect of pulmonary surfactant on respiratory gene transfer may be multimodal and may vary depending on gene vector type.

2.3.2.2. Alveolar macrophages

Alveolar macrophages are phagocytes residing in the airspace that play a critical role in homeostasis, host defense and tissue remodeling [254]. There are 12 -14 macrophages in each alveolus [255], which is increased in individuals who smoke regularly [256]. Alveolar macrophages engulf inhaled foreign substances directly or via an opsonin-dependent mechanism. Particles in the size range of 250 nm to 3 μm are readily phagocytosed by macrophages [257], with increasing phagocytic uptake with increase in the particle size within this range [258]. In contrast, particles smaller than 250 nm are taken up less efficiently by macrophages [257], including alveolar macrophages [259]. These findings suggest that alveolar macrophages may not pose a significant hurdle for gene vectors, since widely explored viral and non-viral gene vectors are generally smaller than 250 nm. However, aggregation of gene vectors in the presence of pulmonary surfactants may render them more susceptible to clearance by alveolar macrophages due to their increased size. For example, 20 and 110 nm silver nanoparticles delivered via aerosol to rat lungs were found as large aggregates that were engulfed by macrophages collected from broncheoalveolar lavage fluid (BALF; Fig. 3C) [260].

Other properties of gene vectors, including surface charge, composition and particle geometry, play a critical role in phagocytosis [261]. In the case of polymeric nanoparticles, studies have shown that particles with hydrophobic surfaces are more readily internalized by macrophages than those with hydrophilic surfaces, while surface

charge did not appear to significantly alter the phagocytosis [261, 262]. Surfactant proteins such as SP-A and SP-D can opsonize inhaled gene vectors [263], which may facilitate opsonin-dependent phagocytosis by alveolar macrophages. Immunoglobulin (i.e. antibody) and complement are primary opsonins present in the lungs [264]. It has also been found that some viral vectors, including retrovirus and AdV, can be rapidly internalized by alveolar macrophages [265, 266]. It is possible that viral vectors are recognized by alveolar macrophages via Fc receptors on the surface of macrophages that bind to antibodies attached to the virus. Although it is a different organ, transient depletion of Kupffer cells, resident macrophages in the liver, enhanced AdV-mediated gene transfer *in vivo* [267]. In addition, it has been shown that complement cleavage fragment C3□ and C3□ opsonize non-viral gene vectors composed of cationic polymers or lipids [268], which may facilitate macrophage uptake via complement receptors.

Vector-mediated inflammation through recruitment of neutrophils and macrophages [269] may further reduce gene transfer efficiency upon repeated administration. Alveolar macrophages may pose a more challenging barrier in the lungs of patients afflicted by diseases characterized by chronic infection and inflammation, such as CF [270] and COPD [271], where alveolar macrophages are activated. It has been shown that alveolar macrophages significantly reduce retrovirus-mediated gene transfer to human bronchial epithelial cells; the inhibitory effect is elevated by lipopolysaccharide-induced macrophage activation [265].

2.3.3. Cellular barriers

Gene vectors that overcame the aforementioned extracellular barriers must then be taken up by target cells to introduce nucleic acid payloads to the intracellular gene expression machinery. Airway epithelial cells are a primary target for treatment of CF, and are of general importance to most muco-obstructive lung diseases. The epithelial surface of the airways poses an additional barrier to inhaled gene therapy that is due to low efficiency of endocytosis across the apical membrane [188] and tight junctions between cells that prevent access of gene vectors to the basolateral side [272] (Fig. 3D). This is a particularly formidable challenge for AdV since its receptor, CAR, is selectively localized on the basolateral membrane of airway epithelium [86]. Preclinical studies of lentivirus-mediated gene therapy in the lungs have also shown limited transduction through the apical membrane [273].

It has been reported that the barrier property of the airway epithelium is altered by muco-obstructive lung diseases, primarily due to structural perturbations of tight junctions [274-279]. In asthma, the barrier function of the airway epithelium is impaired through defective tight junction formation [274]. Likewise, cigarette smoke exposure, the major risk factor for COPD, disrupts tight junctions and increases epithelial permeability [275]. In contrast, it has been hypothesized that tight junction proliferation in CF results in increased epithelial resistance [276]. The transepithelial electrical resistance (TEER) is higher and the paracellular permeability is lower in CF airway epithelial cell cultures compared to cultures expressing wild-type CFTR [277]. However, pro-inflammatory cytokines [278] and bacterial toxins [279] reduce the permeability of tight junctions, suggesting that the barrier property of the airway epithelium may vary with disease state.

Tight junction proteins in the alveolar epithelium, including claudins [280], also limit access of inhaled gene vectors to the basolateral surface.

Once taken up by target cells, gene vectors must overcome several intracellular barriers, including but not limited to acidic vesicles (i.e. endosomes and lysosomes), the molecularly crowded cytoplasm, and the nuclear envelope. These barriers are shared in numerous organs and tissues in addition to the lungs and, thus, are widely reviewed elsewhere [281, 282].

2.4. Strategies to overcome the barriers to inhaled gene therapy

2.4.1. Modification of gene vectors

Tuning gene vectors to overcome one or more important physiological barriers can enhance the efficacy of pulmonary gene therapy. Inhaled gene vectors deposited on conducting airways first encounter the luminal mucus gel layer that serves as a highly adhesive and steric barrier, as described in section 4.1.1. Thus, gene vectors must be small enough to traverse through the mesh spacing of airway mucus, while possessing particle surface resistant to muco-adhesion [133]. It has been reported that nanoparticles as large as 200 nm efficiently diffuse in human airway mucus freshly collected from individuals with [211] or without [206] muco-obstructive lung diseases, but only if particle surfaces are densely passivated with hydrophilic and neutrally charged PEG polymers. Of note, high PEG densities that yield brush conformations, as opposed to mushroom shapes, provides muco-inert particle surfaces [229, 283].

Based on these findings, polymer-based gene vectors capable of efficiently penetrating human airway mucus, namely mucus-penetrating DNA nanoparticles (DNA-

MPP), have been introduced [214, 215, 284]. Suk et al. demonstrated that DNA-MPP based on PEI and PLL, unlike the otherwise identical counterparts without dense surface PEG coatings, efficiently percolated through pathological human airway mucus *ex vivo* [214], followed by a similar observation with DNA-MPP formulated with biodegradable poly(β–amino ester) (PBAE) (Figs. 4A, B) [284]. Likewise, the dense surface coatings with PEG corona may improve the penetration of inhaled gene vectors through another mucin-based meshwork found in PCL, if the particle diameters are small enough to fit through the PCL pores [240, 285]. The PEG surface coating may also minimize the particle aggregation in physiological environments [229], thereby providing another means of improving penetration through the steric barriers, including the mucus gel layer and PCL. Importantly, rapid *ex vivo* diffusion of DNA-MPP in airway mucus was translated to widespread distribution (Figs. 4C, D) and/or prolonged retention [214] in the mouse lungs, leading to approximately 25-fold greater *in vivo* pulmonary transgene expression compared to leading non-viral gene vectors without dense surface PEG coatings, including PEI- and $CK_{30}PEG_{10k}$ –based systems [284].

Given that several viral vectors possess muco-adhesive surfaces [210, 211], their diffusion within the airway mucus is likely enhanced by a dense surface shielding with PEG, similar to the findings with non-viral gene vectors. The enhanced lung gene transfer efficacy of AdV by surface PEG conjugation has been previously reported [286, 287]; however, the benefit of PEG in these studies was experimentally determined to be either reduced immunogenicity or resistance to the neutralization by pre-existing antibody. Thus, the effect of PEG on the ability of viral vectors to overcome key physiological barriers to inhaled gene therapy is yet to be determined. It should be noted though that

interference of PEG with antibody binding will reduce Fc-mediated trapping of viral vectors in airway mucus. Other than the PEGylation approach, Schuster et al. have recently shown that AAV2 mutant possessing the capsid with reduced heparin binding exhibit significantly enhanced diffusion in human CF mucus compared to that of native AAV2 [211]. This is most likely attributed to the decrease in heparan sulfate-mediated adhesion of AAV2 to CF airway mucus rich in these proteoglycans [211].

Targeting cells of interest via specific ligands is certainly a viable approach to enhance gene transfer to the lung. Although the majority of studies involves cancer targeting due to numerous well-established pathways upregulated in cancers [288], several groups have reported feasibility of specifically targeting parenchymal cells in the lung, including airway and alveolar epithelial cells. Most of the relevant studies involve identifying targeting ligands for airway epithelial cells, reflecting the dominance of CF-related research. Following the confirmation of urokinase plasminogen activator receptor (uPAR) expression on the apical surface of differentiated HAE, Drapkin et al., coupled a 7-mer peptide derived from a respective ligand to the surface of AdV via PEG [289]. They found that the targeting AdV provide 10-fold greater gene transfer to airway epithelium *in vitro* compared to native and PEGylated AdV. Employing the phage display technology, Jost et al, identified a 7-mer peptide, THALWHT, that targets human epithelial cell lines [290]. Subsequently, an independent group genetically engineered AAV2 decorated with THALWHT and found that this mutant provided a significantly greater *in vitro* transgene expression both in undifferentiated and polarized (i.e. differentiated) HAE [291]. However, they were unable to identify any ligand harboring the sequence that targets polarized HAE and the mutant did not provide significantly

increased transgene expression *in vivo*, implying that the sequence may not be airway-specific or may be species-dependent. Tagalakis et al. evaluated *in vivo* gene transfer efficacy of a lipid-based gene vector decorated with a peptide sequence previously identified to target intracellular adhesion molecule-1 (ICAM-1), a receptor for rhinovirus which causes the common cold [292]. They demonstrated that while their ICAM-1 targeting formulation provided 99% of airways with evidence of bronchial epithelial cell transfection, 73% and 38% of airways showed epithelial expression with 22 kDa PEI and GL67, respectively, following intratracheal administration [292]. Similarly, lactoferrin [293] and lactose [294] have been shown to enhance the transgene expression of PEI- and PLL-based gene vectors, respectively, in the HAE. However, both studies were conducted using immortalized cells, and thus the validity of the approach should be confirmed with ALI culture of primary HAE. There are relatively few studies describing the targeted gene transfer to alveolar epithelial cells. In one study, □2-adrenoceptor agonist, clenbuterol (Clen), which is used as bronchodilator for COPD and asthma treatment, was incorporated onto the surface of PEI-based gene vectors [295]. The Clen-decorated system provided 14- and 3-fold higher transfection efficiency compared to non-targeted counterpart in alveolar epithelial cells *in vitro* and in mouse lung *in vivo*, respectively.

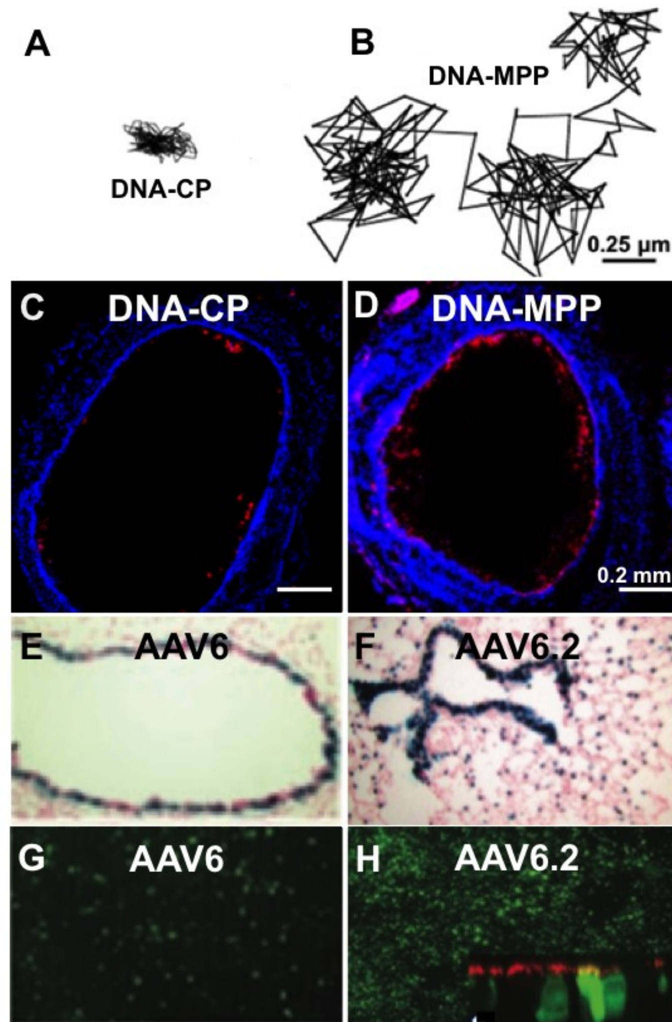

Figure 2.4. Modification of gene vectors to overcome physiological barriers. Representative trajectories of (A) conventional DNA nanoparticles (DNA-CP) and (B) mucus-penetrating DNA nanoparticles (DNA-MPP) based on biodegradable PBAE polymers in freshly expectorated CF mucus. Representative images of gene vector distribution in large airways following intratracheal administration of (C) DNA-CP and (D) DNA-MPP. Reprinted from [284] with permission from PNAS. Copyright © 2016 National Academy of Sciences, USA. (E, F) *In vivo* and (G, H) *in vitro* transduction of AAV6 and a mutant variant, AAV6.2, engineered with a single amino acid substitution in the heparin binding domain. Comparison of intratracheally administered (E) AAV6 and (F) AAV6.2 showing a stronger β-gal transgene expression in the mouse lung with AAV6.2. Comparison of (G) AAV6- and (H) AAV6.2–mediated GFP expression in ALI cultures of primary HAE. AAV6.2 treated cultures show stronger GFP expression as well as transduction of both ciliated and non-ciliated cells (H, inset). Reprinted from [109] by permission from Macmillan Publishers Ltd.

In addition to incorporating specific ligands to the surface by covalent conjugation [289] or genetic engineering [291], other approaches have been widely explored to endow viral vectors with ability to be internalized by cells of interest. Directed evolution approaches have been used to generate libraries of alternative viral capsid types and screened for tropism based on transduction efficiency *in vitro* or *in vivo* [296-298]. Rational design, specifically capsid pseudotyping, has also been used where viral capsids are replaced by those from other virus types or serotypes known to efficiently infect cells in the lung to provide or enhance lung tropism. Given the promising preclinical and clinical results, AAV has been the most extensively engineered viral vector to date using both directed evolution and rational design approaches [296-298]. The rational design approach has also been used to engineer novel AdV and lentiviral vectors for inhaled gene delivery applications [299-301].

Libraries of mutant AAV variants have been generated using a variety of methods, including error-prone polymerase chain reaction, DNA shuffling or random insertion/deletion of peptide-encoding sequences [302]. Specifically for inhaled gene therapy applications, AAV mutant vector libraries can be screened *in vitro* using ALI cultures of HAE and/or *in vivo* with suitable animal models to determine which variants successfully mediate gene transfer. After multiple rounds of selection in ALI cultures of HAE, a mutant vector with shuffled cap genes from both AAV2 and AAV5 was identified with enhanced airway tropism and mediated 100 and 10-fold greater *in vitro* transgene expression as compared to native AAV2 and AAV5, respectively [303]. Using a similar approach, a mutant AAV vector containing shuffled cap genes from AAV1, AAV6, and AAV9 was identified by screening in polarized HAE and showed 3-fold

greater *in vitro* production of CFTR mRNA transcripts than native AAV6 and ~25% restoration of CFTR as compared to healthy controls [304]. By mutating single amino acids in the heparin binding domain of AAV6 capsid, Limberis et al. discovered a novel AAV6 mutant gene vector, AAV6.2, outperforming other native and mutant AAV at mediating gene transfer in mouse airways (Figs. 4E, F) and polarized HAE cultures (Figs. 4G, H) [109].

Using the pseudotyping approach, hybrid viral vectors have been engineered that contain envelope or capsid proteins from other viruses with alternative tissue tropisms to change gene transfer properties [296-298, 301]. AdV and AAV hybrid vectors have been generated with capsid proteins from alternative viral serotypes that use receptors expressed on the apical surface of airway epithelium for entry. For example, a pseudotyped AdV vector was engineered to incorporate AdV35 fibers into the AdV5 capsid, redirecting tissue tropism towards airway epithelium with viral entry mediated by CD46 receptors expressed on the apical membrane [305]. A pseudotyped AAV vector containing an AAV2 genome packaged in AAV5 capsid proteins (i.e. AAV2/5) allowed for targeting of apically-expressed sialic acid receptors [116]. Using aerosolized AAV2/5, Fischer et al. demonstrated efficient gene transfer to the airways of monkeys to a level 20-fold greater than what achieved in historical AAV2 studies [116].

Incorporating components from the capsid of other respiratory viruses, such as bocavirus [112], baculovirus [9] and sendai virus (SeV) [9, 130, 306], is also a common strategy to introduce lung tropism. In addition to improving the packaging capacity, AAV2 pseudotyped with human bocavirus-1 (HBoV1) capsid demonstrated 5.6- and 70-fold greater efficiency at transducing polarized HAE culture *in vitro* compared to native

AAV1 and AAV2, respectively [112]. They were able to transduce via the apical surface of primary HAE isolated from a F508del/F508del homozygous CF patient, resulting in ~30% restoration of CFTR-mediated chloride currents [112]. Initial inhaled gene transfer studies with lentivirus focused on vectors pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G) [125-129]. However, this approach was generally developed to broaden the tropism of lentivirus rather than to specifically design a system for inhaled gene therapy applications [124]. It was later found for VSV-G-pseudotyped lentivirus that pre-treatment with compounds disrupting tight junction is required for gene transfer to the airway epithelium, suggesting that the transduction was achieved through the basolateral surface [125, 129]. To bypass the need for adjuvant treatments, hybrid lentiviral vectors were engineered by incorporating envelope proteins from SeV or baculovirus that have demonstrated tropism for the apical surface of airway epithelium [9]. Alton et al. were able to apically transduce *in vitro* in polarized primary CF HAE cultures and *in vivo* in CF mouse nasal epithelium using SeV-pseudotyped SIV [130]. The transgene expression in mouse nasal epithelium lasted up to 1 year after a single administration with no sign of immune response (Figs. 1C, D). This approach has significantly advanced the use of lentiviral vectors for inhaled gene therapy applications.

2.4.2. Modulation of biological barriers

Adjuvant agents that reduce airway and/or cellular barrier properties provide a relatively simple means to enhance the efficacy of inhaled gene therapy, potentially without the need of modifying gene vectors. However, it is crucial to ensure that any adjuvant approaches do not cause significant toxicity or disrupt normal lung function. This is of even greater concern for patients afflicted with muco-obstructive lung diseases

having impaired lung function. To our knowledge, specific strategies to reduce the barrier properties of the airspace are yet to be introduced, and as described in section 5.1, gene vector modification serves as the primary method to address this issue.

The most widely explored approach in this category is the use of mucolytic agents that degrade primary macromolecular components of airway mucus. Two primary compounds of selection may be recombinant human DNase (rhDNase, dornase alfa, Pulmozyme®) and N-acetyl cysteine (NAC, Mucomyst®), which are currently or previously utilized in the clinic to help CF patients clearing accumulated mucus in their airways. NAC's mode of action is to cleave disulfide intermolecular crosslinks between mucin fibers, which can significantly reduce the viscoelasticity of airway mucus [307]. As previously discussed, airway secretions in patients with muco-obstructive diseases can also carry high levels of DNA, further enhancing its barrier properties. Enzymatic degradation of DNA by rhDNase reduces entanglements within the mucus gel to further decrease the viscoelasticity [307, 308].

