Overcoming Challenges to Development of a Lentiviral-Mediated Airway Gene-Addition Therapy for Cystic Fibrosis

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Abstract

Cystic fibrosis (CF) is the most common genetic disorder in the developed world and is caused by defects in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) channel. Lung disease is the leading cause of illness and premature death among CF patients, therefore developing efficacious airway treatments will undoubtedly improve life expectancy and quality. While highly effective CFTR modulator drugs are now available for the majority of CF patients, recent estimates suggest that up to 30% of patients are unable to use modulator therapies. Thousands of patients are being left behind and alternative treatments are needed for these individuals.

Addition of a functioning *CFTR* gene into the airway cells is one strategy for preventing or treating CF lung disease in all patients. This concept, known as airway gene-addition therapy, aims to restore CFTR-mediated ion transport in the lungs thus ameliorating the disease phenotypes. This work employed use of a lentiviral (LV) vector as the airway gene delivery vehicle.

While there has been significant progress towards bringing a CF airway gene therapy to the clinic, considerable challenges remain. Challenges include, but are not limited to, (1) producing sufficient quantities of gene vector for human clinical use, (2) developing and using appropriate test models to assess airway gene therapies to obtain translational outcomes, and (3) overcoming inherent barriers of the lung to achieve efficient gene transfer. Solutions to these challenges were addressed in this dissertation.

To achieve therapeutic levels of gene correction, *in vivo*-directed airway gene transfer in humans is likely to require large quantities of LV vector. Current methods of LV vector production lack scalability and cannot sustain clinical demands. To address this unmet need for large-scale LV production, two LV production methods were developed. One approach employed cell factories and is suited to producing high titre LV vector for preclinical studies. The second method is a novel approach that used a packed-bed bioreactor system, where concentrated titres of up to 10^9 TU/mL were achieved. This packed-bed system provides capabilities for scalable LV production and is amenable to use in large-scale manufacturing settings.

Limited availability of CF animal models in Australia has impeded progress in trialling airway gene therapies. Consequently, two new CF rat models were generated using CRISPR/Cas9 gene editing. One rat model harbours the common Phe508del mutation, the first mutation-specific rat model to be generated worldwide, while the second is a *CFTR* knockout strain. Characterisation studies revealed a range of CF phenotypes present in both rat models, however, spontaneous lung disease was not observed. The availability of these new CF rats provides an alternative model for testing airway gene-addition therapies.

Efficient delivery of the gene vector to the epithelial cells remains one of the greatest hurdles to developing an airway gene-addition therapy. Chemical and physical epithelial perturbation methods were investigated as a means to enhance gene transfer in rat airways. Chemical conditioning with lysophosphatidylcholine significantly improved lung reporter gene expression levels when assessed 1-week following gene transfer but did not enhance the longevity of expression. Use of physical perturbation in conjunction with LV-mediated gene transfer significantly increased airway gene transduction, indicating the promising potential of this strategy.

While the outcomes presented here show considerable progress, developing an effective airway gene-addition therapy remains challenging feat. However, with growing investment and innovation in the gene therapy sector, and a healthcare paradigm shift towards the adoption of personalised medicines, the development of a safe and effective airway gene-addition therapy for CF patients is now closer than ever before.

Declaration

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Works arising during candidature

Peer reviewed publications

*Denotation with a * indicates the publications that are included in this thesis*

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Chapter 1: Introduction

1.1. Cystic fibrosis

Cystic fibrosis (CF) is a life-shortening disease that significantly affects patient quality of life. Currently 70,000 individuals worldwide are affected by CF, with approximately 3,400 patients residing in Australia [1, 2]. CF has an autosomal recessive mode of inheritance and predominantly affects Caucasians, with other ethnic populations affected at lower rates. Among Caucasian populations the carrier frequency is 1 in 25, and one birth in every 2,500 – 3,500 will result in CF [3]. Continuing improvement in treatments has significantly increased the median predicted survival age of CF patients, which is now in the fifties [4].

CF is caused by mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein. In epithelial cells, the CFTR protein acts as an ion channel that is responsible for cyclic-AMP-dependent chloride and bicarbonate secretion, as well as the regulation of epithelial sodium channels (ENaCs) [5]. CF differs from other genetic disorders in that a large number of mutations (>2000) have been identified, though not all are disease-causing [6]. This diversity in *CFTR* mutations means the clinical phenotype varies significantly in terms of the symptoms and their severity [7]. Accordingly, *CFTR* mutations are generally categorised into six classes based on the resultant cellular phenotype. Classes I to III tend to be more severe mutations that result in little to no CFTR expression or function, while classes IV to VI are typically milder with the CFTR protein retaining some function [8]. The most common CF mutation is the class II mutation Phe508del, with approximately 90% of patients carrying at least one copy [9].

1.2. Disease manifestations

The *CFTR* gene is expressed in epithelial cells throughout the entire body, and as a result the disease presents in a diverse range of organs. The gastrointestinal (GI) tract, lungs, liver, pancreas and reproductive organs are all affected. Table 1 outlines the major disease manifestations of CF and the current symptomatic treatment options available. The lung disease aspects will be discussed in more detail later in this chapter.

Table 1: Summary of the major manifestations of CF clinical syndrome and available symptomatic treatments (excluding lung-related presentations).

Organ	Manifestation	Symptomatic treatment(s)	References
Exocrine pancreas	Exocrine pancreatic insufficiency (85% of newborns)	Pancreatic enzyme replacement therapy; diet supplementation with fat-soluble vitamins	[10]
Endocrine pancreas	Insulin deficiency; CF-related diabetes (40 - 50% of adult patients)	Insulin; high-fat diet	[11]
Liver	CF-associated liver disease (30% of patients)	Ursodeoxycholic acid; end stage disease requires liver transplantation	[12, 13]
Gastrointestinal tract	Gastro-oesophageal reflux	Proton-pump inhibitors	[14]
	Meconium ileus (20% of patients)	Gastrografin enema; surgery	[15]
	Distal intestinal obstruction syndrome (DIOS); constipation	Oral laxatives; enema; polyethylene glycol lavage	[16]
Rectum	Rectal prolapse	Manage pancreatic enzymes; rarely surgery	[17]
Upper airway	Polyps; sinusitis	Topical steroids; antibiotics; surgery	[18]
Bones	Osteopenia	Prevention with regular exercise, vitamin D supplements and diet management	[19]
Male reproductive tract	Infertility due to bilateral absence of vas deferens	Assisted reproductive technology	[20]

1.2.1. Airway disease pathophysiology

The pathophysiological processes underlying CF airway disease are complicated and have long been subject to debate. The most widely accepted theory with a large body of supporting evidence is known as the “low airway surface liquid (ASL) volume” hypothesis [21-23] (Figure 1). In CF airway epithelia, chloride transport via CFTR is reduced and sodium absorption is

increased due to an absence of CFTR-mediated inhibition of ENaC. The low volume hypothesis postulates that this ion imbalance diminishes fluid secretion across the epithelia and in turn, reduces the ASL volume [24]. The ASL is comprised of two layers, a mucus layer and beneath it, the periciliary liquid (PCL) layer. Water is depleted from both layers causing collapse of the PCL and dehydrated mucus that subsequently adheres to the airways [25].

A shallow PCL prevents normal ciliary motion, therefore mucociliary clearance (MCC) and cough-clearance are reduced [24]. Thick airway mucus plaques are impenetrable to neutrophils, and this in combination with compromised innate defences creates an environment that is ideal for colonisation by opportunistic bacteria [26]. As lung disease progresses, persistent biofilms form and more atypical and problematic species including *Pseudomonas aeruginosa* colonise the airways [24, 25, 27]. Ongoing cycles of chronic infection and neutrophilic inflammation lead to mucus plugging, airway obstruction, permanent destruction of the respiratory tissues, bronchiectasis, respiratory failure and ultimately, death [24].

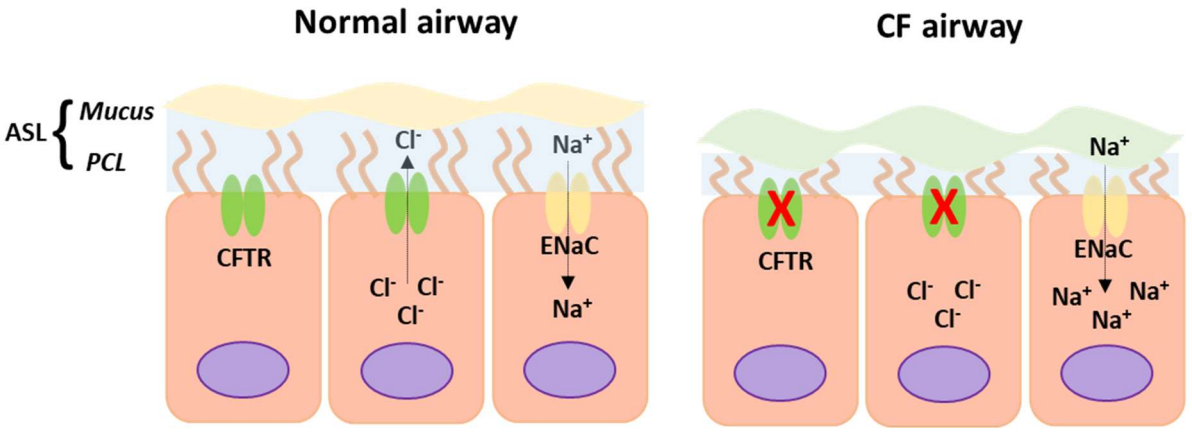

Figure 1: Schematic representation of the low ASL volume hypothesis. In normal airway epithelia (left) there is regulated chloride (CFTR) and sodium (ENaC) transport that maintains optimal ASL height and normal ciliary movement. In the CF airway epithelium (right), CFTR-mediated chloride secretion is reduced and there is hyperabsorption of sodium ions via ENaCs. Water is depleted from the ASL causing collapse of the PCL, mucus dehydration, and diminished MCC, thus creating a lung environment that is susceptible to infection by opportunistic pathogens.