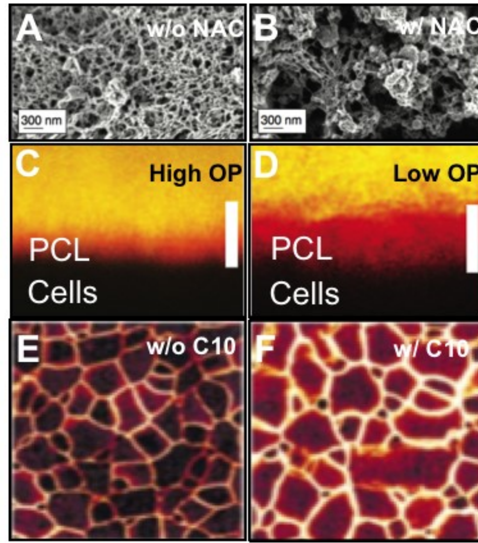

Figure 2.5. Modulating physiological barriers to inhaled gene therapy. (A, B) N-acetyl cysteine (NAC) treatment increases mucus mesh spacing, thereby facilitating gene vector penetration through airway mucus. Reprinted from [309] with permission from Nanomedicine as agreed by Future Medicine Ltd. (C, D) Reducing the osmotic pressure (OP) of mucus that can be achieved inhaled hypertonic saline rehydrates and restores the collapsed PCL. Reprinted from [238] with permission from AAAS. (E, F) Sodium caprate (C10) disrupts tight junctions in epithelial layer allowing gene vectors to access the basolateral compartment where specific receptors required for viral gene transduction are present. Reprinted from [272] with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society. The *American Journal of Respiratory Cell and Molecular Biology* is an official journal of the American Thoracic Society.

In the context of inhaled gene therapy, NAC and rhDNase can enlarge the mesh spacings of airway mucus, reducing the physical obstruction of inhaled gene vectors. For example, NAC was shown to increase the average pore size of CF mucus from 145 ± 50 nm to 230 ± 50 nm (Figs. 5A, B) [309]. It has been reported that pre-treatment with NAC leads to more rapid diffusion of leading gene vectors, including AAV1 [211] and $CK_{30}PEG_{10k}$ –based system [213], through CF mucus. Accordingly, NAC significantly improved $CK_{30}PEG_{10k}$ –mediated gene transfer in the lungs of a lipopolysaccharide-induced mouse model of mucus hypersecretion [213]. Likewise, a NAC derivative was shown to enhance gene transfer efficacy of AdV [310] and non-viral gene vectors [216], including EDMPC-Chol and PEI-based systems, in the mouse lung *in vivo* and in an *ex*

vivo sheep trachea model, respectively. Suk et al. previously demonstrated that diffusion of nanoparticles as large as 200 nm in CF mucus was significantly improved by NAC, but the effect was greater when nanoparticles possessed muco-inert surface coatings [309]. This finding suggests that simultaneously modulating gene vectors and the mucus barrier may synergistically improve mucus penetration and thus the efficacy of inhaled gene therapy. In addition to clinically used agents, Yuan et al. showed methyl 6-thio-6-deoxy-α-D-galactopyranoside reduced levels of reactive oxygen species in CF mucus and provided a greater reduction of disulfide crosslinks in CF mucus compared to NAC [204]. A novel alginate oligosaccharide compound, currently in a Phase IIb clinical trial for CF, was also shown to widen the pores within CF mucus by disrupting mucin-DNA interactions [311]. While safety must be confirmed, these newly developed mucolytic agents may also be useful as adjuvants for improving the penetration of gene vectors through the airway mucus barrier.

Osmotic agents are another type of agent that may effectively reduce the barrier property of the airway mucus blanket. In particular, hypertonic saline is a clinically used osmotic agent to rehydrate the airways of CF patients and improve MCC [312]. Although it is yet to be tested, hypertonic saline may be used as an adjuvant to inhaled gene therapy, as it can potentially reduce the barrier property of airway mucus by diluting this gel layer. Improved MCC is likely achieved by the restoration of the collapsed PCL in the CF airways (Figs. 5C, D) [238], and thus inhaled hypertonic saline treatment may also render the PCL more permeable to inhaled gene vectors. Graeber et al. demonstrated that 3% and 7% hypertonic saline administered via aerosol greatly reduced mucus accumulation in the airways of a ENaC-overexpressing transgenic mouse model of muco-

obstructive lung diseases [313], presumably by increasing airway hydration and improving MCC. Mannitol (Bronchitol™) is another hypertonic osmotic agent that hydrates airways [314] and thus may also be useful as an adjuvant to inhaled gene therapy. The kinetics of hydration by inhaled hypertonic agents must be carefully evaluated in relevant preclinical or clinical settings in order to determine an adequate time interval between the pre-treatment and gene vector administration. Administration of gene vectors in hypotonic solution as a vehicle may also improve gene vector penetration through the mucus barrier via convective flow generated by the osmotic gradient established between the airway lumen and epithelial cells. Ensign et al. demonstrated that by using hypotonic solution as delivery vehicle, muco-inert (i.e. densely PEGylated) nanoparticles were able to rapidly penetrate the luminal mucus layer and reached the immediate surfaces of vaginal [315] and colorectal [316] epithelium *in vivo*. However, the effect and safety of this approach is yet to be established for delivery of inhaled therapeutics, including gene vectors.

Use of hypotonic vehicle solution has shown potential of reducing the cellular barrier to inhaled gene therapy as well. Huang et al. demonstrated that hypotonic shock enhanced uptake of plasmid DNA by nasal epithelium *in vivo* via the regulatory volume decrease (RVD) mechanism [317]. Specifically, they showed that sodium- and sucrose-based hypotonic vehicles provided enhanced transgene expression in the mouse nasal epithelium compared to isotonic vehicles with the greater effect observed with lower osmolality. Similarly, other studies have shown that fluorescently labeled compounds administered to airway epithelium in hypotonic vehicles were efficiently internalized by cells, whereas the same molecules in isotonic vehicle were not [318-320]. In addition to

the observations in conducting airways, Sawa et al. showed an enhanced transgene expression in the lung airspace of rats when plasmid DNA was intratracheally instilled in hypotonic, rather than isotonic, vehicle solution [321]. RVD occurs after cell swelling driven by hypotonic shock [322], stimulating the fusion of numerous intracellular vesicles with the plasma membrane to prevent cell lysis [323, 324]. The response involves release of intracellular ions and subsequent water loss by osmosis [325], leading to the internalization of excess apical and basolateral membrane to reform the lost intracellular vesicles. During this endocytic process, particulates, including inhaled gene vectors, in the vicinity of the apical membrane can be taken up by epithelial cells. The RVD effect has been shown to last up to 30 minutes [317, 319]. Potential safety concern resulting from hypotonic shock would need to be addressed before clinical implementation of this approach.

As discussed earlier, several viral vectors possess natural tropism towards the basolateral surface of airway epithelium [85]. Thus, transient disruption of epithelial tight junctions may enhance transgene expression mediated by these vectors. Further, both viral and non-viral gene vectors may benefit from this approach due to the low rates of endocytosis across the apical membrane [188]. It has been shown that pre-treatment with fatty acid surfactants, including polidocanol (PDOC), sodium caprate (C10) and lysophosphatidylcholine (LPC), can increase paracellular permeability by transiently opening the epithelial tight junctions (Figs. 5E, F). Transduction by AdV was greatly enhanced *in vivo* in mouse nasal and airway epithelium pre-treated with PDOC [326] and C10 [327]. Likewise, pre-treatment with LPC has been shown to enhance VSV-G-pseudotyped lentivirus transduction *in vitro* in polarized HAE culture [273] and *in vivo* in

marmoset [125] and ferret [129] airways. Calcium-chelating agents, such as ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), can also be used as adjuvants to reduce the intracellular concentration of calcium ions, thereby disrupting calcium-dependent formation of tight junction protein complexes [328]. Pre-treatment with EGTA has been shown to enhance efficacy of AdV and retrovirus gene transfer *in vitro* in polarized HAE cultures and *in vivo* in rabbit tracheal epithelium [329]. However, there have been concerns over the safety of this approach and potential adverse effects. To investigate this, Johnson et al. evaluated the safety of EGTA, C10 and sodium laurate (C12), *in vitro* in primary HAE cultures and *in vivo* in the lungs of mice where they found evidence of causing a minimal to mild inflammatory response, based on histopathological analysis [330]. However, this study revealed that EGTA increased cell counts and the levels of pro-inflammatory cytokines in BALF, while C10 and C12 altered airway responsiveness after methacholine stimulation *in vivo* in the lungs of mice [330]. LPC has been used most extensively as an adjuvant to VSV-G-pseudotyped lentivirus-mediated inhaled gene therapy to enhance gene transfer with minimal adverse effects reported [125, 129].

Table 2.2. Physiological barriers to inhaled gene therapy of muco-obstructive lung diseases and strategies to overcome them

Regions of Lung	**Primary Barriers**	**Gene Vector Modification Strategies**	**Barrier Modulation Strategies**
Conducting Airway	Adhesive and/or steric trapping of inhaled gene vectors by mucus [202] and PCL [238]	Engineer gene vectors with surface coatings to reduce adhesive interactions with mucus (e.g. PEG [214, 215, 284], mutant AAV2 [211]) Sizes below characteristic mucus (≤150 nm) [202] and PCL (≤40 nm) [238] mesh pore size	Mucus-altering agents (e.g. rhDNase [307, 308], NAC [309]) to increase mucus mesh pore size Osmotic agents (e.g. hypertonic saline [312], mannitol [314]) to increase mucus and/or PCL mesh pore size by hydration

Airspace	De-stabilization of inhaled gene vectors by pulmonary surfactants [248-251] Uptake of inhaled gene vectors by alveolar macrophages [257-267]	Engineer gene vectors with surface coatings (e.g. PEG [214, 215, 284]) to enhance particle stability and/or to reduce macrophage uptake	None reported
Cellular	Low endocytic rate on apical surface [188] Restricted access to basolateral surface due to the presence of tight junctions [86, 273, 280]	Introduce targeting ligands to enhance apical transfection [288-295] Engineer viral gene vectors to endow apical epithelial tropism (e.g. pseudotyping [296-298, 301], directed evolution [302])	Tight-junction disrupting agents (e.g. C10 [327, 330], C12 [330], EGTA [328, 330], LPC [125, 129, 273], PDOC [326]) to provide an access to the basolateral surface Hypotonic vehicles to enhance uptake by the RVD mechanism [317-324]

2.5. **Preclinical models**

As discussed in previous sections, we have now learned through past successes and failures of preclinical and clinical studies the potential hurdles towards achieving therapeutically relevant inhaled gene therapy of obstructive lung diseases. Relevant preclinical models are needed in order to reliably evaluate newly developed strategies to better predict their performances in clinical trials. First, those models should replicate the physiological barriers found in the lungs of respective diseases to confirm whether the approaches of interest provide the benefits as designed. In addition, general pathology of respective human diseases should be reproduced in the animal models for assessing the pharmacodynamics (i.e. therapeutic gene transfer efficacy). In this section, we discuss the most advanced animal and tissue culture models available for evaluating gene delivery strategies.

2.5.1 Cystic fibrosis

Development of genetic animal models has greatly enhanced our understanding of CF lung pathophysiology. The CFTR-null (i.e. knockout) mouse was the first type of CF animal model established that results in a complete loss of functional CFTR [331-333]. This was followed by development of transgenic mouse models that produce low levels of CFTR [334, 335] and specific-mutant CFTR including but not limited to F508del [336], G551D [337] and G480C [338]. However, spontaneous generation of CF lung phenotype with mucus accumulation as well as chronic infection and inflammation is rare in knockout and transgenic mouse models with only one reported case [339]. This may in part be due to the lack of submucosal glands in the lower airways beyond the trachea of mouse lungs [340, 341]. Of note, submucosal glands are the primary source of the airway mucus secretion in the human lungs [200] and plays critical roles in innate immunity by secreting antimicrobials [342]. A recent study also found an alternative chloride channel is present in the mouse lung that can compensate for the lack of CFTR activity [93].

It has been found that defective CFTR leads to hyperabsorption of sodium through dysregulated ENaC channels present on the apical surface of CF airway epithelium [343-345], and has been implicated as an initiating pathological event in the CF lung. Based on these findings, Livraghi-Butrico et al. have shown that transgenic mice overexpressing β-subunit of ENaC spontaneously established CF-like lung disease, which resembles the pathological features of human CF lungs, including airway inflammation and dehydration as well as mucus obstruction [346-348]. Chronic lung infection, a hallmark of CF, has been established in normal mice and transgenic mouse model with specific CFTR mutation, including R117H, S489X, Y122X and F508del,

using *Pseudomonas aeruginosa*-laden agarose beads to prolong the bacterial residence time within the lung [349, 350]. Interestingly, inflammatory response to *P. aeruginosa* infection did not appear to depend on mutation type of the CF transgenic mouse models included in the study [349]. Mice intranasally challenged with endotoxin derived from *P. aeruginosa* have been shown to establish lung inflammation, mucus cell metaplasia and mucus hypersecretion [213], providing another relevant mouse model that mimics common features of the CF lung.

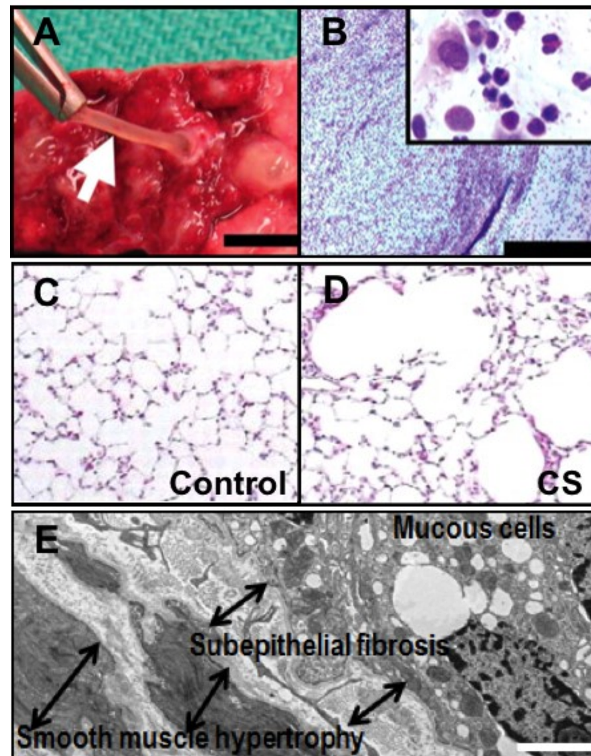

Figure 2.6. Animal models of obstructive lung diseases. Airway obstruction develops in CF pig due to highly viscoelastic mucus (A; white arrow) in the airway where mucus cytology further revealed presence of neutrophils, macrophages, and bacteria (B). Reproduced from [351] with permission from AAAS. Airspace in (C) healthy guinea pigs and (D) guinea pigs exposed to cigarette smoke (CS) for 6 months that show airway enlargement characteristic of emphysema in COPD. Reproduced from [374] with permission from APS. (E) Mucous cell hyperplasia, subepithelial fibrosis, and smooth muscle hypertrophy in ovalbumin (OVA)-induced allergic asthma mouse model. Reproduced from [77] with permission from Elsevier.

Larger genetic animal models have been developed using pig [351, 352], ferret [353-355] and rat [356] as background species, offering physiological features better reflecting the lungs of human CF patients [357-359]. It has been shown that pigs with mutated CFTR and/or CFTR knockout develop lung defects resembling those found in human CF lungs, including inflammation, impaired bacterial clearance, airway remodeling, and mucus hypersecretion (Figs. 6A, B) [351, 352]. A CFTR-null ferret model also shares features similar to humans with CF, showing altered airway chloride transport, mucus hypersecretion and propensity for lung infection [353-355]. These larger animal models are ideal for testing inhaled gene therapy, as they better recapitulate the physiological barriers and pathological events established in the human CF lungs. However, larger animal models are relatively costly, and accompany greater ethical challenges, and thus may be best suited for the later stages of preclinical development. A recent development of CFTR-knockout rats provides a model with more extensive development of submucosal glands in the trachea which may lend itself towards development of CF-like lung phenotype [356], thereby potentially serving as an alternative to aforementioned models.

2.5.2 α-1 antitrypsin deficiency

The most widely employed animal models for AATD to date were established in the 1980's [360-366]. These models are transgenic mice constitutively expressing a mutated version of an essential anti-protease, human AAT (hAAT), with a glutamate-to-lysine mutation at position 342 of the hAAT [367]. The mouse models harboring mutant hAAT exhibit low levels of hAAT in serum, caused by accumulation of the mutant protein in the

endoplasmic reticulum of hepatocytes, similar to what is found in AATD patients [363]. However, none of these studies clearly demonstrated the development of emphysema, a key pathological characteristic in the lungs of human AATD patients. Thus, an advanced preclinical model that more closely mimics AATD lung phenotype is needed to reliably evaluate therapeutic outcomes of inhaled gene therapy. For example, emphysema can be established in mouse and rat lungs by disrupting the protease/anti-protease balance via an instillation of different types of proteases into the lungs, including, but not limited to, papain, human neutrophil elastase, porcine pancreatic elastase and galactosamine [368-371]. It should also be noted that imbalance in protease activity is a common characteristic of a broader disease, COPD [372]. However, the lack of significant inflammation and airway changes [373] limit their use for studies that require general pathology of COPD, including chronic bronchitis. Other clinically relevant COPD animal models are discussed in section 6.3.

2.5.3. Chronic Obstructive Pulmonary Disease

There are a number of mouse-based COPD models, including cigarette-smoke (CS), protease-induced models, and genetic models. The most widely utilized models are generated by exposing rodents including mice and guinea pigs to CS [372, 374]. CS is exposed daily either only to the nose or whole body [375] for an extended period of time, usually weeks to months, to establish COPD-like pathology, including elevated oxidative stress [376], chronic inflammation [377], small airway remodeling and/or emphysema (i.e. enlargement of the airspace compartment; Figs. 6C, D) [378]. The nose-only administration allows for more direct control of amount of CS exposure compared to

whole body exposure, but mandates prolonged restraint of the animals, which may cause stress. Using either administration modality, the effects of CS exposure can be closely monitored based on levels of CS exposure markers such as carbon monoxide-hemoglobin complexes (i.e. carboxyhemoglobin) and nicotine metabolites (e.g. cotinine) present in serum [375].

Using nose-only CS exposure, Beckett et al. recently demonstrated that an 8-week exposure of CS to mice twice per day and 5 times a week resulted in COPD-like pathology, including airway inflammation, emphysema and impaired lung function (determined by forced oscillation and forced maneuver techniques), which was not resolved during the following 4 weeks of CS cessation [379]. The lungs of this model also became susceptible to acute bacterial infection, as evidenced by decreased clearance of *Streptococcus pneumoniae* and influenza virus by 2-fold in comparison with wild-type mice [379], presumably replicating pulmonary exacerbation often observed with COPD patients [380]. However, mucus accumulation and airway obstruction are not established in this model, despite the presence of goblet cell metaplasia and airway remodeling [379]. As a result, this model may be limited in evaluating therapeutic intervention of the chronic bronchitis phenotype.

CS models have been also generated using other larger species such as guinea pigs [381, 382]. Guinea pig-based CS models have been favored by several groups due to its unique advantages, including relatively close resemblance to human lung physiology and anatomy [383-385] as well as lung alveolarization at birth which may be beneficial for pre- and neonatal studies [386]. In addition, hallmark features of COPD including inflammation [387, 388], goblet cell metaplasia [389, 390], small airway

remodeling [391, 392], airway obstruction [393] and emphysema [394], have been successfully demonstrated in guinea pigs. COPD has been extensively studied over the years with the use of various CS-induced animal models reported by numerous groups, but development of a standard CS exposure protocol seems necessary as it is currently difficult to directly compare studies by different groups.

The COPD animal models based on genetic variations include natural mutant, transgenic and knockout models. Some of these models may offer the advantage of consistency, however they do not always reflect the COPD pathogenesis specific to the lungs and may adversely affect other organs [373]. Interestingly, some naturally occurring mutant strains of C57BL/6 mice, such as tight skin and pallid mouse models, are known to have abnormally large airspace, lower serum level of AAT and impaired alveolar septa [369, 395]. Tight skin mouse model is characterized by a mutation in the fibrillin-1 gene, which affects formation of elastic fibers, leading to abnormal airspace development and progressive alveolar enlargement with age [396, 397]. Pallid mouse model is known to have lower level of AAT in serum and gradually develops mild emphysema late in life [369]. The pallid mouse model may also be utilized for AATD studies [398]. Transgenic and knockout models may be more useful for studying a particular pathway of the COPD pathogenesis, as exact roles of specific genes can be elucidated in these models.

2.5.4 Asthma

The ovalbumin (OVA)-challenged mouse model is by far the most widely utilized preclinical model for allergic asthma, exhibiting the characteristic Th2-type immune

response [399]. The models are generally established by intraperitoneal sensitization with OVA, followed by repeated intratracheal OVA challenges, but exact dosing schedule varies among individual studies [400]. The model reliably recapitulates asthma-like pathophysiology, including recruitment of eosinophils, elevated levels of OVA-specific IgE and Th2 cytokines, mucus accumulation by mucous and goblet cell hyperplasia, airway hyperresponsiveness and airway remodeling (Fig. 6E) [401, 402]. Although the OVA mouse model is the most widely used, there are questions regarding its clinical relevance as OVA is not a naturally occurring allergen. Hence, it may not properly mimic how asthmatic patients become sensitized to allergens [403]. Numerous groups using this model have reported that chronic asthmatic symptoms do not exacerbate over time; the symptoms are actually ameliorated by natural development of tolerance and/or termination of OVA challenge [404, 405]. More recently, the development of tolerance to repeated challenges has been addressed by optimizing amount and frequency of OVA administration [77, 400]. Although not as frequently used, rats and guinea pigs are also utilized as background species of OVA models. Rat models share similar immunological cascade as the mouse model when sensitized with OVA and may perhaps be used as an alternative [406]. Guinea pigs, while closely resembling the human lung anatomy and physiology, have been reported that IgG is the primary antibody produced post-sensitization and the baseline level of eosinophils is high, which may somewhat limit their use [406, 407].

Mouse models have been also developed using naturally occurring allergens such as house dust mite (HDM) [408]. Many groups favor HDM models over OVA models as it yields pathophysiology similar to that exhibited by the more widely-studied OVA-

sensitized model, while also having greater environmental/clinical relevance [409]. For example, Johnson et al. administered HDM intranasally for 5 days per week for up to 7 weeks and observed eosinophilic inflammation, elevated levels of Th2 cytokines, as well as airway hyperresponsiveness and remodeling [401, 409]. Additionally, in contrast to OVA-challenged animal models, re-exposure of HDM to mice resulted in sustained inflammatory response, including elevated eosinophil and Th2 effector cell counts, suggesting that tolerance to HDM did not develop [409]. However, after cessation of HDM exposure, airway inflammatory response fully recovered, while alterations in airway remodeling and hyperreactivity persisted [409]. HDM consists of a mixture of allergen subtypes including *Der p I* and *Der p II* and other biomolecules such as endotoxins, proteases, proteins and peptides, making it difficult to identify the component(s) responsible for specific allergic responses [401, 410, 411]. Moreover, many commercially available HDM extracts are different in composition and proteolytic activities [412], and thus direct comparison of various HDM studies can be difficult.

Use of transgenic mice overexpressing GATA3 transcription factor may be considered. GATA3 is known to play significant roles in Th2 cell differentiation and activation by controlling Th2-driven cytokine production [413]. Several groups demonstrated that mice with GATA3 overexpression, in comparison to its normal counterparts, showed upregulation of Th2 cytokines, airway remodeling, airway smooth muscle hyperplasia, eosinophilic inflammation and subepithelial fibrosis, upon sensitization and challenge with allergens [414-417]. Overexpression of GATA3 alone induces production of Th2 cytokines and leads to lung inflammation and mucus hypersecretion, but not to the extent exhibited after allergen challenge [413].

Sensitization of GATA3-overexpressing transgenic mice with even relatively low amount of OVA or HDM cause further increases in airway inflammation, while no significant inflammation was observed in wild-type C57BL/6 mice [413]. In another study, Ano et al. sensitized and subsequently challenged GATA3-overexpressing transgenic mice with OVA and were able to observe enhanced airway inflammation and goblet cell hyperplasia [414]. A Phase IIa trial of an inhaled DNAzyme targeting GATA3 mRNA have been recently initiated for Th2-driven asthma [418], underscoring the relevance of these transgenic mouse models.

Larger animal models based on non-human primates, ponies, and Basenji greyhounds have been developed since they develop natural allergies as well as persistent respiratory allergic responses; however, they have not been widely explored primarily due to cost issues [419].

2.6. **Human Tissue Culture Models**

Therapeutic efficacy and safety of novel delivery strategies must be ultimately demonstrated in preclinical animal models prior to their clinical evaluation. However, recent studies have shown species-specific differences in tropism of AAV by comparing transduction *in vitro* in ALI cultures of human lung epithelium to those of mouse [420], monkey [359], ferret [359] and pig [421] lung epithelium. These findings underscore the importance of confirming the efficacy in relevant human cell-based models as compared to preclinical models. Studies in human tissue culture models are also beneficial for high-throughput screening of gene vector candidates and further optimization of vector design. Human lung epithelial cells grown in ALI cultures have the ability to differentiate into

polarized epithelial layers, developing key physiological barriers including tight junctions between neighboring cells and mucus secreted from the apical surface within ~4 weeks [422, 423]. Ussing chamber experiments can be conducted to confirm the presence of intact tight junctions determined by the measured TEER [422, 423] and also to assess CFTR correction via quantifying the chloride conductance [26, 115, 277].

ALI cultures have been generated from lung epithelial-derived cell lines, including Calu-3, CFBE, NuFi-1, and CuFi-1 (for complete list of available cell lines, see [424]). However, concerns arise around the use of ALI cultures established with a single cell type, as there are several different cells present in the lung (i.e. ciliated epithelial cells, goblet cells, etc.) [424-426]. Mucus production and PCL development may also vary greatly depending on the cell lines used [424-426]. Thus, the properties of relevant delivery barriers encountered by gene vectors may not fully recapitulate their characteristics in the human lung. Primary ALI cultures are an attractive alternative as they are established with airway cells directly collected from human lung explants and thus retain many features of the physiological human lung epithelium [238, 241, 427-430]. Another benefit is that epithelial cells can be harvested directly from specific patients of interest for screening gene delivery strategies, potentially enabling personalized medicine. Shortcomings of primary ALI cultures include limited availability of fresh explants, short cell lifespan, and variability among patient donors. It should be also noted that submucosal glands, an important target tissue in obstructive lung diseases, are not present in ALI cultures regardless of the source of constituting cells.

Recent developments in nasal brushing-derived primary lung epithelial ALI cultures could provide a more readily accessible tissue source [431]. However, there are

concerns over its use as a surrogate tissue source as studies have shown marked differences in genomic profiles of primary human nasal and bronchial epithelial cells [432, 433]. Primary human organoids differentiated from patient-derived stem cells are also an attractive *in vitro* model for high-throughput screening [434]. Specifically for CF, the organoid's degree of swelling in response to the treatment with a well-documented CFTR activator, forskolin, was found to correlate with CFTR function, providing a means to assess CFTR activity and/or correction [435]. The organoid systems remain viable after long periods of storage in liquid nitrogen and could be passaged up to 40 times, beneficial for long-term studies. More recently, this approach was expanded to human lung organoid systems consisting of proximal lung epithelium surrounded by mesenchymal smooth muscle tissue and have been shown to generate airway-like features with both club and ciliated cell development [436]. However, the barrier properties of organoid systems have not been thoroughly characterized and may differ from those observed in the human lung.

2.7. **Conclusion**

Over two decades of preclinical and clinical evaluations of inhaled gene therapy have provided valuable lessons building towards the ultimate goal of developing curative treatments for patients with obstructive lung diseases. We have now established better understanding of disease pathology, genetic targets and physiological barriers. The accumulated knowledge has driven the development of advanced gene delivery systems, nucleic acid engineering tools and human disease-like preclinical models by scientists, engineers and clinicians in various settings. A recently completed clinical trial of inhaled

CF gene therapy has left some questions to be addressed, but has certainly rejuvenated the field with more clinical evaluations anticipated in the near future. Through this review, we hope to provide a comprehensive overview of the knowledge gained and encourage collaborative efforts for realizing therapeutically effective gene therapy of obstructive lung diseases. We began our review by overviewing the types of obstructive lung diseases and potential genetic targets for respective diseases. We then highlighted gene delivery systems, primarily those tested in clinical trials of inhaled gene therapy, followed by challenging physiological barriers that have hampered translation into the clinic. We next introduced strategies to overcome those hurdles and preclinical disease models resembling the pathological lung environments of diseased human patients. Future preclinical studies of the next-generation of gene vectors in animal models that present all these critical features will help better predict therapeutic outcomes and thus facilitate clinical development of inhaled gene therapy.

3. An adeno-associated viral vector capable of penetrating the mucus barrier to inhaled gene therapy

3.1. Introduction

Past clinical trials testing viral gene vectors, including adenovirus (AdV) and adeno-associated virus (AAV) serotype 2, for inhaled gene therapy have failed to provide clinically significant benefits. These disappointing outcomes have been attributed to inefficient gene transfer to airway epithelium and generation of therapy-inactivating host immune responses [437]. Accordingly, recent advances in viral vector engineering have focused on enhancing the transduction of airway epithelium [107, 109, 112, 438-443] and reducing immunogenicity [444, 445]. While underappreciated in early clinical studies, airway mucus has been recently recognized as another critical biological barrier to inhaled therapeutics [133, 446-453]. The airway epithelium is protected by a secreted mucus gel layer that traps most inhaled foreign materials, including gene vectors, and facilitates their removal from the airways via mucus clearance mechanisms [446, 454, 455]. In previous studies, we found that many clinically tested and commonly used viral vectors, including AdV, AAV1, AAV2 and AAV5, bind adhesively to human airway mucus [456, 457], impeding access to the epithelium that is required for successful transduction.

Recent studies comparing AAV serotypes for inhaled application revealed that AAV6 provided superior gene transfer efficacy *in vitro* and *in vivo* to other serotypes [109, 458-460]. However, the underlying mechanisms are not fully understood with one report suggesting a potential role of its ability to escape the proteasome degradation

pathway [458]. In addition, many prior studies have been conducted using *in vitro* and *in vivo* models that lack the lung pathophysiology similar to that of patients with muco-obstructive lung diseases, such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and asthma. To this end, we sought to evaluate the performance of AAV6 in the lung using physiologically relevant experimental conditions, including human CF sputum, a mucus-covered air-liquid interface (ALI) culture of human CF airway epithelium, and an advanced transgenic mouse model closely mimicking the lung environment found in muco-obstructive lung diseases. Using multiple particle tracking (MPT), we first investigated whether AAV6 was capable of avoiding entrapment within the mucus meshwork of CF sputum, unlike previously tested AAV serotypes [456, 457]. We then determined the impact of the airway mucus barrier on the ability of AAV6 to mediate transgene expression in airway epithelium *in vitro* and *in vivo*, and compared AAV6 to AAV1, a serotype shown to outperform the clinically tested AAV2 [106, 461] but unable to efficiently penetrate human CF sputum [457].

3.2. Materials and Methods

3.2.1. Production of recombinant AAV gene vectors and fluorescent labeling

Recombinant AAV1, AAV6 and an AAV6 mutant, AAV6-K531E, vectors were packaged with either self-complementary enhanced green fluorescent protein (scAAV-EGFP) or single-stranded firefly luciferase-yellow fluorescent protein fusion (ssAAV-Fluc-YFP) gene as described previously [462, 463]. Both reporter transgenes are expressed under the control of the ubiquitous chicken β-actin promoter (CBA). To engineer AAV6-K531E, a two-step PCR with reading-proof Turbo Pfu Polymerase

(Stratagene) was used on plasmid pACGr2/c6 to introduce a single mutation;[464, 465] primers were designed to make a change in an amino acid coding triplet from lysine (K; codon: **A**AG) to glutamic acid (E; codon: **G**AG) at 531 position of AAV capsid. The titers of DNase I-resistant vector particles were determined by qPCR using the primer-pair specific for the CBA promoter, F-5’-TCCCATAGTAACGCCAATAGG-3’ and R-5’-CTTGGCATATGATACACTTGATG-3’, and SYBR GreenER PCR Master Mix (Invitrogen). Of note, two separate batches of AAV vectors were constructed and used for *in vitro* and *in vivo* transduction studies to ensure reproducibility. For visualization and tracking of individual viruses, AAV was labeled with the amine-reactive fluorescent dye, Alexa Fluor 647 carboxylic acid succinimidyl ester (AF647; Life Technologies), as previously described [457]. AF647-labeled AAV were stored at −80 °C and thawed on ice prior to use.

3.2.2. Formulation of synthetic mucus-penetrating gene delivery nanoparticles

For comparison with AAV diffusion in CF sputum, synthetic mucus-penetrating gene delivery nanoparticles were formulated as described previously.[452] Briefly, 5 kDa methoxy polyethylene glycol (PEG) N-hydroxysuccinimide (Sigma-Aldrich) was conjugated to 25 kDa branched polyethylenimine (PEI; Sigma-Aldrich) to yield PEG_{5k}–PEI copolymer. Nanoparticles were formed by the drop-wise addition of 9–10 volume of 0.2 mg/ml GFP-encoding plasmid DNA controlled by the cytomegalovirus promoter (Clontech) to 1 volume of a swirling polymer solution containing 25% unmodified PEI and 75% PEG_{5k}–PEI copolymer, at an optimized nitrogen to phosphate ratio of 6. Twenty percent of the plasmid DNA used to assemble nanoparticles was fluorescently labeled by

Cy3 dye using the Mirus Label IT Tracker Intracellular Nucleic Acid Localization Kit (Mirus Bio).

3.2.3. CF sputum sample collection and biochemical assay

CF sputum samples were collected under written informed consent, in accordance with the Johns Hopkins Institutional Review Board (IRB). Spontaneously expectorated sputum samples were collected from patients visiting the Adult Cystic Fibrosis Center at Johns Hopkins University. Particle tracking experiments were conducted using samples stored at 4°C immediately after collection for up to 24 hours, based on our prior confirmation that the barrier properties of freshly collected sputum are maintained in this condition [447, 466]. To reduce the effects of salivary contamination, any sample with a visible quantity of saliva was excluded. Patients involved in this study received no mucolytics other than Pulmozyme® (i.e. dornase alfa) and/or hypertonic saline as part of their standard treatment regimen.

The percent solids content and mucin concentration of sputum samples were determined using previously reported methods [466]. For solids content, sputum samples were frozen in liquid N_2 and placed in a lyophilizer (FreeZone 4.5 Plus; Labconco) for at least 12 hours to completely extract water within individual samples. The percent solids content is defined as the ratio of mucus mass before and after the lyophilization. Mucin concentration was determined based on the reaction between 2-cyanoacetamide (Sigma-Aldrich) with O-linked glycoproteins (i.e. mucin) followed by a fluorometric assay, as previously described [466].

3.2.4. *Multiple particle tracking in CF sputum*

The motions of AAV or synthetic mucus-penetrating gene delivery nanoparticles in sputum were tracked by fluorescent video microscopy and quantified by MPT analysis using a software custom-written in MATLAB (Mathworks), as previously reported [457, 467].

3.2.5. *In vitro transduction*

Human CF bronchial epithelial immortalized cell line[468] (CFBE41o$^-$; gift from Drs. Cebotaru and Guggino from Johns Hopkins University) and primary CF human bronchial epithelial cells expressing the F508del mutant cystic fibrosis transmembrane conductance regulator (CFTR) (CF HBE) were used for *in vitro* transduction studies. The University of Alabama at Birmingham IRB approved the use of primary cells. CFBE41o$^-$ cells were grown in Minimum Essential Media with 10% fetal bovine serum, L-glutamine, penicillin/streptomycinin and puromycin (5 µg/mL). Primary CF HBE cells were expanded using co-culture with irradiated fibroblasts (R&D systems) and conditional reprogramming via media containing a rho-associated protein kinase inhibitor.[469, 470] CFBE41o$^-$ or primary CF HBE cells were grown in 0.33 cm^2 polyester membrane transwell inserts (Corning) at an ALI for 4-6 weeks until terminally differentiated. Once the ALI was established, CFBE41o$^-$ or primary CF HBE cells were maintained in the above-described growth media or PneumaCult-ALI Maintenance Medium (Stemcell Technologies), respectively. A 5 µL suspension of 2 x 10^9 AAV vector genomes, either AAV1, AAV6 or AAV6-K531E, packaged with a gene encoding EGFP (i.e. scAAV-EGFP) were administered to the apical surface of the fully

differentiated ALI cultures at a multiplicity of infection (MOI) of 5000 to evaluate *in vitro* transduction efficiency. After incubation overnight with AAV, 100 µL of PBS was added to the apical chamber of inoculated cultures, incubated for 30 minutes at 37ºC, and washed via aspiration.

We adapted a previously reported protocol to induce mucus hypersecretion in CFBE41o^{-} ALI cultures.[471] Briefly, 5 µL of CSS was added to both the apical and basolateral compartments and incubated overnight. The CSS solution was prepared by high speed centrifugation (21,000 x*g*) for 1 hour of 2.5 mL sputum pooled from 5 CF patients, followed by filtration through a 0.2 µm ELF spin-filter (Molecular Probes). Apical mucin content of CFBE41o^{-} ALI cultures was fluorometrically determined using the identical method used for the analysis of CF sputum. To remove apically secreted mucus from primary CF HBE ALI cultures, the apical chamber was incubated with 100 µL of PBS at 37ºC for 30 minutes, followed by removal via aspiration. GFP transgene expression was imaged using a confocal LSM 510 microscope (Zeiss) at 10× and 20× magnification, and quantified using a custom-written software in MATLAB. To avoid artifacts presented by differences in focus between images, we used automated focusing (available in Zeiss Zen software) of DAPI nuclear staining and captured all images at this focal plane. Six or more randomly selected image regions were taken of each insert and used for quantification. The percentage of GFP coverage was defined as the GFP-positive area divided by the total cell-covered surface area and the total GFP intensity was quantified as the average of total pixel intensity units for each data set. Prior to quantitative analysis, all images were normalized by fluorescence of untreated control ALI cultures to eliminate the contribution of autofluorescence.

3.2.6. In vivo transduction

All animals were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee. Four-week old, congenic C57BL/6N transgenic mice with airway-specific overexpression of the epithelial sodium channel β-subunit (βENaC, gene *Scnn1b*) (i.e. *Scnn1b*-Tg mice)[472] were used for *in vivo* transduction studies. To evaluate the distribution and overall level of transgene expression in the lungs of *Scnn1b*-Tg mice, a 50 μL solution of 2 x 10^{10} AAV packaged with a gene encoding luciferase-YFP fusion protein (i.e. ssAAV-Fluc-YFP) were delivered intratracheally using a microsprayer (MicroSprayer Aerosolizer Model IA-1C; Penn-Century; n = 3 and 6 mice per group for assessing the distribution and overall level of transduction, respectively). Animals were sacrificed 2 weeks post-administration to determine the distribution of YFP transgene expression in airways/airspace as well as luciferase activity of whole lung homogenates, a measure of overall transgene expression level. For the distribution study, lungs were harvested, flash-frozen in OCT and cryosectioned using a CM1950 cryostat (Leica Biosystems). Slides were immunologically stained using an AF488-conjugated GFP monoclonal antibody (Santa Cruz Biotechnology) and DAPI, and subsequently imaged using a confocal LSM 510 microscope under 10× and 20× magnification. Four or more randomly selected image regions were taken from 3 lung tissue sections from regions of the airways and airspace (total images per animal = 4 x 3 = 12) for the quantification of gene expression distribution. To quantitatively assess the coverage of YFP transgene expression in airways/airspace, custom software written in MATLAB was used where images were

blinded and airway regions were manually determined based on DAPI staining. The boundaries of airway and non-airway regions (i.e. anywhere outside of selected airway regions) were further refined using automated image thresholding. Percentage of YFP coverage was defined as the YFP-positive airway or airspace area divided by the total area of a respective compartment. Prior to quantitative analysis, all images were normalized by fluorescence of untreated *Scnn1b*-Tg lung tissue to eliminate the contribution of autofluorescence. To directly visualize mucus plugs in the airways of *Scnn1b*-Tg mice, slides were immunologically stained with anti-MUC5B (Santa Cruz Biotechnology; 5B#19-2E:sc-21768) and anti-MUC5AC (ThermoFisher; MA5-12178) antibodies. For fluorescent tagging of MUC5AC and MUC5B, a biotinylated anti-mouse IgG (Sigma-Aldrich) was used as a secondary antibody, followed by a staining with ExtrAvidin-Cy3 (Sigma-Aldrich). Luciferase activity on lung tissue homogenates was measured using a standard luciferase assay kit (ThermoFisher Scientific) and a 20/20n luminometer (Turner Biosystems) [450, 452]. The relative light intensity was normalized by total protein concentration measured by a standard bicinchoninic acid assay (ThermoFisher Scientific).

3.2.7. Statistical analysis

Data were analyzed with statistical tests including student's t-test using SigmaPlot 10.0 and a non-parametric Mann Whitney test using MATLAB R2014b. Multiple comparisons were performed by one-way analysis of variance (ANOVA) using MATLAB R2014b. Data were considered statistically significant when $p < 0.05$. The statistical tests used in each data set are indicated in the figure legends.