The "low ASL pH" hypothesis has also recently gained traction with supporting evidence from CF pig and rat models [28, 29]. This theory proposes that impaired CFTR-dependent bicarbonate secretion leads to reduced airway surface pH, impaired antimicrobial function of the ASL, and decreased bacterial killing. CF pig secretions appear to have reduced bacterial killing, which can be restored by raising the ASL pH with sodium bicarbonate [28]. Similarly, *CFTR* knockout (KO) rats demonstrate a hyperacidic airway [29]. Interestingly however, a

recent study in humans revealed that ASL pH in children with CF is similar to those without CF, suggesting that low ASL pH does not drive early disease progression in humans. These contradictory findings may be explained by inherent physiological differences between species [30].

1.2.2. Symptomatic airway therapies

Progressive lung disease is the leading cause of morbidity and mortality among CF patients [1, 24], so researchers and clinicians have focused on developing therapies that address the airway disease symptoms. One treatment approach is to restore airway surface hydration and mucus clearance using inhaled hypertonic saline. This compound deposits onto the airway surface and creates an osmotic gradient that draws water into the ASL. Mucus clearance can also be improved by using mucolytics such as Dornase alfa (recombinant human DNase I). Mucus contains very high concentrations of extracellular DNA that is released by degrading neutrophils that accumulate in the airways in response to infection. Dornase alfa cleaves the free DNA in sputum thereby reducing its viscoelasticity and improving airway clearance [31]. These aerosolised medications are often used in conjunction with chest-physiotherapy to aid physical sputum removal from the lungs. Antibiotics remain central to the treatment of CF lung disease and are used in a range of contexts including prophylaxis, eradication of early infection, suppression of chronic infection, or treatment of exacerbation. Combinations of oral, inhaled, and intravenous antibiotics are used depending on the circumstances [7, 32, 33]. When respiratory failure is imminent, lung transplantation can be used to extend lifespan. However, many patients die on the organ-transplant waiting list and not all will be successful, with a 70% post-transplant survival probability at the 5-year mark [7, 34].

1.2.3. CFTR modulators

A recent advancement to these symptomatic treatments is the introduction of small molecule drugs known as CFTR modulators, which act to correct the underlying molecular defect. Broadly, two classes of modulators exist, potentiators and correctors. Potentiators increase ion conductance by binding to the CFTR protein at the plasma membrane and increasing the open state probability of the channel, while correctors facilitate correct protein folding thereby increasing the amount of mature CFTR protein that is trafficked to the cell surface [32, 35].

1.2.3.1. Monotherapy

Ivacaftor (Kalydeco®) is a potentiator molecule that was the first CFTR modulator agent to reach clinical application. Ivacaftor improves CFTR-mediated chloride conductance in individuals with class III gating mutations (e.g. Gly551Asp), where CFTR protein is already present at the cell surface. Ivacaftor has demonstrated many clinical benefits including improved lung function (on average a 10% increase in predicted forced expiratory volume in 1 second or FEV_1), reduced pulmonary exacerbations, and improved overall health including weight gain. However, only a small proportion of CF patients (~5%) have *CFTR* mutations that respond to this medication, though it is possible that patients with certain class IV and V mutations may also benefit clinically from ivacaftor [32, 36].

1.2.3.2. Combination therapy

Double combination therapy lumacaftor/ivacaftor (Orkambi®) is designed to treat homozygous Phe508del patients by partially rescuing traffic of Phe508del-CFTR (lumacaftor) and increasing its function at the cell surface (ivacaftor). Orkambi® has been shown to improve lung function and reduce pulmonary exacerbations, though its effects appear modest when compared to ivacaftor used in patients with class III mutations. Ivacaftor/tezacaftor (Symdeko®/Symkevi®) has also been approved for clinical use in homozygous Phe508del patients and those with qualifying residual function mutations. Tezacaftor appears to be superior to lumacaftor, with fewer drug-drug interactions and reduced incidences of respiratory side-effects [35].

Recent phase III clinical trials with new triple combination therapy, ivacaftor/tezacaftor plus next-generation corrector elexacaftor (Trikafta®/Kaftrio®) have shown highly encouraging results. Robust clinical benefit was observed in patients homozygous for Phe508del and those heterozygous for the Phe508del mutation and a minimal-function variant. Previous generation CFTR modulators were ineffective in the latter group of patients [37, 38].

1.3. Emerging corrective strategies

Over the next few decades, it is likely that CFTR modulator therapies will significantly improve patient prognosis. While a majority of CF patients now have access to highly effective modulators, approximately 7% of individuals have extremely rare or unrescuable CFTR mutations (e.g. class I mutations) where little to no CFTR mRNA is produced and thus cannot be treated by these pharmacological rescue approaches [39]. There are also patients that

cannot tolerate available CFTR modulator drugs due to adverse side effects or drug-drug interactions, some patients in which these drugs provide no clinical benefit despite having amenable mutations, as well as individuals that do not have affordable access to these medications due to their location [40]. Factoring these considerations, recent estimates suggest up to 30% of CF patients are currently unable to receive modulator therapies. Alternative approaches are necessary for these patients remaining without modulators and an intensive search is now underway to develop novel corrective therapies to address this unmet need [32, 41].

For nonsense mutations, premature termination codon (PTC) read-through drugs are being explored. These medications induce ribosomal read-through of PTCs, thus increasing the presence of functional CFTR protein [42]. One of these drugs, ataluren (PTC124), underwent a phase III trial in CF patients. This study failed to meet its primary endpoints with no significant improvements in lung function or pulmonary exacerbations observed when compared to placebo [43]. Novel anticodon engineered transfer RNAs (ACE-tRNAs) is an emerging technology that is also being investigated for suppression of PTCs [44].

Antisense oligonucleotides are short, single-stranded nucleic acid molecules that can be used to repair abnormal *CFTR* mRNA or target transcripts for degradation, for example *ENaC* mRNA [45-47]. Another corrective strategy that is mutation agnostic is mRNA therapy, which involves delivering *CFTR* mRNA to the airway cells using a vector such as a lipid nanoparticle to increase CFTR protein levels [48]. Other strategies involve bypassing the CFTR channel altogether. These include stimulating alternate chloride channels or using inhibitors to reduce ENaC activity [49, 50].

With the advent of gene editing technologies such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), it may be possible to permanently correct *CFTR* mutations *in situ* [51]. Although this approach is still in the early development phase, gene edited CF patient-derived intestinal organoids have already shown restored CFTR function [52]. However, these “classic” CRISPR/Cas9-mediated homology-directed repair approaches tend to be inefficient and may introduce deleterious off-target double-stranded breaks in the DNA, thus reducing their clinical applicability. Such drawbacks can be overcome by using more recently developed Cas9 fusion proteins known as adenine and cytosine base editors. Adenine editors have already demonstrated efficient repair of *CFTR*

nonsense mutations in intestinal organoids with no off-target effects observed [53]. Despite the significant value of these gene editing technologies, it is important to note that any lung-targeted gene editing strategies are likely to face most of the same challenges as gene-addition therapy, as will be discussed below.

While these emerging corrective therapies are promising, many are in preclinical or early clinical phases. Success of these therapies is not guaranteed, and it could be many years before CF patients will be able to benefit from them.

1.4. Airway *CFTR* gene-addition therapy

Effective treatment of pulmonary disease will significantly improve the lives of those living with CF. One potential treatment option that is currently being pursued is airway gene-addition therapy. Gene-addition therapy involves delivering wild-type *CFTR* cDNA to the relevant airway epithelial cells, so that functioning CFTR protein is produced. The goal is to halt or prevent lung disease progression by producing therapeutic levels of CFTR protein. A vector is employed as the vehicle to deliver the *CFTR* gene to the airway cells [54]. Vectors can be non-viral or viral in origin, as will be discussed in further detail below.

Lung-targeted gene therapy has become one of the main contenders for airway disease treatment as it has many potential benefits over conventional medicines. Gene therapy is mutation-agnostic therefore it is suitable for treating lung disease in all patients, irrespective of their *CFTR* mutation type. Moreover, unlike conventional CF therapies that work to address the downstream effects of the disease, gene therapy corrects the disease at its source – the *CFTR* defective airway cells. If administered early in life, gene therapy has the potential to not only treat lung disease but prevent it from occurring altogether.

An understanding of the levels of CFTR restoration needed to ameliorate CF phenotypes is necessary to develop an effective airway-gene addition therapy. It has been suggested that moderate levels of *CFTR* mRNA expression will be sufficient to produce clinical benefit, and while research is advancing in this area, there is still uncertainty surrounding this concept. Heterozygotes carry one healthy *CFTR* allele and have approximately 50% wild-type *CFTR* expression levels [55]. Carriers do not exhibit CF clinical syndrome, indicating that restoration to 50% *CFTR* mRNA levels in the lungs would be considered curative [56]. Moreover, CF patients with mild mutations that retain low-level *CFTR* expression do not appear to suffer from lung disease, suggesting that as little as 10% of normal *CFTR* expression may provide

therapeutic benefit for the lungs [1]. Recent studies have shifted attention to not only the proportion of cells that will require correction, but the cell type. Until recently it was thought that ciliated cells provided a significant source of *CFTR* mRNA expression in the human airway epithelium, however, more recent studies employing single cell RNA sequencing indicate that ionocytes, secretory and basal cells are the major contributors. In order to more closely mimic physiological patterns of *CFTR* mRNA expression, it will be desirable to correct CFTR expression in the cell types that naturally express it [57].

1.4.1. Non-viral vectors

Numerous viral and non-viral vectors have been evaluated as vehicles to deliver *CFTR* cDNA to the lungs [58]. Non-viral vectors consist of two components: (1) the therapeutic DNA, and (2) the carrier molecule that binds to the DNA. The most frequently investigated formulations for CF airway gene therapy include cationic lipids and cationic polymers [1]. Non-viral vectors are an attractive option for CF gene therapy, as they are considered safer than viral vectors, can be easily produced in large quantities, do not have transgene packaging capacity restrictions, and allow for repeat administration due to low immunogenicity [59].

Nine early phase (I/IIa) clinical trials have been performed in CF patients using non-viral vectors, with a majority employing cationic lipid formulations [60-67]. For safety and ease of outcome measures, many of these early trials used the nose as a surrogate for the lung therefore clinical benefit could only be assessed in the nasal airways. Clinical studies that were performed in CF patient lungs revealed that non-viral formulations can induce modest inflammatory responses that result in transient gene expression [67]. This has been attributed to immunostimulatory unmethylated cytosine and guanine (CpG) dinucleotides that are present in bacterial plasmid DNA [67, 68]. Accordingly, CpG-free pDNA vectors have been developed that do not appear to elicit host inflammatory responses, leading to more sustained expression [69].

More recently, a phase IIb clinical trial was performed where liposome *CFTR*-cDNA complexes were administered monthly to CF patient airways. The treatment was well tolerated and significant albeit modest improvements in lung function were observed, with a 3.7% increase in predicted FEV_1 when compared to the placebo group at the 12-month follow-up [70]. Despite these encouraging results, when compared to viral vectors, non-viral vectors tend to be less efficient gene delivery vehicles and lack the ability to target specific cell types. Poor

gene transduction from non-viral vectors can be attributed to lower-level transport of cDNA from the cytoplasm to the cell nucleus [71]. Although clinical studies employing non-viral vectors have been essential and significant in their ability to demonstrate proof-of-principle for CF airway gene therapy, they highlight the need for more potent gene delivery vehicles.

1.4.2. Viral vectors

In general, viral vectors are more effective gene transfer vehicles as, unlike non-viral vectors, they have intrinsic mechanisms to overcome extra- and intra-cellular barriers, and can more efficiently enter the host cell and nucleus [71]. However, with improved efficiency also comes a higher risk profile including potential for adverse immune reactions, and for integrating vectors, the possibility of random integration disrupting normal genes, insertional mutagenesis, and activation of proto-oncogenes [72, 73]. Viral vectors are typically separated into two categories: DNA viruses and RNA viruses. These categories will be discussed below in the context of CF airway gene therapy.

1.4.2.1. DNA viruses: adenoviruses and adeno-associated viruses

Adenoviruses (AdVs) are non-enveloped, non-integrating viruses that were developed as early CF gene therapy viral vectors due to their natural tropism for the lung [74]. AdVs can transduce quiescent and proliferating cells, but following nucleus entry they remain episomal [75]. The first clinically tested viral vector for CF was an AdV-*CFTR* vector that corrected the chloride transport defect in the nasal epithelium of a small number of CF patients [76]. Since this initial proof-of-principle trial, a further nine AdV clinical trials have been performed in the nose and lungs of CF patients [77-85]. Combined, these early AdV clinical trials taught us: (1) *CFTR* mRNA and protein can be detected in some CF patients following AdV-mediated gene transfer, (2) the chloride transport defect can be partially corrected in the nasal epithelium of CF patients, (3) AdV receptors are difficult to access as they are only present on the basolateral membrane of human airway epithelium, and (4) AdVs provoke immune responses that reduce the efficacy of repeat administration [86].

More recently, a new generation of helper dependent (HD) AdVs were developed. These vectors are devoid of all coding viral genes, considerably reducing their immunogenicity and improving long-term transgene expression however, they are yet to be tested clinically for CF [75]. Although AdVs and HD-AdVs are still being investigated preclinically, for example in pig airways [87, 88], the field is now favouring other types of viral vectors.

Recombinant adeno-associated viruses (AAVs) have been investigated extensively for CF airway gene therapy. AAVs are non-enveloped DNA parvoviruses that naturally infect airways, and differ from AdVs in that they are non-pathogenic, have fewer viral genes, and have a much smaller packaging capacity. AAV vectors mostly remain episomal, although integration into human genes does occur at low frequency [89]. To achieve persistent gene expression with AdV and AAV vectors, hybrid vector systems have now been developed with the DNA transposon *piggyBac*. Co-delivery of the *piggyBac* transposase allows AdV and AAV vectors to integrate into the host genome, enabling potential for sustained gene expression with these systems [90, 91]

Serotype 2 (AAV2) has been the most comprehensively studied for CF airway gene therapy. The most recent clinical trial in 2005 was a placebo-controlled, phase IIb repeat-dosing study using an AAV2-*CFTR* vector that was aerosolised into CF patient lungs. Repeated doses were safe and well tolerated, but no significant improvements in lung function were observed [92]. The lack of positive results from these trials have been attributed to poor transduction of AAV2 vectors via the apical membrane, the use of weak transgene promoters due to limited packaging capacity, and host immune responses to AAVs [86, 89].

Although these trials failed to produce clinical benefit, they demonstrated the feasibility of viral-mediated airway gene transfer and highlighted the limitations of AAVs that need to be addressed. Research is now ongoing to overcome poor cell transduction and gene expression levels associated with AAVs. New serotypes with improved airway tropism are being investigated preclinically, stronger promoters are being used to drive *CFTR* expression, and truncated versions of *CFTR* have been designed to allow larger promoters to be packaged [89].

1.4.2.2. RNA viruses: lentiviruses

Lentiviruses (LVs) are enveloped RNA viruses from the family Retroviridae. LVs offer many advantages for CF airway gene therapy. They can transduce both dividing and non-dividing cells (including terminally differentiated airway cells) and have a relatively low immunogenicity. Unlike other retroviruses, extensive *in vivo* preclinical testing has shown that LVs do not favour integration into oncogene hotspots, therefore the theoretical risk of oncogene transformation is very low [72]. Importantly, LVs provide sustained transgene expression in the transduced cell and any subsequent progeny as they integrate stably into

the host-cell genome [93]. By targeting the stem cell niches, gene-corrected cells have the potential to continually replenish the airways, resulting in long-lived gene expression and reducing the required frequency of repeated administrations. Currently human immunodeficiency virus (HIV) is the most commonly researched LV for gene therapy, although simian (SIV) [94, 95], feline (FIV) [96] and equine infectious anaemia virus (EIAV) vectors [97] have also been developed.

LVs do not naturally infect airway cells, however, this can be overcome by pseudotyping the LV with other viral envelope proteins. A broad tissue tropism can be achieved by using vesicular stomatitis glycoprotein (VSV-G), or for a lung-specific tropism, influenza (HA) and Sendai (F/HN) pseudotypes can be employed [74]. For apical entry into the airway epithelial cells pseudotypes such as GP64 and Ebola envelope glycoprotein can be used [98, 99]. Some of the commonly used LV pseudotypes have receptors located on the basolateral membrane (e.g. VSV-G) therefore airway conditioning with tight-junction openers may be required to enable access to those receptors. One compound that has been explored extensively for its tight-junction-opening properties is lysophosphatidylcholine (LPC). LPC exhibits detergent properties and naturally exists in airway surfactant. When used at suitable concentrations, LPC transiently disrupts cell-cell tight-junctions allowing vector particles access to the airway cells via basolateral receptors [100]. Tight-junction opening also enables vector particles to transduce the underlying airway basal cells, known stem cells of the airway epithelium [101]. Transduction of the basal cell population has the ability to produce sustained transgene expression, as the subsequent daughter cells that repopulate the airway epithelium following cell turnover will be gene corrected.

LVs have been employed in gene and cell therapies for a range of diseases with encouraging results. LV vectors are now routinely used in chimeric antigen receptor T-cell therapies for cancer treatment [102-106], however, the use of LVs for CF airway gene therapy remains preclinical. It has been established that LVs (with varying pseudotypes) have an effect that is not species limited and can successfully transduce the airway epithelia of mice [107, 108], rats [109], sheep [110], pigs [98, 111], ferrets [112], marmosets [113], as well as *in vitro* human primary airway epithelial cells and airway liquid interface (ALI) cultures [107, 114, 115]. Delivery of LV-*CFTR* to the nasal epithelium of CF mice has also resulted in long-term partial correction of CFTR function for up to 12 months [108]. Unlike other viral vectors, successful repeat administration of LVs to the nose and lungs of mice has been demonstrated

without significant loss of efficacy [95, 96, 107]. These promising preclinical studies employing LV vectors have warranted a first-in-human LV-mediated airway gene therapy clinical trial in the United Kingdom for patients with CF. This phase I/IIa clinical trial is a single dose, doubled blinded, dose-escalating trial designed to assess the safety and efficacy of a SIV-based vector pseudotyped with the Sendai virus envelope proteins F/HN [114].

1.5. Challenges to clinical translation of CF airway gene therapy

While preclinical studies of CF airway gene therapy continue to show promising results, there are several hurdles to achieving successful translation to the clinic. Three of these challenges include (1) developing LV manufacturing methods that can adequately supply product for a clinically-approved airway gene therapy, (2) generating and employing suitable CF animal models for preclinical trials in order to obtain clinically-relevant outcomes, and (3) efficiently delivering the *CFTR* gene to the airway cells to achieve therapeutic levels of gene expression. These aspects of airway gene therapy development are the focus of this dissertation and will be discussed in more detail below.

1.5.1. Up-scaling LV production

A lack of large-scale LV manufacturing processes is a major barrier to both the preclinical development of LV-based gene therapies in large animal models and translation to clinical phases. Standard LV production methods are based on two-dimensional culture technologies such as flasks and multilayer cell factories [116]. The most common method of production involves transient transfection of adherent Human Embryonic Kidney (HEK) 293T cells with a multi-plasmid LV system. Traditionally, flask or cell factory vessels are used for production, which are systems that often result in high levels of batch-to-batch variability. Substantial manual human input is also required to operate these vessels, which can increase the risk of contamination events and results in higher production costs.