3.3. **Results**

3.3.1. AAV diffusion in freshly collected CF sputum

To assess the abilities of different AAV vectors to penetrate human airway mucus, we measured using MPT the diffusion rates of AF647-labeled AAV in freshly collected sputum samples spontaneously expectorated by CF patients.

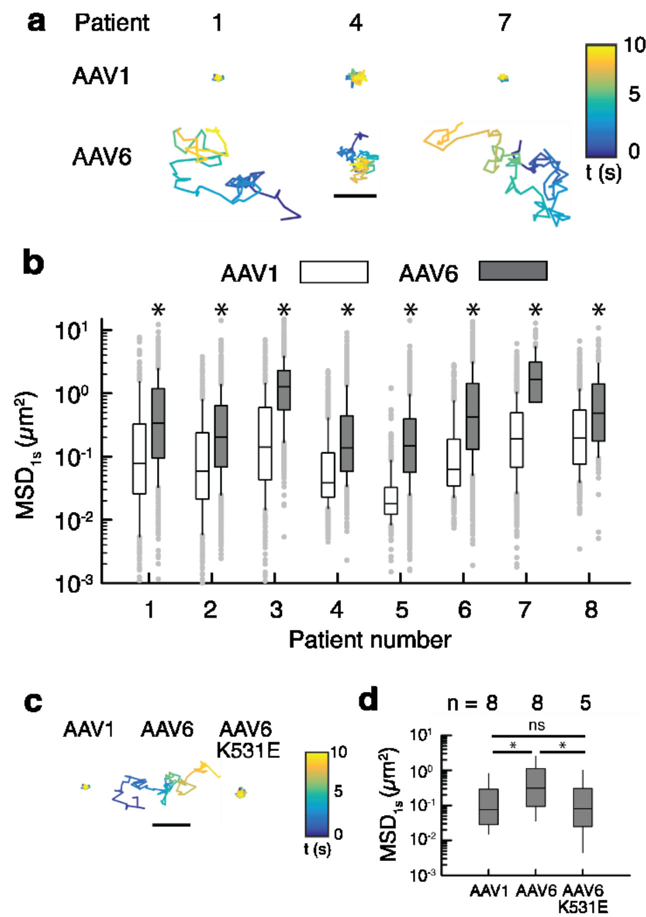

Figure 3.1 AAV diffusion in spontaneously expectorated CF sputum. (a) Representative trajectories of AAV1, AAV6 and AAV6-K531E in CF sputum. Trajectories show 10 seconds of AAV motions. Color bar indicates the time (t) of the trajectory in seconds. Scale bar = 1 µm. (b) Box-and-whisker plots of MSD_{1s} of AAV1 and AAV6 in sputum samples collected from 8 individual CF patients. Maximum whisker length is 1.5 times the interquartile range; outliers are shown as dots. (c) Box-and-whisker plots of MSD_{1s} for AAV1, AAV6 and AAV6-K531E in all patient samples tested. Outliers are not shown. *$p < 0.05$; Mann-Whitney test.

The percent solids content and mucin concentration of collected sputum samples were 7.4 ± 4.9% and 5.9 ± 1.6 mg/mL, respectively, in good agreement with previously reported values [466, 473]. We have previously confirmed that our method of AF647 labeling does not significantly alter the natural surface property and transduction efficiency of AAV [457]. Consistent with our prior observation [457], AAV1 displayed highly confined trajectories, indicating their entrapment within CF sputum. In contrast, AAV6 traveled far greater distances over time (Figure 3.1a). Quantitatively, AAV6 consistently exhibited significantly greater diffusion rates, measured by mean squared displacement at a time scale of 1 second (MSD_{1s}), compared to AAV1 in sputum samples collected from 8 different CF patients (Figure 3.1b). MSD_{1s} represents the average squared distances individual AAV travel within a 1 second time interval and is directly proportional to their diffusion rates [467].

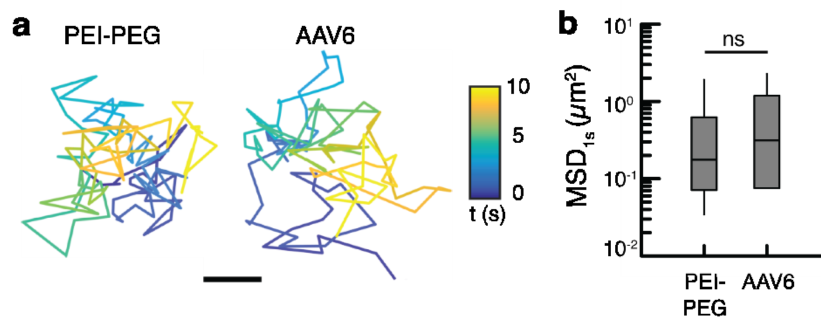

Figure 3.2. Synthetic and viral gene vector diffusion in spontaneously expectorated CF sputum. (a) Representative trajectories of synthetic mucus-penetrating gene delivery nanoparticles based on polyethylene glycol-conjugated polyethylimine (PEI-PEG)[19] and AAV6 in CF sputum. Trajectories show ~10 seconds of gene vector motions. Color bar indicates the time (t) of the trajectory in seconds. Scale bar = 1 µm. (b) Box-and-whisker plots of MSD_{1s} of PEI-PEG and AAV6 in sputum samples collected from 3 individual CF patients. Maximum whisker length is 1.5 times the interquartile range; outliers are not shown. A Mann-Whitney test was used to compare MSD_{1s} and there was no significant difference ($p>0.05$).

Based on the median of MSD_{1s}, AAV6 diffusion in CF sputum was enhanced by 3–10 fold in comparison to AAV1. Of note, we found that AAV6 diffusion in CF sputum was

comparable to that of a synthetic gene delivery nanoparticle previously confirmed to efficiently penetrate CF sputum [452] (Figure 3.2). In order to further explore the differences in diffusion behaviors of AAV1 and AAV6 in CF sputum, we measured the diffusion rate of an AAV6 mutant, AAV6-K531E, which has been previously shown to confer AAV6 with an AAV1-like binding affinity to glycans [465]. The diffusion rates of AAV6-K531E in 5 different CF sputum samples were significantly lower than those of wild-type AAV6, and comparable to those of wild-type AAV1 (Figure 3.1c,d). We found that diffusion rates of AAV1 and AAV6 did not significantly correlate with solids content (i.e. percent dry weight) of sputum samples ($R^2 = 0.05$), consistent with our prior observation with AAV2 [457].

3.3.2. Impact of apical mucus on in vitro AAV transduction

Based on the significantly enhanced diffusion rates of AAV6 compared to AAV1 in CF sputum (Figure 3.1), we next sought to determine the impact of secreted mucus on the abilities of different AAV vectors to penetrate to the underlying airway epithelial cells and mediate transgene expression *in vitro*. We first examined *in vitro* transduction efficiency of AAV1, AAV6 and AAV6-K531E in CFBE41o$^-$ cells, derived from a F508del homozygous CF patient, grown at an ALI where cells are differentiated into a mucus-secreting lung airway epithelium. Once fully differentiated, AAV-mediated GFP transgene expression was evaluated 2 weeks post-infection using confocal microscopy (Figure 3.3a-c). We found that AAV6-treated CFBE41o$^-$ cultures exhibited ~2-fold greater coverage and overall level of GFP transgene expression compared to AAV1-

treated cultures, whereas both the coverage and overall level were comparable between AAV6-K531E- and AAV1-treated cultures (Figure 3.3d,e).

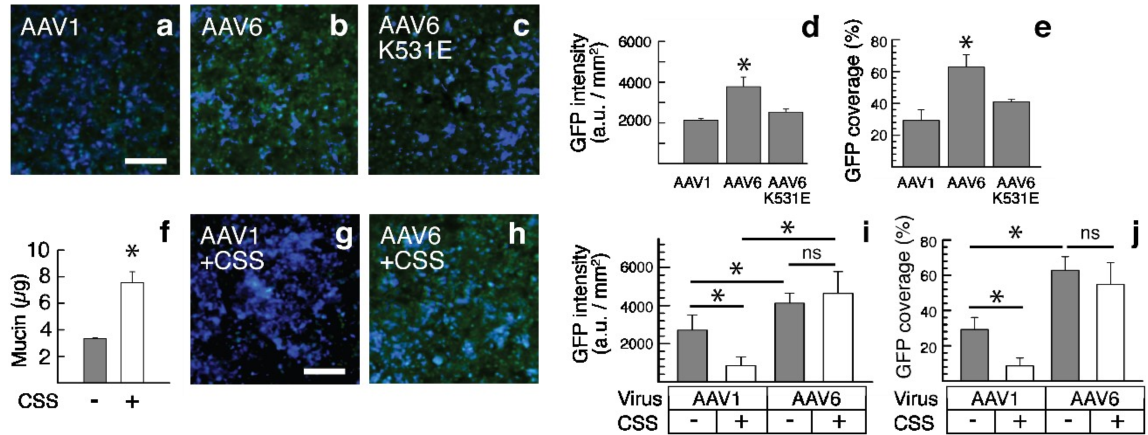

Figure 3.3. *In vitro* transgene expression in human CFBE41o⁻ ALI cultures. (a-c) Representative images of CFBE41o⁻ ALI cultures transduced with AAV1, AAV6 and AAV6-K531E. Scale bar = 50 μm. (d,e) Image-based quantification of AAV-mediated GFP transgene expression in terms of (d) overall level and (e) relative coverage in CFBE41o⁻ ALI cultures (n = 6). (f) Mucin contents of CFBE41o⁻ ALI cultures before and after CSS treatment. (g,h) Representative images of CFBE41o⁻ ALI cultures transduced with AAV1 and AAV6 where cultures were pretreated overnight with CSS. (i,j) Image-based quantification of AAV-mediated GFP transgene expression in terms of (i) overall level and (j) relative coverage in CSS-pretreated CFBE41o⁻ ALI cultures (n = 6). *$p < 0.05$; student's t-test or one-way ANOVA.

We then repeated the experiment using CFBE41o⁻ ALI cultures treated to induce mucus-hypersecretion. Mucus hypersecretion was established by incubating the cultures overnight with CSS solution rich in pro-inflammatory cytokines such as IL-1β, neutrophil elastase, and TNF-α [474], which has been previously shown to induce mucus hypersecretion in human airway epithelial ALI cultures [471]. Mucus hypersecretion was confirmed by measuring the quantity of mucin collected from the apical surfaces of CFBE41o⁻ cultures where the mucin level was ~3 fold greater in CSS-treated cultures than in untreated control cultures (Figure 3.3e). Subsequently, we compared the transduction efficiency of apically administered AAV1 and AAV6 in CSS-treated versus untreated CFBE41o⁻ cultures. The AAV1-mediated coverage and overall level of GFP

transgene expression were significantly reduced in CSS-treated CFBE41o^{-} cultures compared to untreated cultures, whereas AAV6 retained those readouts regardless of the CSS-induced mucus hypersecretion (Figure 3.3f-j).

To further test the effect of secreted mucus on AAV transduction, we evaluated AAV transduction in primary CF HBE cells, harvested from F508del homozygous CF patients, grown at an ALI with or without the removal of apically secreted mucus. We found that while the coverage and overall level of GFP transgene expression mediated by AAV1 was significantly enhanced by washing the apical mucus with PBS, mucus removal did not affect the readouts when treated with AAV6 (Figure 3.4). Of note, the removal of apically secreted mucus restored AAV1-mediated transduction to levels comparable to AAV6 (Figure 3.4).

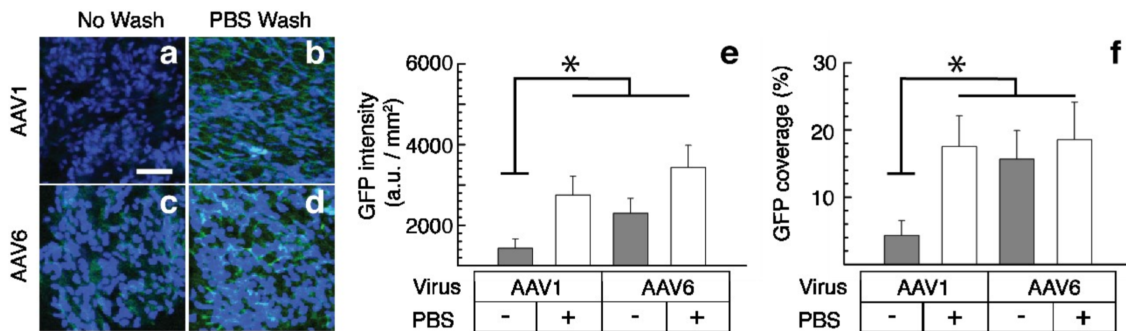

Figure 3.4. *In vitro* transgene expression in primary CF HBE ALI cultures. (a-d) Representative images of GFP expression in primary CF HBE ALI cultures 2 weeks post AAV transduction. Prior to the treatment, cultures were either left unwashed (No Wash) or washed by PBS for the removal of the apically secreted mucus layer (PBS Wash). Scale bar = 50 µm. (e) Image-based quantification of AAV-mediated GFP expression in primary CF HBE ALI cultures with (+) or without (-) PBS wash (n = 3). *$p < 0.05$; one-way ANOVA.

3.3.3. In vivo AAV transduction in a mouse model of muco-obstructive lung disease

To determine if the mucus-penetrating property of AAV6 led to enhanced *in vivo* lung gene transfer, we compared the transduction efficiencies of AAV1 and AAV6

following administration via an aerosol-generating microsprayer to the lungs of *Scnn1b*-Tg mice overexpressing βENaC in the airways. *Scnn1b*-Tg mice spontaneously develop disease symptoms reminiscent of muco-obstructive lung diseases, such as mucus hyperconcentration/plugging, chronic airway inflammation and airspace enlargement [472, 475]. We confirmed the presence of mucus plugs in the airways of *Scnn1b*-Tg mice via immunohistochemistry, which was also distinguished through luminal DAPI staining of immune cells and DNA found within mucus plugs (Figure 3.5). For *in vivo* transduction studies, 2 x 10^{10} ssAAV-Fluc-YFP, either AAV1 or AAV6, in 50 µL PBS were administered into the lungs of *Scnn1b*-Tg mice.

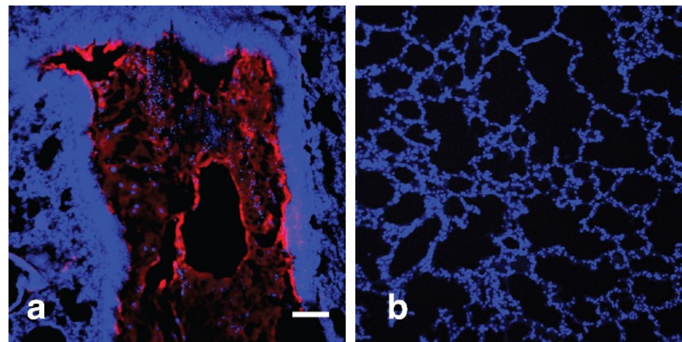

Figure 3.5. Mucus in the lungs of *Scnn1b*-Tg mice. Representative confocal images of mucus in the airways (a) versus the airspace (b). Cell nuclei and DNA are stained with DAPI (blue) and mucins are stained with anti-MUC5B/5AC Ab (red).

The lung distribution of YFP transgene expression was visualized 2 weeks post-infection with AAV1 or AAV6, as shown in the representative confocal micrographs of proximal and distal airways (Figure 3.6a-h). The relative coverage of YFP transgene expression in the airway and airspace was quantitatively assessed using a custom-written MATLAB software. In comparing different, randomly selected regions of the lung, we observed a significantly greater YFP coverage in the mucus-covered/plugged airways (Figure 3.5a) of *Scnn1b*-Tg mice that intratracheally received AAV6 (28%), compared to the mice that received dose-matched AAV1 (13%) (Figure 3.6b, d and i). There was no significant

difference in YFP coverage following the treatment with AAV6 (5%) and AAV1 (3%) in the airspace devoid of mucus secretion (Figure 3.6i and E1b).

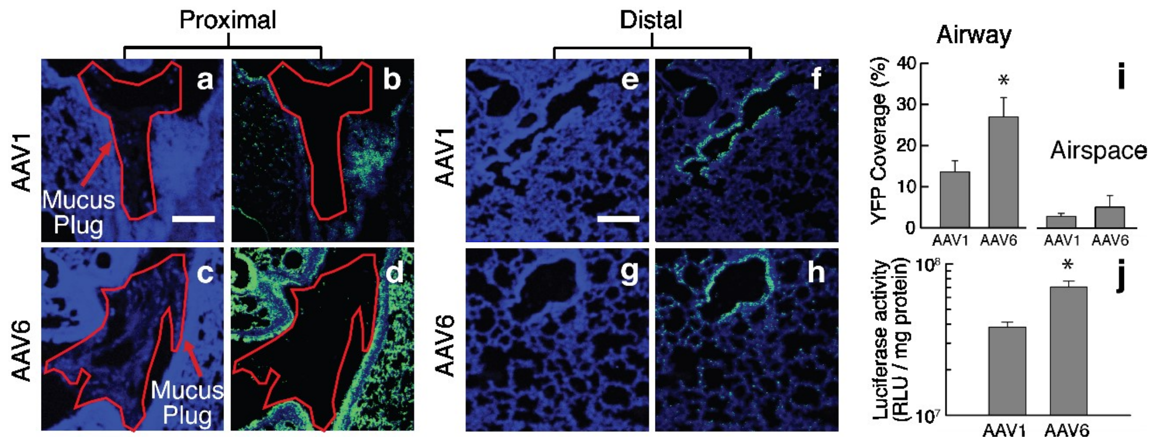

Figure 3.6. *In vivo* transgene expression in the lungs of *Scnn1b*-Tg mice following intratracheal administration of AAV1 or AAV6. (a-h) Representative images of YFP expression (green) in the proximal (a-d) and distal airways/airspace (e-h). Cell nuclei are stained with DAPI (blue). Regions of the airway lumen where mucus plugs were observed are outlined in red. Scale = 200 µm. (i) Image-based quantification of YFP expression coverage (n = 3 mice with 3-4 lung sections per mouse). (j) AAV-mediated luciferase expression quantified for whole lung homogenates (n = 6 mice). *$p < 0.05$; student's t-test.

We also determined the luciferase activity of the whole lung homogenates, an indicator of overall level of reporter transgene expression, from AAV1- and AAV6-treated *Scnn1b*-Tg mice, which revealed a ~2-fold greater bioluminescence intensity in the lungs of AAV6- versus AAV1-treated *Scnn1b*-Tg mice (Figure 3.6j).

3.4. **Discussion**

We have discovered that AAV6 is capable of efficiently diffusing through human CF sputum (i.e. mucus), which presents a critical extracellular barrier to effective inhaled gene therapy. *In vitro* studies in human non-primary and primary CF airway epithelial ALI cultures revealed that AAV6 gene transfer efficacy was not affected either by the presence of an endogenously secreted mucus layer or by induced mucus hypersecretion.

In contrast, AAV1 transduction was markedly inhibited by both steady-state or induced mucus, presumably as a result of its inability to penetrate this barrier. The mucus-penetrating property of AAV6 was further confirmed *in vivo*. Inhaled AAV6 provided widespread and high-level transgene expression in the mucus-covered/plugged airways and the whole lungs, respectively, of a mouse model with airway mucus obstruction. This result agrees with our prior observations using synthetic mucus-penetrating drug and gene delivery nanoparticles that, unlike conventional mucoadhesive particles, exhibited widespread distribution and/or transgene expression in mouse lungs [447, 450-452, 476]. Importantly, the inhaled AAV6 mediated roughly 30% airway coverage of transgene expression, which is comparable to the percentage of human CF airway epithelial cells with CFTR transgene expression (~25%) that was previously shown to require for restoring normal mucus transport rates *in vitro* [37]. Overall, efficient mucus penetration is likely a crucial mechanism by which AAV6 may achieve therapeutically relevant lung gene transfer.