While traditional methods are suitable for producing LV for preclinical and early phase clinical trials, they cannot sustain the commercial demands for LV vector. Standard two-dimensional culture systems have limited scalability and increases in production capacity can only be achieved by increasing the vessel surface area or number of production units. High capital expenditure is required to establish manufacturing facilities with suitable infrastructure and size to support two-dimensional production systems, and operating costs are also increased

due to high manual labour demands. Accordingly, up-scaling LV manufacturing with the existing production scaffold is extremely labour intensive and uneconomical.

Bioreactor-based production methods are now being pursued to overcome the scalability issues associated with two-dimensional culture technologies. A range of approaches are currently under investigation including use of packed or fixed-bed bioreactors for adherent cells [117, 118] and suspension systems such as stirred-tank and wave reactors [119, 120]. A bioreactor-based approach has many benefits for large-scale manufacturing when compared to conventional LV production methods. Firstly, bioreactors allow for process automation, which reduces the costs associated with labour. Bioreactors are also closed systems and require fewer manual manipulations, reducing the chance of batch contamination and overall risk profile. Use of a controller system allows for online monitoring and tight regulation of conditions in the reactor, creating an optimal environment for growth and production phases, and reducing batch-to-batch variability. Moreover, increases in production capacity can be achieved by employing either scale-out (addition of parallel vessels) or scale-up (increasing the size of the vessel) models. These advantages warranted investigation into bioreactor-based approaches for up-scaling LV production. The intention of this work was to develop a bioreactor-based method for use in academic settings that would subsequently inform the development of a production approach suited to future human clinical trials.

1.5.2. Using the right animal model for trailing airway gene therapies

Choosing the right animal model for preclinical trials of airway gene-addition therapies is critical as the data from these studies will inform the safety, efficacy, and success of a future clinical trial. Moreover, demonstrating the efficacy of a therapeutic in multiple animal models can provide additional predictive value for human trials. A range of animal models have been developed for CF research including mice, rats, pigs, ferrets, sheep and rabbits [121-125]. These models have proven useful for a range of studies including investigations of CF disease pathophysiology [29, 126] and evaluations of therapeutics including gene therapies [111, 127-129].

Each CF animal model has advantages and limitations that must be evaluated for the specific research application. Firstly, practicalities such as housing, breeding, and costs and labour associated with animal husbandry need to be considered. Rodents, unlike non-laboratory

animals can be housed in a standard research environment, have relatively low husbandry costs, and are easy to breed due to their short gestation and large litter sizes.

Recapitulation of human-like immune responses is imperative when validating the safety of and longevity of therapies. It is important to consider species-dependent differences in immune responses, as the structure and function of the immune system can vary significantly. For example, some early studies of viral-mediated airway gene therapies in mice were found to poorly predict the magnitude of the immune response to the gene vector [130].

Lung size is also an important factor. While pig and sheep lungs closely resemble the size of humans, producing sufficient quantities of viral vector for an effective dose is challenging and highly expensive at present. Airway cell architecture of the species should also be considered when assessing the cell-targeting properties of gene vectors. For example, the cell type distribution of murine airway epithelium differs from that of human [131].

The lung disease phenotypes displayed by available CF animal models also vary significantly between species. Consequently, the pathological, electrophysiological, and anatomical lung disease characteristics present in each model need to be understood and suited to the therapeutic being trialled to enable relevant outcome measures to be performed. The genotype also differs between CF animal models. A majority of CF animals have CFTR completely knocked-out, which does not reflect the human disease where patients display major variation in the levels and function of the CFTR protein, based on the combination of CFTR mutations that they carry. As such, species carrying human disease-causing mutations may provide more value for certain research investigations.

While a number of CF mouse models have been generated, their use in airway gene therapy studies has been somewhat limited. CF mice do not appear to develop spontaneous lung disease and the CFTR electrophysiological defect is present only in the nasal epithelium. Mice also have naturally low levels of *CFTR* gene expression in the lower airways and the cAMP-mediated CFTR pathway appears to have a less dominant role in murine respiratory epithelium when compared to humans [132]. β-ENaC mice have been developed to overexpress ENaC in the lower airways thus mimicking the sodium hyperabsorption abnormality observed in human CF lungs. While β-ENaC mice been found to recapitulate key features of lung disease, CFTR expression and function are not altered, therefore these mice are not suited to *CFTR*-gene addition studies [133]. Existing CF animal models including ferrets

and pigs develop human-like lung disease manifestations, which is useful for assessing phenotype correction in response to gene therapy. However, these animals develop severe disease, therefore the animal husbandry required during trials is very costly and labour intensive. Moreover, longitudinal assessments of gene therapies are unfeasible with these models of severe CF disease due to reduced survival.

CF rat models are appealing for use in airway gene therapy preclinical trials for a number of reasons. The gene vector volume requirements are significantly lower than those needed to dose the airways of large species, thus allowing for well-powered and cost-effective studies. Rat airways have a more similar cellular architecture to humans when compared to mice. In particular rats have an abundance of submucosal glands throughout the trachea, which are known to be implicated in the development of CF airway disease and are a potential target for gene correction [134, 135]. A previously developed *CFTR* KO rat generated in the USA has shown encouraging results, with these rats developing a range of CF manifestations including aspects of lung disease such as reduced PCL depth and impaired MCC [29, 124]. KO rats also appear to exhibit milder disease phenotypes when compared to pig and ferret models, thus enabling longitudinal assessment of gene therapies.

Import of existing CF animal models into Australia is challenging due to strict biosecurity legislation that prevents many species (with the exception of laboratory rodents) from being introduced into the country. Furthermore, licensing agreements limit or prevent the use of some commercially-developed CF models. The lack of available CF animal models in Australia along with the notable advantages of employing CF rats in airway gene-addition therapy trials supported the generation of two new CF rat models in Australia.

1.5.3. Achieving efficient LV-mediated airway gene transfer

An efficacious airway gene therapy needs to produce adequate expression of the therapeutic gene in the desired cell types and should ideally provide a long duration of effect. However, several hurdles can limit successful *in vivo* gene transfer to the airway cells [136]. The airway epithelium has specific properties that confer resistance to viral vector-mediated gene transfer including a particle-trapping mucus layer, polarisation, paucity of viral receptors on the apical membrane, and the presence of airway tight-junctions that prevent vector access to receptors located on the basolateral side [137].

A range of strategies have been explored to overcome these barriers and improve gene transfer. One approach is to condition or transiently injure the airway epithelium prior to gene delivery [131, 138]. This process of epithelial perturbation disrupts the airway tight-junctions and enables more efficient transduction by providing viral vectors access to cells via the basolateral membrane receptors. Other benefits include improved penetration of the airway mucus barrier, and exposure of underlying epithelial cells that are not in direct contact with the airway lumen, particularly the basal stem cells, which function as tissue-specific stem cells [131, 139].

While cell turnover in the lungs is slow compared to other organs [140], transduction of only the terminally differentiated cells will result in inevitable loss of transgene expression over time and the need for readministration of the gene vector. The key to achieving long-term *CFTR* gene correction in the lungs is transduction of the airway stem cells. Multiple studies have indicated that basal cells are multipotent progenitor cells that repopulate the respiratory epithelium under normal conditions and during repair [101, 141, 142]. Permanent integration of the *CFTR* gene into the airway basal cells will correct the gene defect in the subsequent differentiated progeny, thus enabling sustained therapeutic gene expression following cell turnover. While gene vector readministration is unlikely to be completely avoided, a certain level of basal cell transduction could reduce the required frequency of repeat doses.

Chemical and physical strategies have been used to perturb the airway epithelium in research development scenarios. Compounds including sulphur dioxide, ethylene glycol tetraacetic acid (EGTA), perfluorochemical (PFC), sodium caprate, and LPC (as mentioned above) have previously been used as airway conditioning agents in conjunction with a range of viral vectors [137, 143-145]. Physical perturbation prior to gene vector delivery has also shown success in achieving strong gene expression in mouse airways using early AdV vectors [146, 147]. However, physical damage prior to gene transfer has not yet been explored with LV vectors, therefore the efficacy of this combination remains unknown.

While not addressed in this dissertation, the physical format of the therapeutic during delivery is also an important factor that will have significant impact on the efficacy of gene transfer. Gene vectors can be delivered to the airways in three formats: liquid, course spray, or aerosol. Nebulisation offers the advantages of uniform gene vector distribution to the distal lung and is considered a more clinically-relevant method of administration as it is

minimally-invasive and easily repeatable. However, many viral vector types including LV vectors demonstrate substantial reductions in viability when aerosolised due to shear stress experienced during transit through the nebuliser device [148, 149]. Moreover, aerosolisation produces small particle sizes, which tends to favour deposition of gene vector to the alveolar regions. This is undesirable for CF gene-addition therapy as the target is the conducting airways [149].

Use of course spraying devices offers a potential alternative for achieving uniform gene vector distribution to the airways (rather than alveolar regions), while having a decreased effect on vector stability when compared to nebulisation. Unlike use of orally-administered nebuliser devices however, intra-airway spraying approaches will require patient sedation, thus increasing the complexity of the procedure. Liquid delivery is most commonly used in preclinical investigations as it is relatively simple, specialist devices are not required, and there is negligible impact on vector viability. However, some animal studies demonstrate a non-homogeneous distribution of gene transduction when liquid gene vector is delivered to the lungs [149]. For clinical purposes, delivery of liquid formulations using flexible bronchoscopy would allow gene vector to be administered precisely to specific lung regions however, patient sedation would be necessary for this type of procedure [149, 150].