In contrast to our observations, mucin was previously shown not to inhibit AAV1 transduction *in vitro* [465]. The discrepancy is likely due to the markedly lower mucin concentrations employed in this earlier study (≤ 0.5 mg/mL) [477], which is about an order of magnitude or more lower than the concentrations in naturally or pathologically secreted airway mucus collected from human subjects or ALI cultures of primary CF HBE [206, 213, 466, 478, 479]. It should be noted that unlike an aqueous mucin solution, a mucus gel can trap nano-sized objects, such as AAV, via multivalent adhesive interactions as well as steric obstruction [447, 449]. We also showed that, out of the 6 different capsid residues between AAV1 and AAV6, a single amino acid K-to-E

substitution at the 531 position of the AAV6 capsid caused the mutant AAV6 (i.e. AAV6-K531E) to behave like AAV1 [465], a finding confirmed in airway mucus *in vitro* and *ex vivo*. We have previously demonstrated that heparan sulfate binding may be at least partially responsible for the adhesion of AAV2 to CF sputum [457]. However, it is unlikely the case here as it has been shown previously that AAV6 binds to heparan sulfate, whereas AAV1 and AAV6-K531E do not [465, 480]. These findings suggest that glycan-mediated entrapment mechanisms may vary among AAV serotypes. In future studies, we aim to further explore the nature of interactions between different AAV serotypes and the glycans present on airway mucins.

The results of this study demonstrate the importance of using preclinical models that mimic key pathophysiological conditions of patients affected with muco-obstructive lung diseases in evaluating the efficacy of inhaled gene therapy. *Ex vivo* and *in vitro* experiments were designed to evaluate the ability of AAV6 to overcome pathological human airway mucus (i.e. CF sputum) and subsequently achieve efficient transduction of human airway epithelium. Past studies comparing the gene transfer efficacy of different AAV serotypes in human ALI cultures may have been affected by the large volumes (50 - 200 μL) [109, 112, 481-483] applied on the apical surface during AAV administration, which dilute and potentially alter the barrier properties of apically secreted mucus. We thus used a small volume (i.e. 5 μL) in our experiments to minimize this issue. Most preclinical *in vivo* studies have also been conducted using healthy animals lacking critical pathological features in the lung [449]. In contrast, we evaluated the *in vivo* gene transfer efficacies of AAV serotypes possessing different abilities to penetrate the airway mucus

barrier using an established animal model of CF and COPD characterized by airway mucus plugging.

3.5. **Conclusion**

We found that, by using a relevant pre-clinical mouse model of muco-obstructive lung diseases and *in vitro* model of primary CF HBE cells, AAV6 exhibits better mucus penetrability and more widespread transduction of airway epithelial cells compared to AAV1. Future studies should address the persistence of transgene expression as well as the ability to re-administer AAV without concern of therapy-inactivating immunogenicity. Towards this end, development and/or identification of a library of AAV vectors that possess the mucus-penetrating property while retaining high-level transduction efficiency would allow for serial vector administration for life-long therapeutic effects. In summary, we have demonstrated that the ability of AAV6 to penetrate the mucus barrier is a critical mechanism by which it achieves robust gene transfer to the lung airways. Our results underscore the importance of vectors used in inhaled gene therapy to overcome the mucus barrier, which is particularly reinforced in patients with muco-obstructive lung diseases.

4. Efficient non-viral gene delivery in a mouse model of muco-obstructive lung disease

4.1. Introduction

Due to recent advances in identification of genetic loci and modifiers involved in development of muco-obstructive lung diseases, gene therapy has been considered an important, high-potential therapeutic strategy [449]. Muco-obstructive lung diseases (e.g. chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), asthma) has the central pathology of mucus obstruction in the lung airways caused by various non-mutually exclusive factors such as hyper-concentrated mucus and dehydrated airway surface liquid (ASL), impaired mucociliary clearance (MCC), bacterial infection, inflammation, and dysfunctional epithelial ion channels [447, 449, 484]. Mucus obstruction also serves as a biological barrier that hinders delivery of therapeutics to the underlying epithelium [196, 447, 449, 479, 485], limiting the attractiveness of the lungs as one of the most straightforward targets for gene delivery (i.e. inhalation) (Griesenbach, Curr Opin Mol Ther, 2005). As such, inhaled gene therapy of muco-obstructive lung diseases has not successfully translated to the clinics to date [196, 449]. CF, an inherited genetic disease hallmarked by muco-obstructed lungs, has received much attention in gene therapy due to well-defined therapeutic target (i.e. CF transmembrane conductance regulator gene, or CFTR) and aforementioned direct access to the lungs, where the most life-threatening symptoms are manifested [486, 487]. Over 25 clinical trials of inhaled viral and non-viral gene therapy have failed to show clinical benefits largely due to inefficient gene transfer to the airway epithelium [449, 487]. Indeed, many commonly used viral vectors and conventionally formulated non-viral vectors cannot efficiently

penetrate through CF sputum [202, 210, 211], resulting in inadequate transgene expression both spatially and temporally. We believe there are two key considerations that may have been beneficial to their outcome – 1) the use of phenotypically relevant (i.e. muco-obstruction) animal models and 2) mucus-penetrability of gene vectors.

We previously demonstrated that mucus-penetrating DNA nanoparticles (DNA-MPP) composed of poly(β-amino ester) (PBAE) and polyethylene glycol (PEG) provided widespread distribution and transgene expression in the lungs of a healthy mouse model [488]. For clinical relevance, however, it is critical that DNA-MPP perform as such in the lung conditions relevant to muco-obstructive lung diseases. Therefore, the aim of present study was to demonstrate the safety and effectiveness of DNA-MPP in the β-epithelial Na^+ channel-overexpressing transgenic (*Scnn1b*-Tg) mice, a mouse model of muco-obstructive lung disease.

The muco-obstructed lung phenotype in the *Scnn1b*-Tg mice is caused by clara cell-specific overexpression of the *Scnn1b* gene, which encodes for the β-subunit of the epithelial sodium channel (ENaC) [472]. Overexpression of the β-subunit leads to increased interaction with the two remaining subunits of ENaC (i.e. α and γ), subsequently over-production of airway ENaC. The increased flux of Na^+ ion into the epithelium causes CF-like lung disease in this mouse model with features including dehydration of ASL, impaired MCC, mucus hypersecretion, bacterial infection, and inflammation [472, 489]. In human CF epithelium, ENaC is intact or in some cases hyper-activated on top of CFTR deficiency, which leads to an imbalance of ions and fluid that makes CF airway surfaces vulnerable to dehydration and impaired MCC [490]. Exacerbations in many non-CF airway diseases result from impaired MCC as well, which

underscores the therapeutic importance of ENaC modulation [242, 314, 491]. To this end, multiple drugs that target ENaC are currently in the pipeline [492]. Therefore, the *Scnn1b*-Tg mouse model serves as an essential tool for validation of inhaled gene vectors as well as the effects of ENaC modulation *in vivo* for muco-obstructive lung diseases [493]. We hypothesized that DNA-MPP can safely achieve widespread distribution and robust transgene expression in presence of muco-obstruction in the airways of the *Scnn1b*-Tg mice. Further, we aimed to deliver shRNA-encoding DNA plasmid to modulate the expression of ENaC in order to ameliorate the muco-obstruction.

4.2. Materials and Methods

4.2.1. Reagents

Reagents were purchased from different companies as follows: 1,4-butanediol diacrylate, 5-amino-1-pentanol, 4-amino-1-butanol, and 1-(3-aminopropyl)-4-methylpiperazine (C4) from Alfa Aesar; 1,3-diaminopropane (C1) and 2,2-dimethyl-1,3-propanediamine (C2) from Sigma-Aldrich; N-(3-aminopropyl)pyrrolidine (C3) from Acros Organics; 2-(3-aminopropylamino)ethanol (C5) from Oakwood Chemical; N-(3-aminopropyl)diethanolamine (C6) and 1,11-diamino-3,6,9-trioxaudecane (C7) from Tokyo Chemical Industry; and methoxy-polyethylene glycol (PEG)-succinimidyl succinate from JenKem Technology. Solvents were purchased as follows: ethyl ether anhydrous from Fisher Scientific; and dimethyl sulfoxide anhydrous (DMSO) and tetrahydrofuran (THF) from Sigma-Aldrich.

4.2.2. Polymer synthesis

PBAE polymers were synthesized as previously described [488]. Briefly, 1,4-butanediol diacrylate and 4-amino-1-butanol were reacted at a 1.1:1 ratio to synthesize

high molecular weight uncapped polymer (6 ± 0.2 kDa) or at a 1.2:1 ratio to synthesize low MW upcapped polymer (4 ± 0.2 kDa) at 95 °C for 20 hours. Polymers were then purified by dropwise precipitation into cold anhydrous diethyl ether. The mixture was vigorously vortexed and centrifuged at 2,000 rpm for 3 minutes to pellet the polymer. The supernatant was discarded, then the polymer was washed twice more in anhydrous diethyl ether. The resulting polymer was dried under vacuum for at least 72 hours, then the molecular weight was estimated by nuclear magnetic resonance (NMR). The end acrylate groups were capped with 30 molar equivalents of 2-(3-aminopropylamino)ethanol or other capping groups (Figure 4.1) at 100mg/ml in THF overnight at room temperature followed by the cold ether purification and vacuum-mediated solvent removal process. Complete end-capping and purity were confirmed by the absence of the diacrylate peaks in the NMR spectrum. The end-capped PBAE polymer was dissolved in DMSO at 100mg/ml, and stored at -20 °C.

For the synthesis of PEGylated PBAE polymers, the low MW uncapped polymer synthesized with abovementioned steps was end capped with C1, resulting in P1C1 polymer. Then, 2.05 molar equivalent of 5 kDa methoxy-PEG-succinimidyl succinates were reacted with the two terminal primary amine groups at both ends of P1C1 at 100mg/ml in THF for 24 hours at room temperature (Figure 4.1). The resulting product was washed with anhydrous diethyl ether as described above, dried under vacuum, dissolved in DMSO at 100mg/ml, and stored at -20 °C.

Figure 4.1. Chemical structure of monomers and polymers used to create DNA-MPP formulations. Core polymer, PBAE, is comprised of a combination of P1 and a C (capping group). PEG-PBAE is comprised of short molecular weight P1, C1, and 5 kDa methoxy PEG.

4.2.3. ^{1}H nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR spectra of PBAE and PEG-PBAE dissolved in deuterated methanol (MeOH-d4; Cambridge Isotope Laboratories) were recorded on a Bruker spectrometer (500 MHz). ^{1}H chemical shifts were reported in ppm (δ) and the MeOH peak was used as an internal standard. Data were processed using Mest-ReC software.

4.2.4. DNA-NP formulation and characterization

The luciferase-expressing plasmid driven by a human beta-actin promoter (pBAL) was produced by Copernicus Therapeutics, and the green fluorescent protein (GFP)-encoding plasmid driven by a cytomegalovirus (CMV) promoter (pEGFP) was purchased

from Clontech Laboratories. CFTR-expressing plasmid driven by chicken beta-actin promoter was a kind gift from Dr. Liudmila Cebotaru. shRNA-expressing plasmid DNA against alpha subunit ENaC (*Scnn1a* location 1498, target sequence ggcgaattattctcagttcca), driven by H1 promoter, was purchased from Genecopoeia. The plasmids were expanded by transformation into appropriate E.coli strains and isolated by using Endofree Plasmid Giga Kit (Qiagen). Downregulation of alpha ENaC was validated *in vitro*, in M-1 mouse kidney epithelial cell line using Lipofectamine 3000. For assessment of DNA-NP distribution in mouse lungs, Label IT® Tracker Intracellular Nucleic Acid Localization Kit (Mirus Bio) was used to fluorescently label plasmid DNA with a Cy3 fluorophore. DNA-CP and DNA-MPP were formulated as previously described [280]. We characterized the hydrodynamic diameter and polydispersity index of DNA-NPs in water by dynamic light scattering (DLS), and the ζ-potential was measured in 10 mM NaCl at pH 7.0 by laser doppler anemometry using a Zetasizer Nano ZS (Malvern Instruments). To assess the stability of DNA-NP in physiologically relevant conditions, DNA-CP and DNA-MPP were incubated in bronchoalveolar lavage (BAL) fluid at 37 °C for 4 hours, with DLS measurements at 0, 5 minutes, 1, 2, and 4-hour timepoints. After observing rapid aggregation in BALF, DNA-CP was ruled out for further characterization.

To test the DNA compaction of the DNA-MPP, conventional gel retardation assay was performed. 100 ng of various plasmid DNA used in this study (shENaC, pBAL, and pEGFP) and DNA-MPP carrying the respective plasmid DNA were mixed with 6X DNA loading dye (ThermoFisher) and loaded onto a 0.8 % agarose gel pre-stained with SYBR safe (ThermoFisher), and run for 30 minutes at 50 V. Also, DNA protection was assessed

by the same gel electrophoresis method after incubating the plasmid DNA and DNA-MPP with 2 U of DNase I (ThermoFisher) for 15 minutes at 37 °C followed by addition of 50 mM EDTA at 65 °C to deactivate the DNase activity. To induce de-compaction of the MPP, samples were incubated with heparin (Sigma Aldrich) at a 3:1 (w/w) ratio of heparin to DNA at room temperature for 10 minutes, followed by agarose gel electrophoresis. Gels were imaged using a Chemi-Doc imaging system (Bio-RAD).

4.2.5. Multiple Particle Tracking

Multiple particle tracking (MPT) is a well-established method to quantify the mean square displacements (MSDs) of fluorescently labeled particles, as previously described. CF mucus freshly expectorated from CF patients were collected from the Johns Hopkins University Adult Cystic Fibrosis Center under written informed consent, in accordance with the Johns Hopkins Institutional Review Board. All experiments were conducted using samples stored at 4 °C immediately after collection for up to 24 hours. Any sample with a visible quantity of saliva was excluded to reduce the effects of salivary contamination. A 1 μl solution of fluorescently labeled DNA-NPs at a plasmid DNA concentration of 1 μg/ml was added to 30 μl of CF mucus, placed in custom-made microwells, and equilibrated for 30 minutes at room temperature. Movies were recorded over 20 s at an exposure time of 66.7 ms by an Evolve 512 EMCCD camera (Photometrics) mounted on an inverted epifluorescence microscope (Axio Observer D1; Carl Zeiss) equipped with a 100×/1.46 N.A. oil-immersion objective. Movies were then analyzed using a custom-made MATLAB code (MathWorks) to simultaneously extract x, y coordinates of the hundreds of DNA-NP centroids and calculate individual and median mean square displacement as a function of timescale [467]. To minimize the effect of

dynamic error in our measurement, we calculated the MSD at 1 s, a timescale previously established to be long enough to minimize dynamic error while allowing for tracking of DNA-NPs without losing a large number of the fast-moving population in the z direction. The theoretical diffusion rates of DNA-NPs in water were calculated using the Stokes–Einstein equation, as previously described [467].

4.2.6. BAL fluid collection

To collect BAL fluid, mice were euthanized by deep isoflurane anesthesia and the lungs were carefully harvested. A narrow micropipet tip connected to a 1 ml syringe was inserted into the trachea and lavage was performed with 1 ml of PBS, and then repeating twice more to collect total volume of ~3 ml. Cells were pelleted by centrifugation at 300 x g for 5 minutes at 4 °C and the cell-free supernatant was collected and stored at 80 °C for ELISA, or lyophilized for mucin analysis. BAL cells were resuspended in 1 ml of PBS and total number of cells were counted with a hemocytometer. The lyophilized supernatant was reconstituted with 1 ml of ultrapure water for quantification of O-linked glycoproteins to approximate the mucin concentration by using the previously described method [466].

4.2.7. Animal studies

Mice were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee. The *Scnn1b*-Tg mice used in this study has been extensively characterized for use in CF research [346, 472, 494-497] and were kind gift of Dr. Richard Boucher of University of North Carolina at Chapel Hill. The colony was maintained on a mixed genetic background (C3H/HeN x C57BL/6N), and *Scnn1b*-Tg mice were identified by genotyping service from TransnetYX, Inc.

Wildtype (WT) littermates were used as controls in all studies. Mice were anesthetized in 2% isofluorane chamber. 4-week old mice were used for all *in vivo* studies. To assess the DNA-NP distribution and distribution of transgene expression in the mouse lungs, a 50 µl suspension of DNA-NPs carrying Cy3-labeled plasmid DNA or pEGFP, respectively, was administered intratracheally by using a microsprayer (Model IA-1C, Penn-Century) at the plasmid DNA concentration of 0.5 mg/ml (n=3 per group). Mice were killed 1 hour or 48 hours after the administration to assess distribution of DNA-NP or transgene expression, respectively. For DNA-NP distribution studies, lungs were perfused with PBS, fixed with 4% paraformaldehyde (PFA) overnight at 4 °C, and sequentially incubated in 10%, 20%, and 30% sucrose solutions for two days each at 4 °C, prior to embedding in OCT. The lungs were cryosectioned using a CM1950 cyrostat (Leica Biosystems), followed by DAPI staining (ProLong® Gold Antifade with DAPI, ThermoFisher) and imaging with an inverted epifluorescence microscope (Axio Observer D1, Carl Zeiss) at 10x magnification. For transgene expression distribution studies, lungs were perfused with PBS, harvested, then carefully filled with 1:1 mixture of PBS and OCT, immediately followed by embedding in cryomold. The lungs were cryosectioned, fixed in ice-cold 100% acetone, blocked with Block-Aid agent (ThermoFisher), and immunologically stained overnight at 4 °C using an anti-GFP antibody tagged with Alexa 488 fluorophore (Biolegend) diluted in 1% bovine serum albumin/0.3% Triton X-100/PBS solution. After washing with PBS three times, the slides were stained with DAPI for imaging with a confocal LSM 710 microscope (Carl Zeiss) under 10x or 20x magnification. Lung sections from untreated mice were processed the same way and used to determine the background fluorescence with the microscope settings. Image-based

quantification of distribution of DNA-NP and transgene expression was performed with a custom-written JAVA code. To assess the overall level of *in vivo* transgene expression, DNA-CP or DNA-MPP carrying pBAL were administered to WT littermate or *Scnn1b*-Tg mice at 50 μl of fixed volume. After 1 week, the lungs were harvested and homogenized to measure luciferase activity as previously described [488]. The RLU was normalized by total protein concentration measured by BCA assay. For western blotting, lung homogenate was prepared for each mouse and 40 mg of protein were loaded into each well of Novex Tris-Glycine gel (ThermoFisher) and run at 120 V for 1.5 hrs. Protein was transferred to nitrocellulose membranes and blocked with bovine serum albumin (BSA) for one hour, followed by overnight primary antibody incubation at 4 °C. The membranes were washed three times with PBS, and were incubated with secondary antibodies for 1 hour at room temperature. Membranes were washed with PBS three times, and ECL substrate (Promega) was applied for visualization of protein bands with Chemi-Doc imaging system.

4.2.8. Hypertonic saline treatment

7% NaCl solution was prepared and sterile-filtered with 0.22 μm syringe filter. 4-week old *Scnn1b*-Tg mice were anesthetized under 2% isoflurane, then 7% NaCl or equal volumes of vehicle (i.e. ultrapure water) was administered (1 μl/g body weight, three times per day) via intratracheal instillation for 1 week. A day after the last treatment, DNA-MPP containing the shENaC plasmid DNA was delivered via intratracheal instillation with a microsprayer.

4.2.9. In vitro studies

Primary CF human bronchial epithelial cells expressing the homozygous F508del mutant CFTR (CF HBE) were used for *in vitro* transfection studies. The University of Alabama at Birmingham IRB approved the use of primary cells. Primary CF HBE cells were expanded using co-culture with irradiated fibroblasts (R&D Systems) and conditional reprogramming via media containing a rho-associated protein kinase inhibitor. Primary CF HBE cells were grown in 0.33 cm^2 polyester membrane Transwell® inserts (Corning) coated with NIH 3T3 fibroblast conditioned media until fully confluent. Primary CF HBE cells were maintained in differentiation media for at least 4-6 weeks until terminally differentiated. An 8 μL suspension of DNA-CP or DNA-MPP at 0.1 mg/ml concentration was administered to the apical surface of the fully differentiated ALI cultures to evaluate *in vitro* transfection efficiency. After two days, the Transwell® inserts were cut out with a micro-scalpel and placed onto a glass slide and coverslip for fluorescence imaging. To avoid artifacts presented by differences in focus between images, we used automated focusing (available in Zeiss Zen software) of DAPI nuclear staining and captured all images at this focal plane. Six or more randomly selected image fields were taken of each insert and used for quantification. The percentage of GFP coverage was defined as the GFP-positive area divided by the total cell-covered surface area, and the total GFP intensity was quantified as the average of total pixel intensity units for each dataset. Prior to quantitative analysis, all images were normalized by fluorescence of untreated control ALI cultures to eliminate the contribution of autofluorescence.

4.2.10. Immunocytochemistry

Cells grown on Transwell® inserts were stained for detection of transgene expression via confocal microscopy. Each Transwell® insert was washed with PBS three times, and then fixed with 4% paraformaldehyde overnight at 4 °C. Cells were washed with PBS again, and by using a micro-surgical knife (MSP, Cat# 7503), the Transwell® membranes were carefully cut out. For blocking unspecific binding of antibodies and cell permeabilization, each membrane was placed cell-side down on a 20 µl drop of 6% normal goat serum/0.05% Triton X-100 on a piece of parafilm for 30 minutes. Primary antibody was diluted to appropriate concentration in 3% normal goat serum/0.025% Triton X-100, and the Transwell® membrane was again placed cell-side down on a 20 µl drop of the antibody solution on parafilm for overnight incubation at 4 °C. Membranes were washed three times with PBS, and secondary antibody in 3% normal goat serum/0.025% Triton X-100 at 1:1000 dilution was applied for 1 hour. Membranes were washed with PBS three times, and were mounted on a glass slide and coverglass with ProLong™ Glass Antifade Mountant with NucBlue™ Stain (Thermo P36981) for imaging.

4.3. **Results**

4.3.1. Characterization of DNA-MPP

We first engineered a DNA-loaded mucus-penetrating particle (DNA-MPP) platform for inhaled gene therapy of muco-obstructive lung diseases, using a method that we had previously established [488]. Specifically, a mixture of poly(β-amino ester) (PBAE) and polyethylene glycol (PEG)-conjugated PBAE polymers at an optimized ratio

was used to compact plasmids encoding various reporter or therapeutic nucleic acids, including luciferase (~5 kb), GFP (~4.7 kb) or shRNA against ENaC (~6.5 kb). In parallel, we formulated mucus-impermeable DNA-loaded conventional particles (DNA-CP) using PBAE polymers only. We first confirmed that all the DNA-MPP formulations exhibited mucus-penetrating particle properties, including small particle diameters (~ 50 nm) and near neutral surface charges [488], regardless of the type/size of plasmid

Table 4.1. Characterization of DNA-NP. Size and surface charge measurement with Zetasizer. (n=3)

	Hydrodynamic diameter (nm)	Polydispersity index	ζ-potential (mV)
DNA-CP	107 ± 3	0.15	30 ± 4
DNA-MPP	54 ± 1	0.12	0.8 ± 0.1

payloads (Table 4.1). In comparison, DNA-CP possessed larger particle sizes (> 100 nm in diameters) and highly cationic surfaces (ζ-potentials of ~ 30 mV) (Table 4.1). We then conducted gel electrophoretic retardation assay where we found that all three plasmids were stably packaged into DNA-MPP, as evidenced by their complete retention within the wells of the gel (Figure 4.2A). Moreover, DNA-MPP were capable of protecting plasmid payloads from enzymatic degradation by DNase, while free plasmids were completely degraded (Figure 4.2B). We next confirmed that DNA-MPP retained their small particle diameters (~50 nm) in bronchoalveolar lavage (BAL) fluid at 37 ˚C as least up to 4 hours (Figure 4.2C), indicating an excellent colloidal stability in a physiologically relevant lung environment. In contrast, DNA-CP immediately aggregated as soon as the incubation commenced, reaching microns of sizes within an hour (Figure 4.2C).

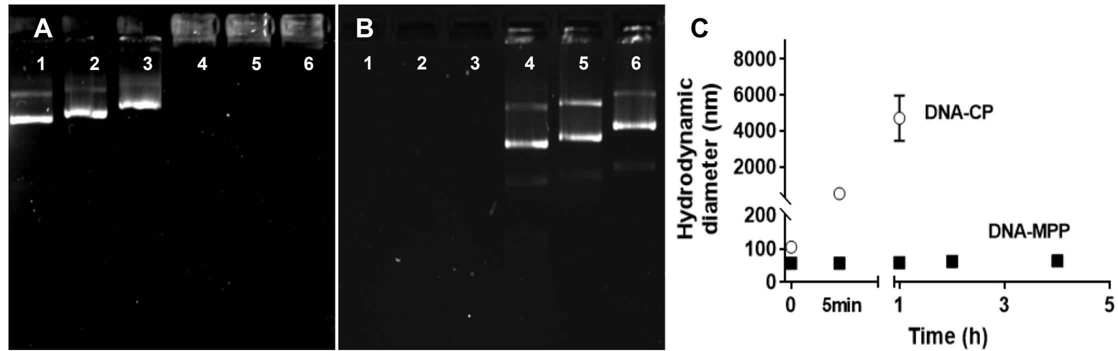

Figure 4.2. *In vitro* characterization of DNA-loaded nanoparticles. Electrophoretic analysis of (A) complexation and (B) protection (against enzymatic degradation by DNase) of plasmids. Lane 1: GFP-encoding plasmid; 2: pBAL-encoding plasmid; 3: shENaC-encoding plasmid; 4: GFP-MPP; 5: pBAL-MPP; 6: shENaC-MPP. (C) Colloidal stability of DNA-CP and DNA-MPP in BAL fluid (i.e. change in hydrodynamic diameter) over time at 37°C. Data represent mean ± S.D.

4.3.2. In vivo distribution of nanoparticles and transgene expression

We previously demonstrated that DNA-MPP provided widespread distribution and transgene expression in the lungs of healthy mice [488]. However, it is critical for its clinical relevance that DNA-MPP perform as such in the lung conditions relevant to muco-obstructive lung diseases. We thus investigated in vivo behaviors of DNA-MPP, in comparison to DNA-CP, in the lungs of *Scnn1b*-Tg mice, a mouse model that emulates the phenotypic hallmarks of muco-obstructive lung diseases, including mucus plugging and chronic inflammation [472]. We first compared lung distribution of Cy3-labeled DNA-CP and DNA-MPP following a single intratracheal administration via a microsprayer. It appeared that some amounts of both formulations were inevitably found entrapped within the mucus plugs established in the airways of *Scnn1b*-Tg mice one hour after the administration (Figures 4.3A-C). However, while DNA-CP were sparsely distributed or rarely observed in the airway lumen (Figure 4.2B), DNA-MPP exhibited widespread and uniform distribution throughout the airways and airspace (Figure 4.3C). We next investigated whether the observed particle distribution pattern translated to the

distribution of transgene expression. To do this, we identically treated *Scnn1b*-Tg mice with a single dose of DNA-CP or DNA-MPP carrying GFP-encoding plasmids and assessed the transgene expression at 48 hours post-administration via confocal microscopy of lung tissue sections (Figure 4.3D-F). In good agreement with the particle distribution profile, GFP transgene expression was sporadic in the lungs of mice treated with DNA-CP (Figure 4.3E), whereas DNA-MPP provided widespread transgene expression throughout the lungs (Figure 4.3F), even in an entire lobe in several cases (Figure 4.4). Of note, no fluorescence was observed in untreated lungs (Figures 4.3A, D). Image-based quantification revealed that the airway coverages of GFP transgene expression mediated by DNA-CP and DNA-MPP were ~5% and ~35%, respectively

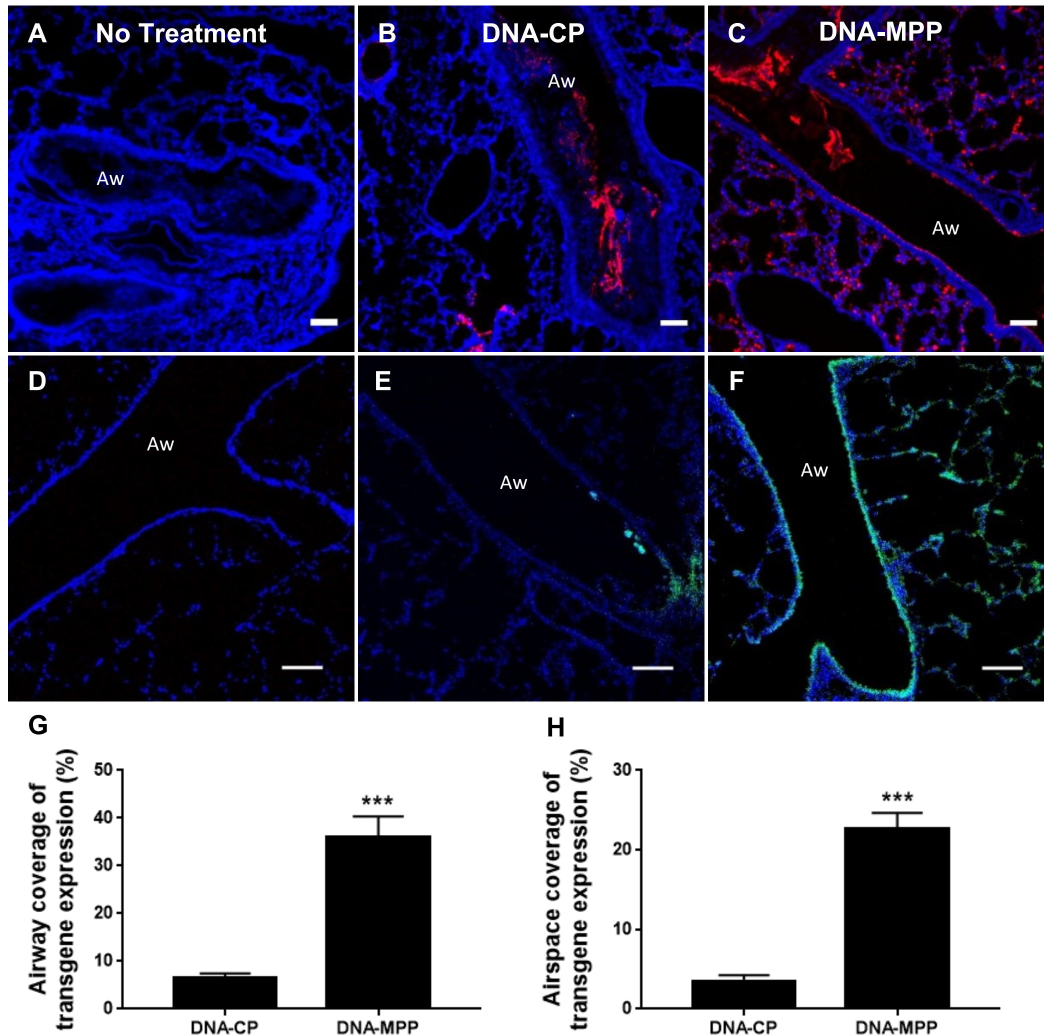

Figure 4.3. *In vivo* particle distribution and transgene expression in the lungs of *Scnn1b*-Tg mice following a single intratracheal administration of DNA-loaded nanoparticles. Representative confocal images demonstrating particle distribution in (A) untreated lung and lungs treated with (B) DNA-CP or (C) DNA-MPP. Red: DNA-CP or DNA-MPP; Blue: cell nuclei; White arrows: mucus plugs. Representative confocal images demonstrating reporter transgene expression in (D) untreated lung and lungs treated with (E) DNA-CP or (F) DNA-MPP. Green: GFP transgene expression; Blue: cell nuclei. Aw: airway; Scale bars = 100 µm. Quantification of transgene expression coverage in (G) airways and (H) airspace. Data represent mean ± S.E.M. Differences are statistically significant as indicated (***$p < 0.001$; student's t-test).

(Figure 4.3G). Likewise, DNA-MPP exhibited approximately 8-fold greater coverage of transgene expression compared to DNA-CP in the airspace (Figure 4.3H).

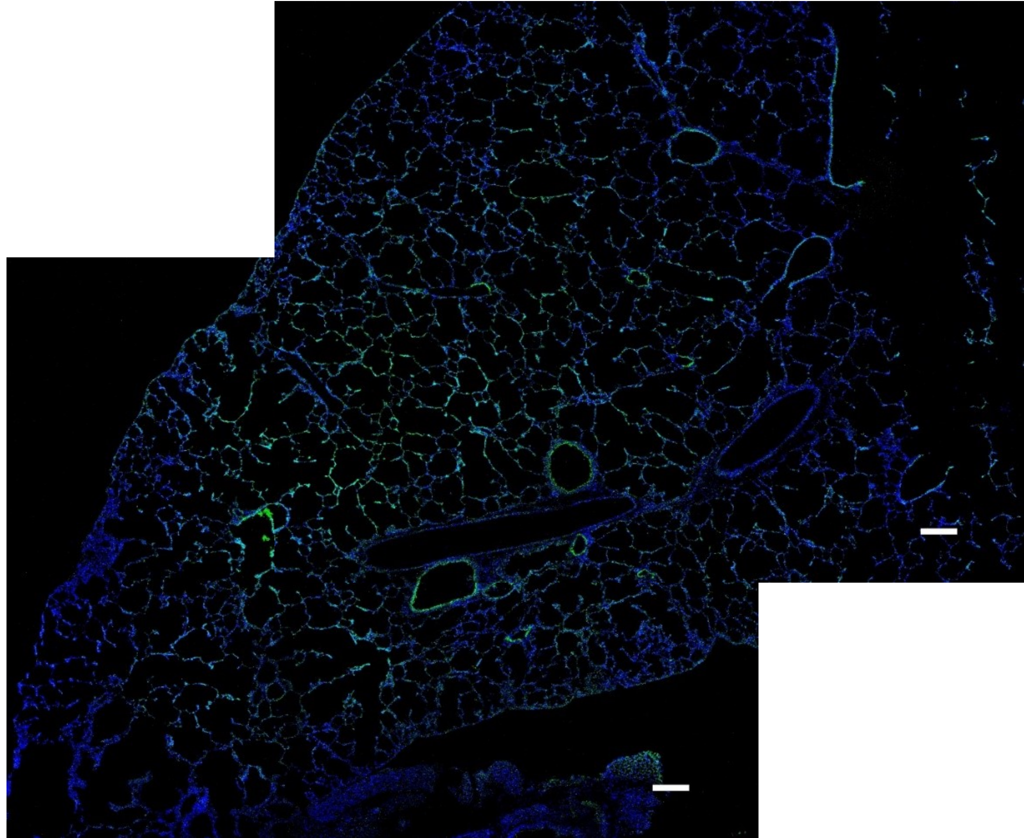

Figure 4.4. Confocal image of a section of left lung of *Scnn1b*-Tg mouse treated with DNA-MPP carrying GFP plasmid DNA. Green: GFP; Blue: Nuclei; Scale bar: 500μm

4.3.3. Overall in vivo transgene expression and safety in chronically inflamed mouse lungs

We subsequently investigated whether the widespread gene transfer enabled by DNA-MPP resulted in high-level overall transgene expression. Specifically, DNA-CP or DNA-MPP carrying luciferase-encoding plasmids (at 0.5 mg/ml plasmids in 50 μl) were administered intratracheally to the lungs of *Scnn1b*-Tg mice, and the lung tissues were harvested for homogenate-based luciferase assay seven days after the administration. We found that DNA-MPP mediated about an order of magnitude greater luciferase activity compared to the level achieved by DNA-CP (Figure 4.5A). We then conducted a dose escalation study where single incrementing doses of DNA-MPP carrying luciferase-

encoding plasmids (i.e. 0.125 mg/ml to 2 mg/ml plasmids in 50 µl) provided dose-dependent increase in the luciferase activity (Figure 4.5B). While the 1 mg/ml plasmid

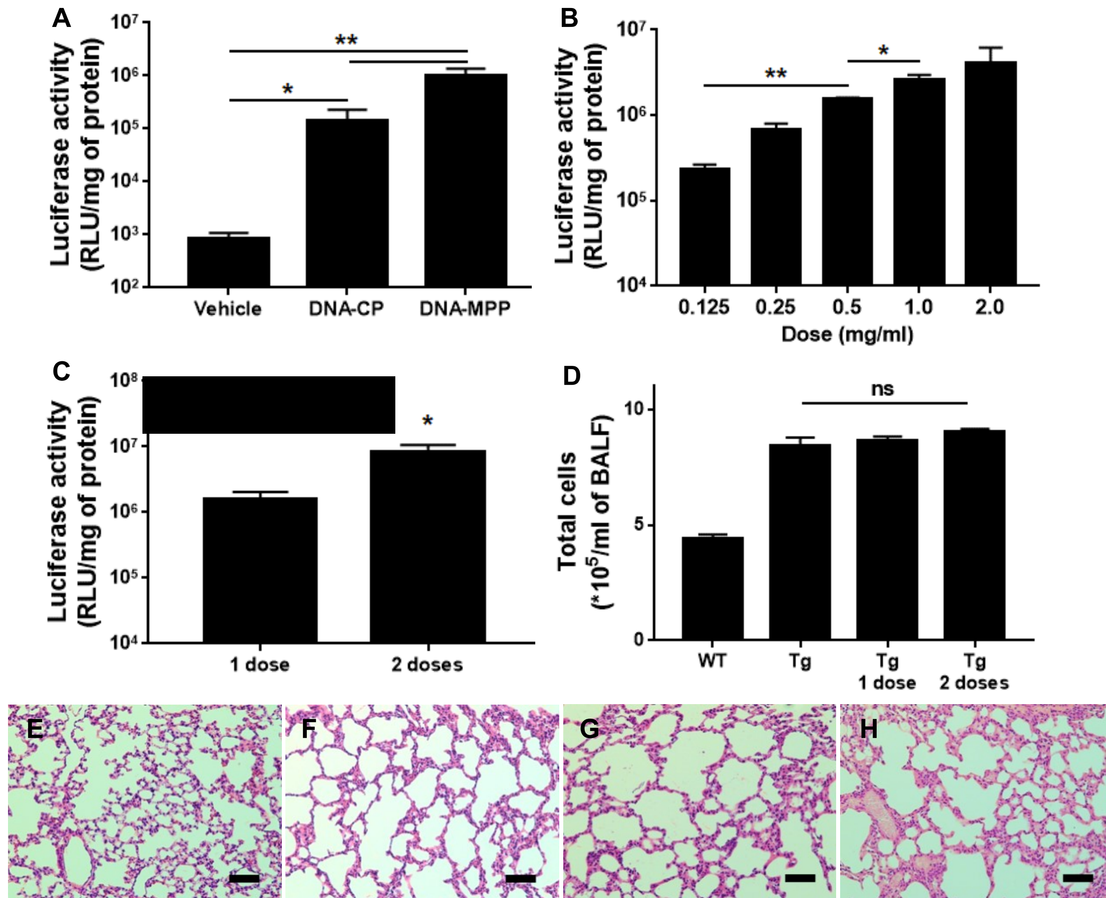

Figure 4.5. Overall level of *in vivo* transgene expression in the lungs of *Scnn1b*-Tg mice following intratracheal administration of DNA-loaded nanoparticles. (A) Comparison of transgene expression mediated by DNA-CP and DNA-MPP following a single administration. Transgene expression mediated by (B) incrementing or (C) repeated doses of DNA-MPP. (D) Total number of cells in BAL fluid following a single and repeated treatment(s) with DNA-MPP. Data represent mean ± S.E.M. Differences are statistically significant as indicated (**p* < 0.05; ***p* < 0.01; One-way ANOVA or Student's *t*-test). Histological H & E staining of lungs of (E) untreated wild-type (WT) littermate mice, (F) untreated *Scnn1b*-Tg mice and *Scnn1b*-Tg mice received (G) a single dose or (H) two doses of DNA-MPP. Scale bar = 200 µm

concentration provided significantly greater overall transgene expression (i.e. luciferase activity) compared to its half dose (i.e. 0.5 mg/ml) (Figure 4.5B), we elected to conduct further studies with the latter due to its *in vivo* safety established in healthy mouse lungs in our prior study [488]. We next evaluated the effect of repeated administration of DNA-

MPP and discovered that two doses given with one-week interval resulted in statistically significant amplification of the overall transgene expression level (Figure 4.5C). It is critical that DNA-MPP provides an acceptable safety in relevant disease conditions, such as in the readily inflamed lungs of patients with muco-obstructive lung diseases. We thus sought to test whether DNA-MPP further aggravated chronic inflammation established in the lung of *Scnn1b*-Tg mice by quantifying the total number of cells in BAL fluid. Consistent with a prior observation [472], the total cell count was markedly greater in the lungs of *Scnn1b*-Tg mice compared to the lungs of littermate wild-type mice (Figure 4.5D). However, a single or two consecutive dose(s) of DNA-MPP did not exacerbate the chronic inflammation in the lungs of *Scnn1b*-Tg mice, as evidenced by the virtually identical total cell counts (Figure 4.5D). In addition, H&E staining of lung tissues from different treatment groups further confirmed the *in vivo* safety of DNA-MPP in the inflamed lungs (Figure 4.5E-H).