It is evident that gene vector delivery issues remain a major obstacle to achieving an efficacious airway gene therapy for CF lung disease. To overcome the physical airway barrier aspects, airway epithelial disruption strategies were investigated in rats. In these trials, physical and chemical (LPC-mediated) approaches were assessed to determine whether LV-mediated gene transduction could be enhanced.

1.6. Thesis overview

In the following chapters some of the challenges that are pertinent to the development of CF airway gene-addition therapy will be unravelled and addressed. Chapter 2 comprises of a review paper published in the Journal of Biotechnology, which details the challenges associated with up-scaling LV production to clinical and commercial scale and discusses some of the potential solutions for overcoming these hurdles. This review sets the scene for Chapters 3 and Chapter 4. Chapter 3 provides a methodology paper published in the Journal of Biological Methods. This paper details an LV production method that employs 10-layer cell factories and is suited to producing LV vectors for preclinical applications including animal

studies. Chapter 4 consists of an original research article published in Human Gene Therapy Methods that presents a packed-bed bioreactor approach for LV production. This novel method can be employed to produce large quantities of LV vector for preclinical studies in academic settings, but importantly, it also has the potential to be adapted to clinical production in the future.

Chapter 5 shifts direction and focuses on the need for suitable CF animal models for lung-related research investigations and therapeutic trials. This review article published in Respiratory Research provides an evaluation of the airway disease phenotypes present in established CF animal models including mouse, rat, pig and ferret models. The advantages and limitations of each animal model for CF airway research are also highlighted. Following on directly from Chapter 5, Chapter 6 presents an original research article published in the American Journal of Pathology. This paper describes the generation, phenotype characterisation and comparison of Australian-developed Phe508del and *CFTR* knockout rat strains.

Developing an efficacious airway gene therapy that produces therapeutic levels of gene expression is critical for moving towards human clinical trials. Accordingly, Chapter 7 presents a research article that explores the use of airway epithelium disruption strategies for enhancing gene transfer. These studies performed in rats assessed both chemical (LPC-mediated) and physical epithelial perturbation approaches for improving airway gene transfer.

The final section, Chapter 8, draws together conclusions from the presented body of work, discusses the strengths and limitations of each study, and proposes potential future work to expand upon the concepts presented.

Chapter 2: Challenges of up-scaling lentiviral vector production for gene therapies

Challenges of up-scaling lentivirus production and processing

By Alexandra McCarron, Martin Donnelley, Chantelle McIntyre and David Parsons

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Preclinical airway gene therapy studies employing LV vectors have revealed promising results, however, a major bottleneck to clinical trials is the lack of scalable LV production methods. Efficacious *in vivo* gene therapy in human-sized airways is likely to require large volumes of concentrated LV vector, and available upstream and downstream methods are not currently able to sustain this demand. To progress airway gene therapy preclinical studies in large animal models with 'human sized' airways (e.g. sheep and pigs) and ultimately clinical trials, scalable LV manufacturing and processing solutions need to be developed.

This review paper provides an update of the state of LV vector production and describes the challenges currently facing the field. Upstream LV production is discussed with a focus on cell cultivation technologies, transient transfection methods, and progress in the development of stable LV packaging cell lines (PCL). The advantages and limitations of common downstream processing methods are presented, along with a proposed workflow for large-scale LV manufacturing. Clinical quality control measures for LV products are also briefly discussed.

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Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100%.

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Review

Challenges of up-scaling lentivirus production and processing

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ABSTRACT

Lentiviruses are becoming an increasingly popular choice of gene transfer vehicle for use in the treatment of a variety of genetic and acquired human diseases. As research progresses from basic studies into pre-clinical and clinical phases, there is a growing demand for large volumes of high purity, concentrated vector, and accordingly, the means to produce such quantities. Unlike other viral vectors, lentiviruses are difficult to produce using stable cell lines, therefore transient transfection of adherent cell lines is conventionally used, and this method has proven challenging to up-scale. Furthermore, with the required increases in the volume of vector needed for larger animal and human use, comes the need for more efficient and sophisticated supernatant purification and concentration techniques. This review presents the challenges of up-scaling lentivirus production and processing approaches, novel systems for overcoming these issues, and the quality assessments recommended for producing a clinical grade lentiviral gene therapy product.

Contents

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1. Introduction

Lentiviruses (LVs) are an attractive gene delivery vehicle for a wide range of applications. LVs offer many advantages including the ability to transduce both dividing and resting cells, and the capacity to permanently integrate into the host cell genome, thereby providing sustained gene expression in the transduced cell and any progeny (Naldini et al., 1996). Furthermore, there is potential for life-long therapeutic benefit when gene-corrected stem cell populations pass on the transgene to daughter cell lineages during the normal cell replacement process. LV vectors are considered safer than many other viral vectors due to their relatively low immunogenicity and toxicity (Sinn et al., 2005), and reduced rate of insertional mutagenesis/tumorigenesis when compared to their oncoretroviral counterparts (Hematti et al., 2004). LVs can also be pseudotyped with envelope proteins from other viruses therefore improving the stability of the viral particles, and conferring the vector with either a broad tissue tropism, or a specificity for target cells (Cronin et al., 2005). These favourable characteristics make LVs a promising tool for use in the treatment of an array of human disorders including cystic fibrosis (Cmielewski et al., 2014), X-linked severe combined immunodeficiency (SCID-X1) (De Ravin et al., 2016), sickle cell anaemia, β-thalassemia (Bank et al., 2005), Parkinson's disease (Palfi et al., 2014), HIV (Gu et al., 2012), and cancer (Neschadim et al., 2012). Consequently, LVs are one of the fastest growing vectors under development amongst gene therapy research communities.

Growing interest in the use of LVs has created a strong demand for large volumes of concentrated vector for use in pre-clinical small and large animal studies, toxicology studies, and clinical trials. Presently, lentiviral vectors derived from human immunodeficiency virus-1 (HIV-1) are most commonly used, although simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anaemia virus (EIAV), and caprine arthritis-encephalitis virus (CAEV) vectors have also been developed (Schweizer and Merten, 2010). Although LVs are routinely produced in laboratories on a small-scale level for *in vitro* experiments and *in vivo* testing in small animal models, translating these methods to large-scale production has proven challenging (Segura et al., 2013). Major obstacles to up-scaling include complications surrounding the use of stable packaging cell lines for LV production, the restrictive nature of adherent human cell lines used in transient transfection, and difficulties implementing downstream processes (DSP) that can efficiently handle large volumes of LV vector supernatant, while maintaining vector functionality and minimising losses.

In this review, LV production will be discussed with a focus on the challenges surrounding large-scale cell cultivation and the up-scaling of purification and concentration processes. The LV quality analytics recommended for producing a clinical grade gene vector will also be addressed.

2. Lentivirus packaging cell lines

Lentiviral vector upstream processes (USP) can be achieved using two approaches: the development of stable vector packaging cell lines (PCL), or the transient transfection of human or monkey derived cell lines (Smith and Shioda, 2009). Stable PCLs are ideal for up-scaling as they can adapt to serum-free media and be cultivated in suspension culture systems. Stable expression systems have been successfully developed for a variety of viruses such as oncoretroviral Moloney murine leukaemia virus, however, the generation of stable producer cells for LVs has proven more complicated (Schweizer and Merten, 2010). Unlike other retroviral vectors, stable cell lines for LV production have not been successful as the cytotoxic effects of proteins such as protease, rev, gag-pol, and the commonly used vesicular stomatitis virus glycoprotein (VSV-G) envelope, all prevent constitutive expression (Sinn et al., 2005). Attempts have been made to overcome this issue by developing inducible expression systems for cytotoxic proteins (Stewart et al., 2009). Although successfully generated, inducible cell lines are not routinely used as the addition of induction agents complicates downstream processing, the cells are often unstable following induction (Sanber et al., 2015), and vector yields remain relatively low due to an observed decline in titre post-induction (Segura et al., 2013).

In most instances, LVs are produced by multi-plasmid transient transfection of adherent HEK 293 cell lines (293T and 293E) that are cultured in the presence of fetal calf serum (FCS) in single monolayer cultures using T-flasks or multi-tray systems such as cell factories (Segura et al., 2007). Traditionally, calcium-phosphate (CaP) co-precipitation is used for LV production, however, it is not easily amenable to up-scaling as it requires large quantities of DNA, the presence of serum or albumin is needed to reduce the CaP cytotoxicity, and it is extremely sensitive to pH variations. In order to minimise CaP toxicity to cells, it is standard to change the media shortly after transfection, a process that is unfeasible in large-scale production both practically and financially. Calcium-phosphate co-precipitation is also considered a 'static' method whereby precipitates settle onto the cell monolayer for a period of time (van der Loo et al., 2012; Lennaertz et al., 2013). For this reason, CaP is unlikely to be effective for dynamic conditions such as suspension culture, as the collisions between cells and precipitates may be too brief to mediate endocytosis of the DNA (Merten et al., 2016).

Many alternative transfection agents have been used for vector production including cationic polymers such as polyethylenimine (PEI) (Reed et al., 2006) and cationic lipid-based reagents such as lipofectamine™ (Cribbs et al., 2013) (ThermoFisher Scientific). Both approaches have proven to be as effective as CaP for the generation of LV, however, lipid-based agents become very costly for use in large-scale production (Segura et al., 2013). PEI, on the other hand, is a relatively inexpensive approach, and is suitable for large-scale LV production. PEI does not require tight regulation of transfection conditions (*e.g.* pH), and it is also substantially less toxic than CaP, meaning that media change following transfection is not necessary. Moreover, PEI has proven to be effective for the transfection of both adherent and suspension cultures, and can be used in the presence or absence of serum (Reed et al., 2006; Segura et al., 2010).