4.3.4. In vivo ENaC modulation mediated by DNA-MPP

We next investigated whether DNA-MPP were capable of delivering therapeutic plasmids and exerting meaningful therapeutic intervention in the lungs of *Scnn1b*-Tg mice. Specifically, we treated mice with DNA-MPP carrying plasmids encoding shRNA against α-subunit of ENaC (shENaC-MPP). While the diseased phenotypes observed in the lungs of the *Scnn1b*-Tg mice are driven by overexpression of β-subunit of ENaC [472], we opted to target the α-subunit due to its essential role on the physiological ENaC functions [498-500]. We also note that α-subunit is currently serving as a primary clinical target for ENaC-based CF therapy [501, 502]. Western blot analysis revealed that a single dose of shENaC-MPP was capable of mediating significant downregulation of both

glycosylated and non-glycosylated forms of ENaC (Figure 4.6A-B). As expected from the earlier reporter multi-dose study (Figure 4.5C), we also confirmed that two consecutive doses of shENaC-MPP further reduced the ENaC expression (Figure 4.7). Quantitatively, a single and two dose(s) resulted in 30% and 60% ENaC downregulation, respectively, compared to the vehicle-treated control mice (Figure 4.6C). To examine whether the shENaC-MPP-mediated ENaC downregulation ameliorated the muco-obstruction in the lungs of *Scnn1b*-Tg mice, we collected BAL fluid and quantified the amount of mucin in the mucus plug. We found that approximately 45% and 60% of mucin content were reduced compared to the vehicle-treated control when shENaC-MPP were administered once and twice, respectively (Figure 4.6D). In parallel, we conducted a control experiment in which we confirmed that the mucin concentration in the lungs of *Scnn1b*-Tg mice remained unchanged when mice received DNA-MPP carrying GFP-encoding plasmids instead of shENaC-MPP (Figure 4.8).

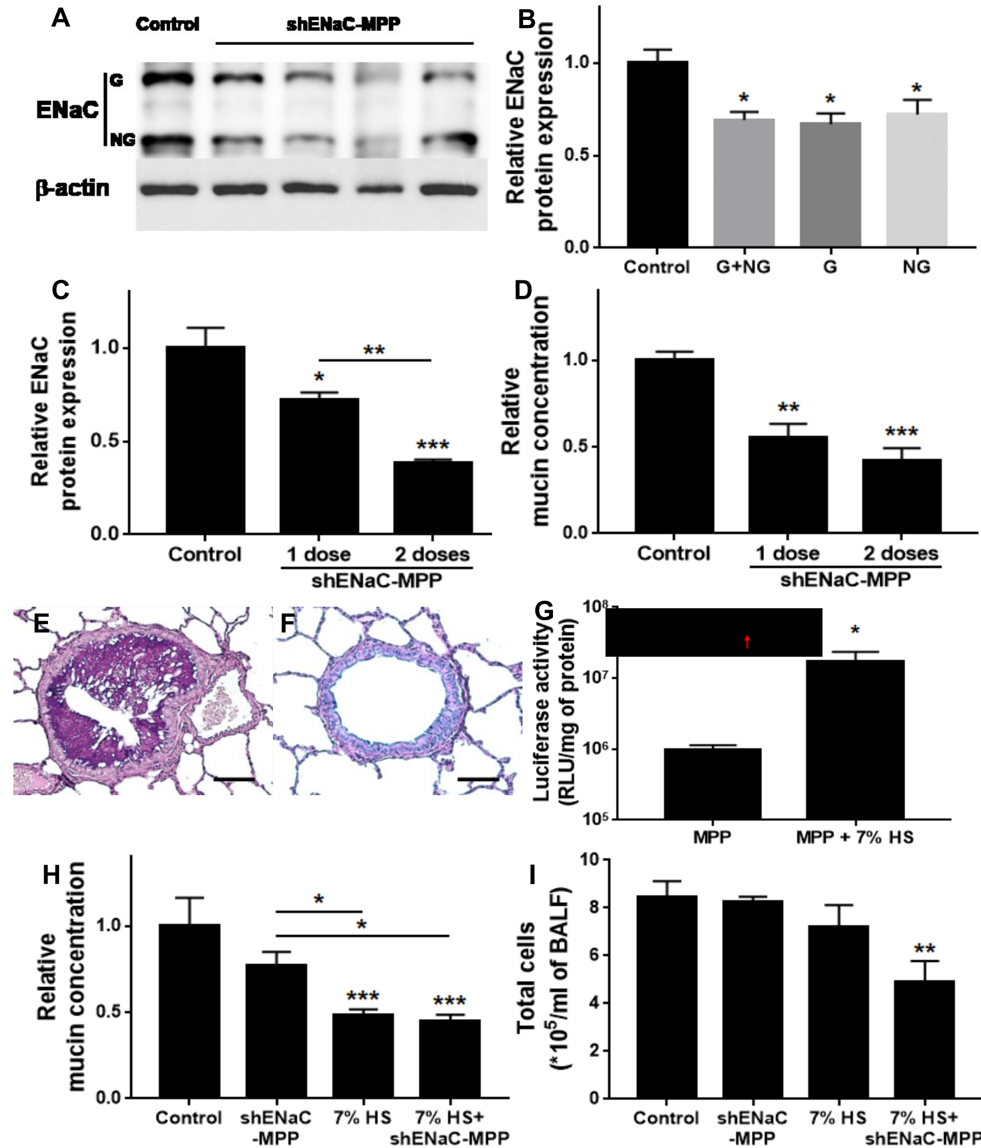

Figure 4.6. Clinically-relevant effectiveness of DNA-MPP carrying shENaC-encoding plasmids (shENaC-MPP) in the lungs of *Scnn1b*-Tg mice. Western blot (A) imaging and (B) quantitative analysis demonstrating downregulation of ENaC in *Scnn1b*-Tg lungs by a single intratracheal dose of shENaC-MPP. G: glycosylated; NG: non-glycosylated. (C) Further downregulation of ENaC after repeated doses of shENaC-MPP. (D) Relative total mucin concentration in BAL fluids from untreated *Scnn1b*-Tg mice vs. *Scnn1b*-Tg mice received a single or two dose(s) of shENaC-MPP. Ab-PAS staining of *Scnn1b*-Tg mouse lungs treated with (E) vehicle (i.e. ultrapure water) or (F) 7% HS. Scale bar = 500 μm (G) Enhancement of DNA-MPP-mediated overall transgene expression in the lungs of *Scnn1b*-Tg mice with 7% HS pre-treatment (1 μl/g body weight, 3 x/day, 7 days). Inset: treatment regimen (Black arrows: 7% HS pre-treatment; Red arrow: DNA-MPP administration). (H) Relative total mucin concentration and (I) total number of cells in BAL fluids collected from *Scnn1b*-Tg mice received single dose of shENaC-MPP, 7% HS, or sequential treatments of 7% HS and shENaC-MPP in comparison to untreated *Scnn1b*-Tg mouse control. Data represent mean ± S.E.M. Statistically significant as indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; One-way ANOVA or Student's *t*-test).

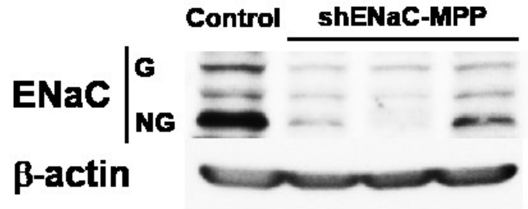

Figure 4.7. Western blotting demonstrating downregulation of ENaC in the lungs of *Scnn1b*-Tg mice by repeated intratracheal administration of shENaC-MPP. G: glycosylated; NG: non-glycosylated.

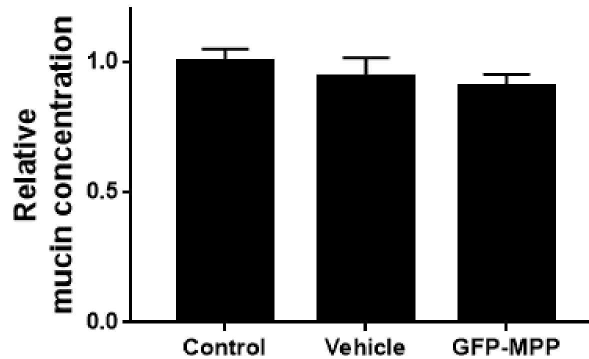

Figure 4.8. Quantification of O-linked mucin in the lungs of *Scnn1b*-Tg mice that were untreated, vehicle treated, and GFP-MPP treated.

4.3.5. Impact of hypertonic saline pre-treatment in vivo

Hypertonic saline (HS; 7% NaCl) is routinely used in clinic to improve pulmonary functions of CF patients by facilitating mucus removal from the lung via physiological mucociliary clearance mechanism [503]. We thus hypothesized that pre-treatments with HS would improve gene transfer efficacy of DNA-MPP due to the reduction in mucus burden. Consistent with a prior observation [504], a week regimen of intranasal HS (three times a day; 1μl/g of body weight) was highly efficient in removing mucus plugs in the lungs of *Scnn1b*-Tg mice (Figure 4.6E). We then confirmed that the HS pre-treatments further enhanced DNA-MPP-mediated transgene expression (i.e. luciferase activity) by over an order of magnitude (Figure 4.6G). Encouraged by this result, we investigated the combined therapeutic effects of HS pre-treatments followed by

a single dose of shENaC-MPP on key pathology in the lungs of *Scnn1b*-Tg mice, including muco-obstruction and inflammation. A modest reduction in the mucin concentration was observed in the lungs of *Scnn1b*-Tg mice received a single dose of shENaC-MPP. However, a week regimen of intranasal HS was more effective in alleviating the mucus burden and subsequent treatment with shENaC-MPP did not lead to further reduction in the mucin concentration (Figure 4.6H). We note that unlike the earlier study (Figure 4.6D), we quantified the mucin concentration in the whole BAL fluid rather than in mucus plugs due to their absence in the lungs of HS-treated mice. Interestingly, the total cell count in BAL fluid was significantly reduced in the lungs of mice that received sequential HS pre-treatments and shENaC-MPP, whereas either treatment alone failed to do so (Figure 4.6I).

4.3.6. Apical transfection of well-differentiated primary CF HBE grown on ALI

To evaluate the relevance of DNA-MPP to human muco-obstructive lung airways, we next established ALI cultures with human primary CF bronchial epithelial cells harvested from an F508del homozygous CF patient. After the tightly sealed monolayer and continuous mucus secretion were confirmed, we treated the ALI cultures apically with either DNA-CP or DNA-MPP carrying GFP-encoding plasmids. Of note, each well received a very small volume (0.1 mg/ml plasmids in 8 μl) to minimize dilution of the apical mucus gel layer [505]. Confocal microscopy revealed that while the transgene expression mediated by DNA-CP was negligible (Figure 4.9A), relatively widespread GFP signal was observed in the well that received DNA-MPP (Figure 4.9B). Quantitatively, DNA-MPP provided marked greater level (i.e. GFP intensity; Figure 4.9C) and coverage (Figure 4.9D) of transgene expression compared to DNA-CP.

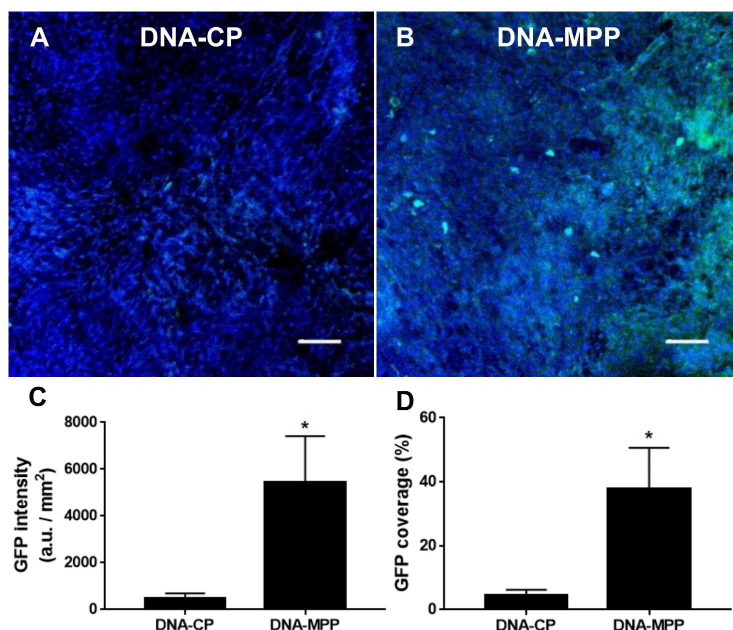

Figure 4.7 *In vitro* transgene expression in ALI cultures of primary CF HBE cells. Representative confocal images of GFP transgene expression mediated by (A) DNA-CP or (B) DNA-MPP. Scale bar = 100μm. Image-based quantification of (C) level and (D) coverage of GFP transgene expression mediated by DNA-CP versus DNA-MPP. Data represent mean ± S.D. Differences are statistically significant as indicated (*$p < 0.05$; Student's *t*-test).

4.3.7. Ex vivo and in vivo characterization of other PBAE-based MPP formulations

As an exploration, we engineered a library of eight DNA-MPP formulations, including the DNA-MPP formulation used in this study, that share the same PEG-conjugated PBAE but different PBAE, in order to elucidate the impact of subtle chemical modifications to colloidal stability, diffusivity in mucus, and gene transfer efficiency. We found that all eight MPP formulations, albeit presence of slight variation, exhibited virtually identical hydrodynamic diameter, ζ-potential, and polydispersity index (PDI) (Table 4.2). However, when their colloidal stability was tested in BALF over the period of four hours, some formulations were more stable than others (Figure 4.10). We next evaluated their *ex vivo* mucus-penetrability and *in vivo* gene transfer efficiency. Due to technical feasibility and the finite resource of CF sputum and *Scnn1b*-Tg mice, we elected to test four formulations – P1C1, P1C2, P1C5, and P1C8 – based off their varying

stability in BALF. Interestingly, we did not find any statistically significant difference in mucus-penetrability and gene transfer efficiency between the four formulations (Figure 4.11).

Table 4.2. Physicochemical properties of various MPP formulations

Core polymer	Hydrodynamic diameter (nm)	PDI	ζ- potential (mV)
P1C1	49 ± 0.5	0.07	2.11
P1C2	51 ± 2.5	0.10	0.90
P1C3	61 ± 1.4	0.09	3.61
P1C4	50 ± 0.4	0.12	2.23
P1C5	55 ± 7.1	0.08	1.65
P1C6	60 ± 1.2	0.12	1.12
P1C7	55 ± 6.5	0.08	1.28
P1C8	50 ± 4.3	0.08	0.93

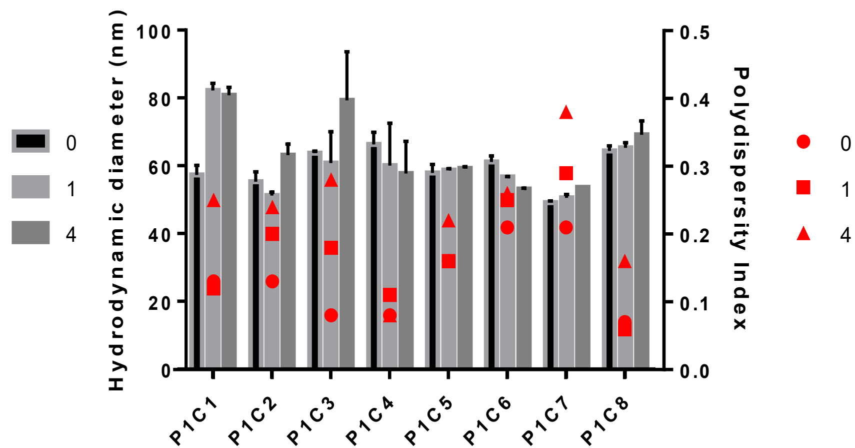

Figure 4.8. Stability of various DNA-MPP formulations in BAL fluid over time course of four hours

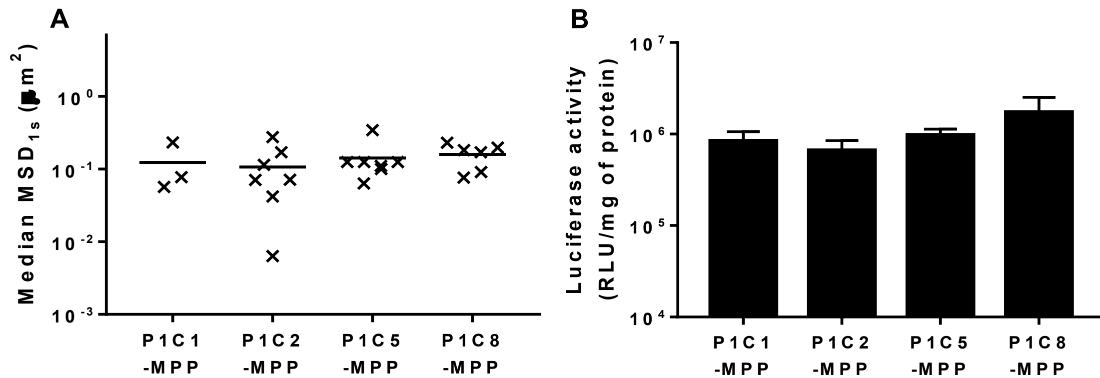

Figure 4.9. *Ex vivo* and *in vivo* validation of four MPP formulations. (A) Dot plot of median of MSD of DNA-MPPs at a timescale of 1 s in individual human CF sputum samples. (B) Overall level of in vivo transgene expression mediated by single administration of various DNA-MPPs in the lungs of *Scnn1b*-Tg mice. Data represent mean ± S.E.M. One-way ANOVA.

4.4. Discussion

We here demonstrate that a synthetic gene delivery platform uniquely designed to efficiently penetrate the airway mucus gel layer (i.e. DNA-MPP) provides widespread reporter transgene expression throughout the lungs of transgenic mice emulating muco-obstructive lung diseases (i.e. *Scnn1b*-Tg). Our image-based blinded analysis revealed that DNA-MPP provided at least 30% airway coverage of transgene expression on average in the lungs of *Scnn1b*-Tg mice and ALI cultures of primary human CF bronchial epithelium. In contrast, an otherwise identical mucus-impermeable formulation (i.e. DNA-CP) exhibited less than 10% coverages in these highly relevant CF airway models. Encouragingly, it has been demonstrated using an *in vitro* model of human primary CF ciliated airway epithelium that functional CFTR protein production in 25% of cells restore normal mucus transport rates [37]. We acknowledge that a next step in the line of

this study would be confirmation of the findings using a more quantitative method, such as flow cytometry.

While the widespread coverage resulted in a high-level transgene expression, DNA-MPP exhibited about an order of magnitude or more lower levels in the lungs of *Scnn1b*-Tg mice compared to an AAV serotype that we previously confirmed for efficient mucus penetration (i.e. AAV6; [505]). However, we found in this study that two doses of DNA-MPP markedly enhanced the level of transgene expression compared to a single administration, underscoring that our formulation did not elicit therapy-inactivating immunogenicity inevitably observed for virus-based gene vectors [506]. To this end, DNA-MPP may provide transgene expression levels *on par* or greater than mucus-penetrating viral vectors by multiple doses. In a previous study, we established a library of DNA-MPP formulations consisting of various core polymer structures but sharing the same PEG-conjugated polymer [488]. Through our well-established *ex vivo* multiple particle tracking in CF sputum [206] and *in vivo* transfection of *Scnn1b*-Tg lungs, we found that four distinct DNA-MPP formulations exhibit similar degree of efficient penetration of CF sputum as well as robust transgene expression (Figure 4.11), presumably due to the same PEGylated surface in all four MPP formulations. Flow cytometric analysis or DNA barcode method [507] to elucidate specific cell types transfected by the four formulations may reveal differences in cell tropism and provide versatile strategies for treatment of muco-obstructive lung diseases.

We have previously shown the DNA-MPP does not trigger an acute inflammatory response in the lungs of healthy mice [488], but it is critical to retain the safety in diseased lungs. We thus demonstrate here that DNA-MPP does not exacerbate chronic

inflammation readily established in the CF/COPD-like lungs of *Scnn1b*-Tg mice. This finding suggests that DNA-MPP would be safe even in the immunologically vulnerable lung environment inherent to muco-obstructive lung diseases.

We discovered that a single dose of DNA-MPP carrying plasmids encoding shRNA against ENaC (i.e. shENaC-MPP) markedly reduced the level of ENaC protein production in the lungs of *Scnn1b*-Tg mice, and the effect was further pronounced when an additional dose was given. Importantly, the mucus burden was significantly reduced by the shENaC-MPP-mediated downregulation of ENaC presumably due to airway rehydration followed by partial restoration of MCC. In support of the scenario, it has been recently reported that three times daily intranasal administration of ENaC-inhibitory peptides significantly increases the ASL height (i.e. airway rehydration) in the airways of neonatal *Scnn1b*-Tg mice [508]. Likewise, ENaC antagonists have been shown to restore and/or increase the ASL height in ALI cultures of human CF bronchial epithelial cells [508, 509]. Of note, Zhou et al. demonstrated that a small molecule-based ENaC inhibitor, amiloride, given at an identical dosing schedule applied to the aforementioned peptides prevented mucus obstruction in the airways of neonatal *Scnn1b*-Tg mice, but therapeutic reversion was not achieved with adult mice [493]. In contrast, we report here a moderate but significant mitigation of mucus plugging in the lungs of *Scnn1b*-Tg mice treated with shENaC-MPP at the ages of 4 weeks when muco-obstructive phenotype is fully established [472, 496].

We report that pre-treatment with HS markedly increases the overall level of transgene expression mediated by the subsequently administered DNA-MPP. Of note, an appreciable amount of DNA-MPP, despite the excellent mucus-penetrating ability [488],

was found within the mucus plugs in the airways of *Scnn1b*-Tg mice that did not receive the pre-treatment. Thus, the further enhancement of transgene expression is most likely attributed to the significant reduction of the particle-trapping mucus plugs via the pre-treatment as evidenced by our histological analysis and a prior report [504]. We found that the airway-rehydrating HS alone failed to ameliorate chronic inflammation inherent to the model, in agreement with prior observations with both *Scnn1b*-Tg mice [504] and CF patients [503]. Further, even a remarkable ENaC downregulation achieved by shENaC-MPP was insufficient in altering the inflammatory profile. However, a combinatory regimen of HS and shENaC-MPP roughly halved the inflammatory burden in the lungs of *Scnn1b*-Tg mice. The findings here suggest that a threshold of ENaC downregulation required for airway rehydration relevant to intervening the chronic inflammation may exist at least in this model. Of note, a synergistic effect of HS and ENaC inhibition on airway rehydration, but not chronic inflammation, has been previously reported using a preclinical model [510].

Success of gene therapy for muco-obstructive lung diseases such as CF has been ever-elusive. Excellent pre-clinical results from numerous viral and non-viral gene vectors have not translated well to the clinics. As mentioned earlier, the disconnect between pre-clinical and clinical studies may be attributed to the lack of animal models that accurately depict the pathobiology. Consequently, the gold standard for testing the effectiveness of a gene vector for muco-obstructive lung diseases has been the use of primary CF HBE grown at ALI, where a gene vector may experience pathological barriers such as mucus, ciliated cells, tight epithelial junctions, and other intracellular barriers. There has been reports of *in vitro* non-viral gene transfer on non-differentiated

airway epithelial cells [511, 512] or normal HBE [513, 514] grown on ALI, or with the use of active gene transfer techniques such as electroporation [515] to address the muco-obstructive lung disease phenotypes exhibited *in vitro*. While these studies met their respective endpoints and have added great value to the field, the barriers to gene therapy of muco-obstructive lung diseases were either bypassed or not adequately addressed. Similarly, *in vitro* studies of viral gene vectors have been conducted on primary cells grown at ALI with apical mucin concentration that is lower than naturally or pathologically secreted airway mucus, or with a large volume of viruses that may dilute and alter the barrier properties of apically secreted mucus [109, 112, 481, 482, 505]. It should be noted that careful recapitulation of biological barriers in both *in vitro* and *in vivo* settings should be considered in order to assess the clinical applicability and relevance of gene transfer agents. Importantly, we found here that apically administered DNA-MPP is capable of mediating widespread transgene expression in mucus-covered ALI cultures of primary CF HBE cells compared to DNA-CP, which is consistent with our observations from *in vivo* studies with the *Scnn1b*-Tg mouse model. From our pilot study of delivering GFP-encoding plasmid via DNA-MPP, we learned that DNA-MPP is capable of apically transfecting fully differentiated primary CF HBE, an unprecedented result in the field of non-viral DNA delivery. Currently we are under optimization process in order to deliver a therapeutic gene (i.e. CFTR, shENaC). Moreover, delivery of other nucleic acid (i.e. mRNA, miRNA) as well as gene-editing components (i.e. CRISPR RNP) will be considered in future studies.

4.5. Conclusion

In this study, we engineered a novel synthetic gene carrier platform capable of penetrating the human CF sputum barrier as well as the mucus barrier in the lungs of a mouse model of muco-obstructive lung diseases. We found that DNA-MPP, compared to mucus-impenetrable DNA-CP, achieves more widespread and uniform distribution in muco-obstructed mouse lungs, and provides higher transgene expression. We found that DNA-MPP is capable of delivering therapeutically relevant gene to this mouse model and assert phenotypic change. we also show that this effect is amplified when the mice are pre-treated with hypertonic saline, a common adjuvant given in the clinic. Lastly, we show that DNA-MPP is capable of apically transfecting mucus-covered primary bronchial epithelial cells harvested from a CF patient.

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Curriculum Vitae

Namho Kim

EDUCATION

Johns Hopkins University Whiting School of Engineering/School of Medicine
Baltimore, MD
Center for Nanomedicine at Wilmer Eye Institute
Ph.D., Chemical and Biomolecular Engineering

Purdue University Weldon School of Biomedical Engineering
West Lafayette, IN
M.S., Biomedical Engineering. 2010

Lehigh University P.C. Rossin College of Engineering
Bethlehem, PA
B.S., Bioengineering. 2008

BUSINESS EXPERIENCE

9th Annual Johns Hopkins Healthcare Case Competition (1st Place out of 28 teams). 2019

Johns Hopkins University | Sponsored by 3M

- Synthesized implementable, data-driven, cost-effective recommendation that will benefit the entire healthcare value chain by reducing surgical site infection
- Presented to 26 judges composed of top 3M executives and consultants from leading global consulting firms (BCG, LEK, Putnam, ClearView, and more)

VentureLabs Summer Fellow/Consultant. 2018

Flagship Pioneering**,** Cambridge, MA

- Selected from over 400 applicants for the coveted fellowship program at the leading life science venture capital firm
- Directly participated in development of first-in-class life science ventures
- Generated over 30 venture hypotheses, directly reported to the CEO and partners
- Post fellowship: Served as a consultant for a portfolio company by running extensive competitive intelligence

Humanitarian Engineering Hackathon: (1st place out of 8 teams). 2018

Center for Bioengineering Innovation & Design (Johns Hopkins), Baltimore, MD

- Inaugural joint US-Middle East project: collaborated with American University of Beirut students to design a solution to aid health needs of Lebanon refugee camps; prototyped an eco-friendly, resources-efficient kit to enhance tent insulation
- Currently pursuing humanitarian grants to further advance the prototype development

Biotech Equity Research Analyst Extern, 2018

Biotech Investment Group, Johns Hopkins University, Baltimore, MD

- Perform fundamental analysis on biotechnology companies; evaluate drug pipeline and IP, conduct industry diligence, and analyze scientific literature and clinical data
- Develop investment recommendation and present stock pitch to T. Rowe Price healthcare executives at externship conclusion

Business Development/Researcher BioActs. 2010 – 2012

BioActs (Leading Korean Fluorescent Probes R&D Company), Incheon, South Korea

- Directly supported global business and strategic initiatives: secured distribution deals and expanded R&D partnerships
- Directly contributed to the development of novel fluorescent probes for cell apoptosis detection (patented)
- Served as company delegate at international conventions and conferences to promote BioAct's visibility and presence in international markets
- Prepared patent applications and actively contributed to research publications

Biomedical Entrepreneurship. 2010

Purdue University**,** West Lafayette, IN

- Formulated an original product concept for a presbyopia topical treatment; assessed market opportunity and analyzed competitive drug/treatment landscape
- Conceptualized an eye drop to absorb UV rays to prevent the onset/decelerate the progression of presbyopia
- Presented product proposal to biotech industry executives and received honorable mention

Product Development Team Leader | Sponsored by B. Braun Medical. 2007

Lehigh University, Bethlehem, PA

- Led a team to develop a sharps containment device for an epidural anesthesia kit with enhanced design and material to improve patient/doctor safety; consulted with local doctors/hospitals to assess unmet medical needs and conducted market research
- Developed proprietary 3D CAD prototype and presented product business plan to B. Braun Medical

LEADERSHIP EXPERIENCE

Team leader | Johns Hopkins Fall Case Competition**.** 2018

- Formed a team to compete in the annual Case Competition and advanced to the final round – conducted market analysis and developed a value-based pricing strategy for a medical device company | Actively participate in the club events to become familiar with consulting and expand network

Mentor | Center for Nanomedicine at Johns Hopkins School of Medicine**.** 2014 – 2019

- Advise and mentor 2 post-Baccalaureate and 3 undergraduate students for MD and PhD programs (mentees have successfully transitioned to medical/graduate schools) | TA for undergraduate lab class

Philanthropic work – Track record of 2 successful fundraisers in 2 countries

Team Leader | Great Strides for Cystic Fibrosis. 2014 – 2019

- Started and lead a fundraising team for the annual Great Strides walk-a-thon held by the Cystic Fibrosis Foundation | Single-handedly raised over $6,000 to date | Recognized as one of top fundraising groups in the US

Chair of Organizing Committee | Dream Field Charity Gala. 2014

- Assembled a team and organized a charity gala to help build a safe soccer field for children with blindness and other disabilities | Acquired 8 sponsors | Raised over $10,000 in 2 months | The field is now functionally operating in Seoul

Republic of Korea Army Reserve

- Fulfilled military obligations 2010-2013, and have been serving as ROK Army Reserve

RESEARCH EXPERIENCE

Johns Hopkins University, Baltimore, MD. 2014 – 2019

Graduate Researcher | Research Focus: Gene Delivery | Advisor: Prof. Justin Hanes

- Directly contribute to two NIH grants ($2MM) for novel gene therapy of (i) cystic fibrosis and (ii) Parkinson's disease
- Conceptualized idea and co-wrote successful grant proposal – 1-year $100K Focused Ultrasound Foundation grant for enhanced intra-urothelium delivery of therapeutics (patent application pending)
- Engineer non-viral polymeric nanocarriers for gene delivery across various biological barriers
- Execute multi-faceted screening of various adeno-associated viruses for obstructive lung diseases

Korea Institute of Science and Technology, Seoul, South Korea. 2012 – 2014

Research Scientist at Korea's Flagship Scientific Research Institute

- Developed method to synthesize long chains of gold nanoparticle and siRNA for photothermal gene therapy of prostate cancer mouse xenograft model; Authored 3 papers
- Led events for visiting scientists and guests

Purdue University, West Lafayette, IN. 2008 – 2010

Graduate Researcher | Research Focus: Atherosclerosis | Advisor: Prof. Kinam Park

- Designed and developed a co-culture model of human aortic endothelial cells and human aortic smooth muscles to mimic the native atherosclerotic arterial model
- Supervised and mentored three undergraduate students on their research
- Thesis: The Effects of Probucol, AICAR, and a MK2 Inhibitor on Human Vascular Cells and Their Possible Implementation on Drug Eluting Stents; authored 1 paper in collaboration with Yale School of Medicine

Lehigh University, Bethlehem, PA. 2007 – 2008

Undergraduate Research Assistant | Research Focus: Gap Junctions

- Modified/engineered photoactivatable fluorescent proteins to track gap junction activity and internalization process; published research work – Advisor: Prof. Matthias Falk

AWARDS

1. Highest-rated abstract in respiratory engineering, Biomedical Engineering Society Annual Meeting, 2018, Atlanta, GA
2. Meritorious Abstract Travel Award, ASGCT 20th Annual Meeting, 2017, Washington DC
3. Nature BME Best Poster Award, NanoDDS'16, Baltimore, MD
4. Johns Hopkins University Graduate Representative Organization Travel Grant

PUBLICATIONS ([1]Co-First Author)

1. **N. Kim**, J. Rodriguez, A. Livraghi-Butrico, S. Shenoy, M. Mazur, S. Birket, S.M. Rowe, R.C. Boucher, J. Hanes, J.S. Suk, Efficient non-viral gene delivery in a mouse model of muco-obstructive lung diseases. In preparation
2. Y.C. Kim, H. Hsueh, **N. Kim**, …J. Hanes, J.S. Suk. Strategy to enhance dendritic cell-mediated DNA vaccination in the lung, Under review
3. B.P. Mead, C.T. Curley, **N. Kim**, … , J.S. Suk, R.J. Price, Augmentation of Brain Tumor Interstitial Flow via Focused Ultrasound Promotes Brain-Penetrating Nanoparticle Dispersion and Transfection, Under revision
4. A. Lopes da Silva, G.P. de Oliveira, **N. Kim**, … , J.S. Suk, M.M. Morales, Nanoparticle-based thymulin gene therapy therapeutically reverses key pathology of experimental allergic asthma, Under review
5. B.P. Mead, C.T. Curley, **N. Kim**, K. Negron, W.J. Garrison, J. Song, G.W. Miller, J.W. Mandell, B.W. Purow, J.S. Suk, J. Hanes, R.J. Price, Focused Ultrasound Pre-Conditioning for Augmented Nanoparticle Penetration and Efficacy in the Central Nervous System. *Small*, 2019, In press
6. S.J. Miller, T. Philips, **N. Kim,** R. Dastgheyb, Z. Chen, J.G. Daigle, M. Datta, E.G. Hughes, J.T. Pham, M.B. Robinson, R. Sattler, R. Tomer, J.S. Suk, D.E. Bergles, J. Hanes, J.D. Rothstein, Molecularly defined cortical astroglia subpopulation modulates neurons via secretion of Norrin. *Nature Neuroscience*, 2019, 22 (5), 741
7. **N. Kim**[1], G.A. Duncan[1], Y. Colon-Cortes, J. Rodriguez, M. Mazur, S.M. Rowe, N.E. West, A. Livraghi-Butrico, R.C. Boucher, J. Hanes, G. Aslanidi, J.S. Suk, Adeno-associated virus serotype 6 overcomes the mucosal barrier to inhaled lung gene therapy. *Molecular Therapy – Methods & Clinical Development*, 2018, 9, 296-304

8. G.Osman, J. Rodriguez, S. Chan, J. Chisholm, G. Duncan, **N. Kim**, A. Tatler, K. Shakesheff, J. Hanes, J.S. Suk, J.E. Dixon, PEGylated enhanced cell penetrating peptide nanoparticles for lung gene therapy, *J. Controlled Release*, 2018, 285, 35-45
9. B.P. Mead, **N. Kim,** G.W. Miller, D. Hodges, P. Mastorakos, A.L. Klibanov, J.W. Mandell, J. Hirsh, J.S. Suk, J. Hanes, R. Price, Novel focused ultrasound gene therapy approach non-invasively restores dopaminergic neuron function in a rat Parkinson's disease model, *Nano Letters*, 2017**,** 17 (6), 3533-42
10. **N. Kim**, G.A. Duncan, J. Hanes, J.S. Suk, Barriers to Inhaled Gene Therapy of Obstructive Lung Diseases: A Review, *J. Controlled Release*, 2016, 240, 465-488
11. J.S. Suk, Q. Xu, **N. Kim**, J. Hanes, L.M. Ensign, PEGylation as a Strategy for Improving Nanoparticle-based Drug and Gene Delivery, *Advanced Drug Delivery Reviews*, **2015**, 99, 28-51
12. **N. Kim[1],** S. Son[1], D.G. You, H.Y. Yoon, J.Y. Yhee, K. Kim, I.C. Kwon, S.H. Kim, Antitumor Therapeutic Application of Self-Assembled RNAi-AuNP Nanoconstructs: Combination of VEGF-RNAi and Photothermal Ablation, *Theranostics*, 2017, 7 (1), 9-22
13. H.K. Han, S. Son, S. Son, **N. Kim**, J.Y. Yhee, J.H. Lee, J.S. Choi, C.K. Joo, H.H. Lee, W.J. Kim, S.H. Kim, I.C. Kwon, H.C. Kim, K. Kim, Reducible Polyethylenimine Nanoparticles for Efficient siRNA Delivery in Corneal Neovascularization Therapy (**Front Cover**), *Macromolecular Bioscience,* 2016, 16, 1583-1597
14. J.Y. Yhee, S. Son, **N. Kim**, K. Choi, I.C. Kwon, Theranostic Application of Organic Nanoparticles for Cancer Treatment, *MRS Bulletin,* 2014, 39 (3), 239-249
15. Muto, A. Panitch, **N. Kim**, K. Park, P. Komalavilas, C.M. Brophy, A. Dardik, Inhibition of Mitogen Activated Protein Kinase Activated Protein Kinase II with MMI-0100 Reduces Intimal Hyperplasia ex vivo and in vivo, *Vascular Pharmacology,* 2012, 56, 47-55
16. S.M. Baker, **N. Kim**, A.M. Gumpert, D. Segretain, M.M. Falk, Acute Internalization of Gap Junctions in Vascular Endothelial Cells in Response to Inflammatory Mediator-induced G-Protein Coupled Receptor Activation, *FEBS Letters,* 2008, 582 (29), 4039-4046

PRESENTATIONS

1. DNA-loaded Mucus-penetrating Particles for Gene Therapy of Muco-obstructive Lung Diseases, BMES, Oct 17-20, 2018, Atlanta, GA
2. Mucus-penetrating Non-viral Gene Delivery Platform for Cystic Fibrosis, *NACFC*, Nov 2 – 4, 2017, Indianapolis, IN
3. Intersections of neuromodulation, focused ultrasound, and gene delivery with brain-penetrating nanoparticles, *The Journal of the Acoustical Society of America*, Oct 2017
4. Airway mucus minimally impedes AAV6 for inhaled lung gene therapy, *American Thoracic Society*, May 2017, Washington DC
5. Mucus-penetrating Non-viral Gene Delivery Platform for Obstructive Lung Diseases, *ASGCT 20th Annual Meeting*, May 10 – 13, 2017, Washington DC
6. Targeted Delivery of Brain-Penetrating Non-Viral GDNF Gene Vectors to the Striatum with MRI-Guided Focused Ultrasound Reverses Neurodegeneration in a Parkinson's Disease Model, *NanoDDS'16*, Sept. 16 – Sept. 18, 2016, Baltimore, MD
7. Fluorescent Probes for Biological Research, *Hangzhou International Molecular Imaging Conference*, Sept 24, 2011 – Sept 25, 2011, Hangzhou, China
8. Novel Peptide-based Fluorescent Probes for Apoptosis Analysis, *World Molecular Imaging Congress*, Sept 7, 2011 – Sept 10, 2011, San Diego, CA
9. ApoFlamma^{TM}, Novel Fluorescent Probes for in vivo and in vitro Apoptosis Detection, *Controlled Release Society*, July 30, 2011 – Aug 3, 2011, National Harbor, MD

10. Novel Cyanine Dyes for in vivo and in vitro Research, *World Molecular Imaging Congress*, Sept 8, 2010 – Sept 10, 2010, Kyoto, Japan
11. Introduction of BioActs, *BIO Convention*, May 3, 2010 – May 7, 2010, Chicago, IL
12. Effect of Probucol, AICAR, and TGF-β1 on Smooth Muscle Cell and Endothelial Cell Phenotype and Proliferation, *NanoDDS'09*, Oct. 5 – Oct. 6, 2009, Indianapolis, IN

SKILLS & INTERESTS

Language: Korean and English (native), Spanish (beginner)
Interests: Johns Hopkins PE & VC Club/Graduate Consulting Club, Hopkins Biotech Network, American Society of Gene and Cell Therapy, Biomedical Engineering Society, golf, skiing, food enthusiast
Other: Korea National Men's Lacrosse Team at 2004 ASPAC Lacrosse Championship (starting goalie, 2-time winner of Player of the Game medal), Theta Xi Fraternity

PATENTS

- Methods for Labeling Biomolecules with Vinylsulfone Dye Compounds, 2011, Korea Patent 10-2011-0079332