Although faster and more efficient than stable production methods, transient transfection is not optimal for large-scale manufacturing due to significant batch-to-batch variations, the requirement for massive amounts of costly plasmid (Segura et al., 2013), and the potential for contamination of the final product with immunogenic residual plasmid DNA (Krieg, 1999). For pre-clinical and clinical studies transient transfection is likely to be sufficient, but for GMP (good manufacturing practice) grade production it is

essential that the vector is produced with consistent quality; therefore a stable approach is desired (Sanber et al., 2015).

The generation of LVs using stably-producing cells remains an ongoing challenge; however, the recently constructed clinical grade, constitutive WinPac cell line has shown potential for up-scaling. Compared to other reported constitutive LV PCLs (Stornaiuolo et al., 2013), WinPac cells can produce third generation, self-inactivating LV at superior titres of 10^6 TU/mL, with the potential for titres of up to 10^8 TU/mL following concentration (Sanber et al., 2015). In contrast to the GPRTG cell line recently used to produce LV for a SCID-X1 phase 1 clinical trial (De Ravin et al., 2016), induction agents are not required therefore avoiding cumbersome purification steps needed to remove the agents and improving scalability (De Ravin et al., 2016; Throm et al., 2009). WinPac cells also overcome safety issues associated with previously developed STAR (Ikeda et al., 2003) and RD2-MolPack (Stornaiuolo et al., 2013) PCLs, which were rendered unsuitable for clinical applications. Although promising, further optimisation of the WinPac cell line is necessary as current vector expression levels are unlikely to provide sufficient yields for human gene therapy applications where large quantities of high-titre vector are required (Sanber et al., 2015).

3. Scaling-up cell cultivation for transient transfection

3.1. Adherent cells

Although current methods are sufficient for the small level production needs in basic research, translational research in animal models demands much larger volumes of vector. The existing adherent cell lines used for transient transfection have limited scalability as there are practical limitations surrounding the huge surface area required for the attachment of cells. There is also an inherent difficulty in up-scaling adherent cultures due to the need for time-consuming manipulation of numerous culture flasks in a biosafety cabinet (Segura et al., 2013).

Attempts to up-scale adherent cultures using multiple surface cultivation systems such as roller bottles (0.175 m^2 surface area), multi-layer flasks (*e.g.* HYPER*flasks*™ 0.172 m^2 surface area), and cell factories (*e.g.* Nunc™ CF-10 tray system 0.632 m^2 surface area), have allowed for modest increases in LV production. These technologies are limited however, as they are low cell density culture systems, therefore up-scaling can only be achieved by increasing the number of culture units, which requires significant amounts of incubator space, and is labour-intensive (Segura et al., 2013; Merten, 2015). In an attempt to overcome the lack of scalability associated with conventional adherent cell culture systems, technologies such as hollow fibre bioreactors, fixed-bed bioreactors, and microcarriers have been developed. It should be noted however, that direct comparisons between the following systems cannot be made from published data due to differences in the titering methods and vector types produced.

3.1.1. Hollow fibre bioreactors

A hollow fibre bioreactor is a closed, fully automated system, consisting of a single-use cartridge containing thousands of porous capillaries (hollow fibres) arranged in a parallel orientation. The cartridge is separated into two spaces: the extra-capillary space where the cells are cultivated, and the intra-capillary space where medium is transported, and nutrients and metabolites are exchanged (Tapia et al., 2014). Previous reports of virus production using hollow fibre bioreactors have involved systems with relatively small surfaces areas. For instance, the Quantum® bioreactor (Terumo BCT) provides a growth area of 2.1 m^2 (Sheu et al., 2015), while the Primer™ bioreactor (Biovest International) only provides an area of 0.5 m^2 (Tapia et al., 2014). Given the limited scalability of these particular bioreactors, it is likely that these systems are more suited to a scale-out approach whereby multiple production units are used in parallel; however, this may result in undesired variation between batches, and requires labour-intensive cell cultivation for seeding.

3.1.2. Fixed-bed bioreactors

Fixed-bed bioreactors (also referred to as packed-bed bioreactors) allow for high-density growth of adherent cells on a densely compacted bed of microfibre carriers. Culture media is then perfused through the bedding allowing for nutrient and oxygen transfer to the cells. Fixed-bed systems such as the iCELLis® nano bioreactor (Pall) can provide surface areas up to 4 m^2, the equivalent of 6 ten-layer cell factories. The iCELLis® nano has been shown to be efficient for the production of retroviral vectors using stable PCLs. In this scenario, up to 25 L of virus supernatant was produced in a single run using perfusion mode, with total yields of 10^{12} TCIU (tissue culture infectious units) obtained (Wang et al., 2015). PEI-mediated transfection has also been successfully performed in the iCELLis® nano for the production of adeno-associated virus (Lennaertz et al., 2013), although there have been no reports of LV production using this system. The bioreactor can also be scaled up to industrial-level production using the iCELLis® 500 m^2 fixed bed, however, this leap from small-scale production to industrial is dramatic, as there are no medium-scale options available.

An alternative bioreactor of interest is the bench-top scale CelliGen® fixed-bed basket device that is packed with Fibra-Cel® disks (Eppendorf). The disks provide a large surface area and are electrostatically treated to attract cells and mediate their attachment. The vessels are either reusable or single-use, and range from 2.5–14 L, with a maximum surface area of up to 60 m^2 for the largest sized vessel (Chaubard et al., 2000). The CelliGen® fixed-bed has demonstrated relatively high-titre stable retrovirus production (10^7 viral particles per cm^3) (Merten et al., 2001), however, there are no reports of its use for transient LV production.

Another system that takes advantage of fixed-bed reactor technology for the cultivation of adherent cell lines is the CellCube (Corning). The CellCube is a small, perfused bioreactor that is contained within a CO_2 incubator and uses a module containing a series of parallel culture plates for the growth of cells, with the largest module having a surface area of 2.1 m^2. Media is then gas-conditioned with the oxygenator chamber, and is continuously supplied to the module using a variable-rate pump. The CellCube has been successfully used for the production of various viral vectors for gene therapy purposes including retroviruses (Merten et al., 2001). However, this system is not ideal for large-scale production, as a scale-out approach is not feasible due to the incubator space requirements (Merten, 2004).

3.1.3. Microcarriers

Microcarriers are generally spherical-shaped growth matrices with a diameter ranging between 100 and 200 μm, with either a solid or porous composition (Merten, 2015). In contrast to conventional two-dimensional planar culture systems (*e.g.* T flasks), microcarriers provide large surface areas for the growth of anchorage-dependent cell lines in three-dimensional suspension systems such as stirred tank and wave bag bioreactors. The ability to cultivate adherent cells in suspension systems with the use of microcarriers is a major advancement in overcoming the scalability issues associated with anchorage-dependence. For example, solid microcarriers such as Cytodex 1 and Cytodex 3 (GE healthcare) have been used successfully in suspension culture systems for the production of both adenoviral and retroviral vectors with

high titres of up to 10^{10} TCID50/mL (50% tissue culture infective dose) reported (Wu et al., 2002).

More recently, there appears to be movement away from the use of microcarriers in vector production. Although they have their advantages, including the ease of separating cells from the desired product, they do not completely eliminate the need for labour-intensive expansion of adherent cells (Merten et al., 2016). As a result, there has been a shift towards adapting adherent producer cell lines to suspension culture where possible.

3.2. Suspension cells

To overcome the need for large surface areas and labour intensive cell cultivation, one approach is to adapt adherent LV producer cell lines to suspension culture. Suspension cells are easily amenable to large-scale LV production when compared to their adherent counterparts, as they can achieve much greater cell densities in vessels such as shake flasks, stirred tank bioreactors, and wave bags (*e.g.* WAVE™ 25 GE healthcare), without the need for attachment surfaces (Ghani et al., 2007). Suspension-adapted cells can also be cultured in serum-free media, thus favouring the move towards a clinical grade product, reducing the risk of contaminating the final product with potentially immunogenic adventitious agents and animal components, and simplifying downstream processing. Replacing two-dimensional scale-out adherent culture technologies with scale-up suspension culture processes will also reduce variability between virus batches (Geraerts et al., 2005).

LV has been successfully produced using transient transfection of suspension HEK 293 cells lines in bioreactor systems including stirred tanks and wave bags. Encouragingly, previous studies suggest that LV titres achieved in suspension systems are comparable to those routinely obtained using adherent cells, with values ranging from 10^6–10^8 TU/mL (Segura et al., 2007; Ansorge et al., 2009). (Ansorge et al., 2009) outlined a method for the production of lentivirus that involved the transient transfection of suspension HEK 293SF-3F6 cell line in a perfusion-based bioreactor system. Although promising, this method demonstrated limited scalability as only three litres of virus supernatant was successfully produced. Furthermore, the use of perfusion culture can be labour-intensive and more technically complex as it requires several harvest stages (Ansorge et al., 2009). Segura et al. (2007) also proposed a LV production protocol that involved transient transfection of 293E suspension cells in a three-litre bioreactor. Although high-titre lentivirus was successfully produced, the process proved time-consuming as it required a number of harvesting steps, as well as total media replacements at days 3, 4, and 5 post-transfection (Segura et al., 2007). More recently, a suspension cell approach was shown to produce lentivirus at a 50 L scale using single-use bioreactor vessels and PEI-mediated transient transfection (Marceau et al., 2013).

4. Downstream processing of lentiviral vectors

Production of high titre, high purity lentivirus is challenging due to a lack of simple methods that are capable of rapidly processing large volumes of vector supernatant. As a result, the selection and integration of the virus purification and concentration steps is an important prerequisite for successful large-scale production of a clinical grade therapeutic vector. For use in an up-scaled setting, the techniques should meet a number of essential criteria: they should be scalable, economical, have a high capacity and throughput, and efficiently remove contaminants, all while maintaining the functionality of the virus and minimising viral particle losses at each step (Rodrigues et al., 2007).

The purpose of downstream processing is to concentrate the vector and remove impurities that are unsafe or may be inhibitory to vector transduction (Schweizer and Merten, 2010). There are several reported methods for the purification and concentration of lentiviral vectors. Most commonly, column chromatography techniques based on ion-exchange (Kutner et al., 2009) or size-exclusion (Transfiguracion et al., 2003) are used. Although other approaches including affinity adsorption chromatography (Segura et al., 2007), ultracentrifugation, and ultrafiltration methods such as tangential flow filtration have also been developed for lentiviral vector processing (Geraerts et al., 2005). However, not all approaches are considered ideal for large-scale applications; accordingly, Table 1 outlines the compatibility of each method for up-scaling.

4.1. Anion-exchange chromatography

Chromatography is considered one of the most promising technologies for large-scale purification of viral vectors (Segura et al., 2007). Anion-exchange chromatography is one approach that involves passing virus supernatant through a column where negatively charged vector particles bind to the positively charged chromatographic matrix. Bound virus particles are then eluted from the column using a high concentration salt buffer. Anion-exchange chromatography is an attractive option as it is relatively straightforward, produces vector of high purity, and has previously been reported to recover up to 68% of vector particles (Scherr et al., 2002). Chromatography columns are also simple to up-scale as they are available in a variety of sizes and can also be connected in series to create a larger binding surface area, with previous studies demonstrating purification of up to 1,500 L per day (Marino et al., 2003). The columns also have the capacity to be re-used thus reducing the costs associated with virus production (Scherr et al., 2002). One drawback of anion-exchange chromatography is that elution of virus particles with a high concentration salt buffer has been shown to significantly reduce the infectivity of the vector, most likely due to an increase in osmotic pressure that results in damage to the viral membrane (Zimmermann et al., 2011). In fact, previous studies have indicated that exposing retrovirus particles to 1 M NaCl for one hour (at room temperature) is enough to reduce the infectivity by 50% (Segura et al., 2005).

4.2. Size exclusion chromatography

Size exclusion chromatography (SEC) (also known as gel filtration) involves using a porous, non-adsorbing material to separate virus particles from medium contaminants on the basis of size and mass (Rodrigues et al., 2007). Infectious particle recoveries of up to 70% and vector purity of >90% have been previously obtained using this purification method. SEC is a powerful separation method, and is particularly suitable for unstable virus particles as the conditions applied during purification are relatively mild compared to other techniques (Transfiguracion et al., 2003). However, SEC may not be appropriate for up-scaling purposes as it has very low throughput, and requires low linear flow rates that increase processing time. Vector particles purified via size exclusion also require further concentration as there is an observed dilution effect during the passage of virus through the column. Due to these factors, SEC is more suitable as a final vector "polishing" step to remove any remaining small impurities, rather than for bulk purification (Rodrigues et al., 2007; Merten et al., 2011).

4.3. Affinity adsorption chromatography

Affinity adsorption chromatography is a relatively inexpensive method that is used widely in the pharmaceutical industry to iso-

Table 1
Comparison of LV vector purification methods and their compatibility with up-scaling.

Method	Recovery (%)	Purity (%)	Titre (TU/mL)	Throughput	Scalability	Reference
Anion-exchange chromatography	68	>95	10^7–10^8	10 mL/min	High	Scherr et al. (2002)
Size-exclusion chromatography	70	≥90	10^7	0.4 mL/min	Low	Transfiguracion et al. (2003)
Heparin-affinity chromatography	53	94	10^9–10^{10}	16 mL/min	High	Segura et al. (2007)
Tangential flow filtration	90–100	–	10^9–10^{10}	17 mL/min	High	Geraerts et al. (2005)

– Not supplied.

late biomolecules. For vector purification purposes, one method, namely heparin affinity, has shown promising results. Heparin affinity chromatography involves loading LV supernatant onto a column where virus particles bind to heparin ligands that are immobilized on a chromatographic gel; desorption of particles is then achieved using a low molarity NaCl solution (*e.g.* 0.35 M) (Segura et al., 2005). A previous study conducted by Segura et al. (2007) demonstrated recoveries of up to 53% of infectious LV particles, and removal of 94% of impurities using heparin affinity. The same report also proved this technique could be used to purify up to three litres of virus supernatant, suggesting that it is suitable for processing large volumes (Segura et al., 2007). However, heparin affinity chromatography is complex and requires a high level of expertise, thus making it difficult to implement in a standard laboratory (Kutner et al., 2009). Impurities such as cellular proteins also have the potential to compete with vector particles for heparin-binding sites, thereby reducing the binding capacity of the column. Heparin-binding impurities can also elute with the virus, therefore further purification is typically required to remove these contaminants, thus increasing processing time (Segura et al., 2007; Summerford and Samulski, 1999).

4.4. Tangential flow filtration

Tangential flow filtration (TFF) is an ultrafiltration technique that involves pumping viral supernatant tangentially along the surface of a membrane with pore sizes varying between 0.001–0.1 μm. TFF can be applied at both the initial bulk purification stages, and the downstream polishing steps. In the example of polishing, a pressure difference across the membrane drives smaller particulates such as proteins and DNA through the pores of the filter, while vector particles are retained on the upstream side. The virus-containing retentate is then recirculated over the membrane surface, resulting in a net concentration of vector. Unlike conventional direct flow filtration systems, the retained particles do not build up at the membrane surface thus preventing fouling and allowing large volumes to be processed (Rodrigues et al., 2007). TFF has proven to be a highly effective method for the purification of lentivirus, with studies reporting vector recoveries between 90 and 100%. TFF also has potential for use in up-scaled LV production due to its high throughput and capacity to rapidly process large volumes of vector supernatant at approximately one L per hour (Geraerts et al., 2005).

4.5. Ultracentrifugation

Ultracentrifugation is a conventional concentration process typically used following preliminary purification steps such as anion-exchange chromatography, in order to achieve small volumes of high-titre vector. The use of ultracentrifugation has been observed to substantially increase concentration with reports of 50–300-fold increases (Reiser, 2000). It is particularly useful in situations where minute volumes of highly concentrated virus are required, such as LV-mediated gene delivery to the brain for the treatment of neurodegenerative disorders (Scherr et al., 2002). Although practical for small to medium scale applications, ultracentrifugation is inherently difficult to up-scale as the volume of virus that can be concentrated in one run is limited by the capacity of the centrifuge rotor (Ichim and Wells, 2011). Ultracentrifugation is also time-consuming and has the potential to co-concentrate impurities that may be inhibitory to vector performance (Transfiguracion et al., 2003).

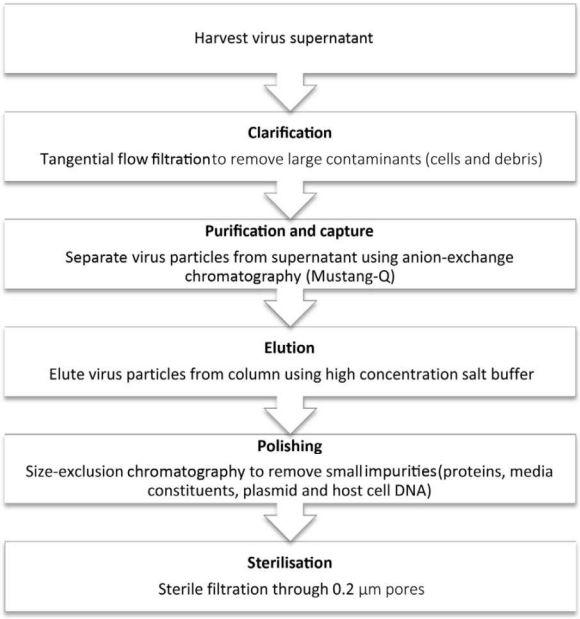

Fig. 1. Proposed protocol for downstream processing of LV vector preparations (applicable to either pre-clinical or clinical applications).

It is evident that there is a wide range of methods available for the processing of lentiviruses. For successful purification and concentration of LVs, it is likely that not only one method will be used, but a streamlined integration of many techniques based on differing separation principles will be necessary. Accordingly, Fig. 1 presents one of many possible protocols that could be used for large-scale downstream processing of LV.

5. LV quality analytics

Producing a GMP grade LV vector preparation for human use requires rigorous characterisation of the final product in order to establish its purity, potency and safety. Unlike other pharmaceuticals, there are particular challenges that arise when analysing a biologically active therapeutic such as a viral vector. There are a diverse range of *ex vivo* and *in vivo* applications for LVs, therefore the production method and desirable quality of the final product will depend on its intended use. For example, in previous LV-mediated gene therapy clinical trials including those for Parkinson's (Palfi et al., 2014), Wiskott-Aldrich syndrome (Aiuti et al., 2013), and metachromatic leukodystrophy (Biffi et al., 2013), LV vectors were produced in GMP certified facilities with

the common multi-plasmid transient transfection of HEK 293T cells using calcium-phosphate. The downstream processes utilised among the trials were relatively crude and consisted of an initial anion-exchange chromatography step followed by filtration using size-exclusion chromatography. Although this production method was deemed suitable for the above-mentioned clinical trials, in all cases, the LV preparations were destined for *ex vivo* gene therapy applications. However, vector for *in vivo* administration is likely to require a greater purity and potency than vector delivered *ex vivo* to somatic cells for subsequent administration to the patient.

At this time, there are no international standards for LV vector products, and a limited number of clinical trials for novel applications have been performed. The currently available regulatory directives for developing a human gene therapy product are generalised, so in practice LV vector products should be assessed on a case by case basis (Segura et al., 2013). Outlined in the following section is a selection of tests recommended for routine batch analysis of purified LV vector product intended for human use.

5.1. Purity

Achieving a clinical grade LV of high titre, purity, and concentration is a difficult task, as one parameter is typically optimised at the expense of the others. Titre can often be compromised in order to achieve a product of greater purity; as the number of purification steps increases, more loss and damage to virus particles occurs. Despite these vector losses, it is essential for clinical grade vector to undergo purification processes in order to meet a number of stringent quality control assessments (Rodrigues et al., 2007).

Each vector batch must be subjected to tests to ensure that contaminants are below levels capable of inducing toxicity and immune responses in the recipients, however, the exact upper limits of each contaminant will depend on the intended use of the vector. Protein impurities arising from host cells and culture medium can be evaluated using an array of methods including SDS-PAGE with silver staining analysis, colourimetric protein assays (*e.g.* Bradford and Lowry), Western blotting directed to specific protein contaminants, and commercially available enzyme-linked immunosorbent assay (ELISA) kits. Contaminating DNA sequences from producer cells such as the SV40 large T antigen and undesirable plasmid DNA sequences (*e.g.* VSV-G and gag-pol) can be determined using quantitative PCR (qPCR). The total DNA content can also be measured using spectrofluorimetry (Merten et al., 2011). Standardised sterility tests must be performed to ensure that each vector batch is free from endotoxins and adventitious agents including bacteria, mycoplasma, yeast and fungi. Analyses of physiochemical characteristics such as pH and osmolality are also required to ensure the final vector batch meets clinical standards (Segura et al., 2013).

5.2. Potency

When compared to other pharmaceutical products, biologicals such as virus-based gene vectors tend to have greater variability between batches, particularly in regards to the ratio of total and functional vector particles (potency). In a clinical setting, it is imperative that every batch of the final product is consistent; therefore, quantification of the functional and non-functional virus particles is necessary to ensure that every dose has an identical transduction efficiency (Segura et al., 2013). If the ratio of defective particles to functional particles is found to be excessively high, the whole batch should be rejected. However, obtaining accurate assessments of LV batch potency has proven to be an ongoing challenge, as the available titering methods are not yet standardised and demonstrate large intra- and inter-assay variations, as well as considerable differences between laboratories and operators (Sinn et al., 2005).

Current LV titering methods include assessment of reporter gene expression following cell transduction, quantification of vector RNA in supernatants (RNA titre), and assessment of vector DNA (provirus) in transduced cells (DNA titre). For developmental vectors, measuring transgene expression in transduced cells is a simple method that can be performed when the vector carries a reporter gene such as GFP or *LacZ*, however, this method tends to underestimate titre as it cannot distinguish cells with multiple vector copies, and is not suitable for titering therapeutic gene vectors. RNA titering of viral supernatants is the most rapid technique, although it is limited in that it does not accurately reflect functional titre due to interference from defective virus particles and DNA carried over from production, with RNA titres previously found to overestimate titre by 1000-fold when compared to DNA titres. Although time-consuming, quantification of proviral DNA integrated in the genome of transduced cells provides the most reliable estimate of functional titre as it is able to detect multiple vector copies per cell, and accounts for factors such as defective vector particles and contaminating plasmid DNA (Sastry et al., 2002).

5.3. Safety

Biological assays must be performed in the case of replication-deficient lentiviruses to demonstrate that homologous recombination has not occurred. Each vector batch must be tested for replication-competent lentivirus (RCL), and if detected, the whole batch should be rejected (Escarpe et al., 2003). Sensitive assays are required to detect RCLs as such recombination events are rare (Sinn et al., 2005). While methods are well established for detecting replication-competent retroviruses, the development of valid RCL assays is difficult as LV safety features are specifically designed to prevent recombination, as such, there are no reports of RCLs in the current vector systems. Therefore, assay positive controls can only be based on theoretical assumptions about the RCLs genomic structure (Sinn et al., 2005; Escarpe et al., 2003).

Nevertheless, a number of assays have been developed for the detection of RCLs. Conventional cell-based assays involve transducing a cell line that is permissive to viral infection with the vector sample and performing several passages in order to amplify any potential RCLs. RCL detection is then achieved by using an ELISA to measure the HIV-1 p24 capsid protein concentration in the culture supernatants from each passage stage; in the absence of RCLs the p24 concentration should decrease overtime (Escarpe et al., 2003; Cornetta et al., 2011). Sensitive and rapid PCR-based techniques have also been developed for detecting RCLs including the product-enhanced reverse transcriptase (PERT) assay that detects reverse transcriptase activity (Sastry et al., 2005), a real-time PCR assay for VSV-G envelope DNA, and the psi-gag PCR assay that detects recombination between the transfer vector and gag-pol plasmid (Cornetta et al., 2011).

6. Conclusions

Growing interest in the use of LVs for the treatment of a range of inherited, acquired, and infectious diseases has resulted in increasing demand for large volumes of concentrated vector. Unlike other viral vectors, LV production has proven challenging to up-scale due to difficulties developing a stable packaging cell line. These complications have driven the development of culture technologies that allow for considerable scale-up of transient transfection processes for both adherent and suspension-adapted cell lines. However, transient transfection methods are only an interim solution, as it is likely that stable cell lines will eventually be required for commercial vector production.

With the up-scaling of cell cultivation and the subsequent production of large volumes of virus supernatant comes the challenge of developing more sophisticated purification and concentration techniques. A review of the current methods available suggests that effective downstream processing of LVs is likely to require a number of different approaches integrated together to achieve clarification, isolation, concentration and polishing of the vector preparation. Techniques of particular interest include TFF for clarification and concentration, anion-exchange for isolation, and size-exclusion for polishing.

Extensive characterisation of purified LV vector batches is required in GMP manufacturing to develop a product suitable for human use. Although numerous tests are available for assessing LV quality including measures of sterility, potency (titre) and detection of RCLs, the lack of international standards for LV vector products creates uncertainty when developing a gene vector intended for human purposes. As leading LV vector gene therapy groups worldwide progress into phase I clinical trials (both *in vivo* and *ex vivo*), it is anticipated that such studies will provide critical knowledge for developing more detailed, application-specific regulatory guidelines.

To conclude, there has been considerable progress in large-scale LV manufacturing over the past decade, however, it is evident that further development of these processes is required in order to produce sufficient quantities of vector for clinical and commercial applications. Accordingly, the advancement of large-scale LV production processes will be critical in hastening gene and cell therapy trials, and ultimately bringing us closer to effective treatments for a wide range of human diseases.

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Chapter 3: Preclinical lentiviral vector production in cell factories

Large-scale production of lentiviral vectors using multilayer cell factories

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Preclinical airway gene therapy trials require high quantities of concentrated LV vector. For these investigations to be economically viable, LV production methods need to yield high titres, while minimising complexity and costs. Producing large volumes of LV vector with standard flask or dish-based methods is a labour-intensive process. Due to the low surface area of a monolayer flask, numerous vessels are required for large-volume production, which demands high levels of manual manipulation. Not only is this time consuming, increased handling of the vessels risks culture contamination. Additionally, incubator space requirements can be a limiting factor when scaling-up with flask-based production methods.

To overcome the limitations of traditional LV production methods, culture technologies such as roller bottles, multilayer T-flasks, and cell factories are being employed. These systems provide greater surface areas for cell cultivation, allowing for high cell density cultures and increased productivity from a single unit. Labour input is also reduced with these methods, as fewer vessels are required. Accordingly, reported herein is a cell factory-based LV vector production method that can be used to generate material for preclinical animal studies. This shift in production capacity is particularly relevant as studies using newly developed CF rat models necessitates greater LV quantities when compared to mice.

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Overall Percentage (%)	50%		
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By signing the Statement of Authorship, each author certifies that:

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PROTOCOL

Large-scale production of lentiviral vectors using multilayer cell factories

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Abbreviations used: BSA, bovine serum albumin; CFTR, cystic fibrosis transmembrane conductance regulator, FCS, fetal calf serum; HEK, human embryonic kidney; LV, lentiviral; PBS, phosphate buffered saline; PEI, polyethylenimine; RT-PCR, reverse transcription-polymerase chain reaction

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ABSTRACT

Lentiviral-mediated gene therapy has been proposed for the treatment of a range of diseases, and due to its genome integration properties, it offers the potential for long-lasting benefit from a once-off treatment. Production methods for pre-clinical studies in animal models, and ultimately for human clinical trials, must be capable of producing large quantities of high-quality lentiviral vector in an efficient and cost-effective manner. We report here a medium-scale method (from 1.5 L to 6 L of vector supernatant) for lentiviral vector production in adherent cell cultures using the NUNC™ EasyFill™ Cell Factory™ from Thermo Fisher Scientific. Downstream purification uses a Mustang Q XT5 anion exchange capsule from Pall, and an ultracentrifugation step to concentrate the vector. This method is capable of producing lentiviral vector with concentrated titres of 10^8–10^9 TU/ml, with reduced manual handling compared to single monolayer flask methods.

Keywords: calcium phosphate, cell factories, lentiviral vector, Mustang Q, ultracentrifugation

BACKGROUND

Gene-addition therapy is a promising therapeutic approach with the potential to treat or cure a range of genetic and acquired diseases by introducing a working copy of the appropriate gene into affected target cells. Lentiviral (LV) vectors are advantageous for therapeutic development because they transduce dividing and non-dividing cells, can be pseudotyped to target specific cell types by altering surface receptor recognition elements, and show high levels of gene transfer efficiency [1].

Phase II trials for metachromatic leukodystrophy [2], Wiskott-Aldrich syndrome [3,4], and Parkinson's disease [5] have reported therapeutically-effective outcomes using LV vectors. Similarly, gene therapies for β-globinopathies (sickle-cell anemia and β-thalassemia) have survived the rigors of pre-clinical safety and efficacy testing [6]; with long-term, multi-institutional, phase III/IV studies now underway in Europe and the USA [7]. The VSV-G pseudotyped, LV vector described here [8] has been applied to gene therapy studies for Sanfilippo syndrome, Sly syndrome, methylmalonic aciduria, and cystic fibrosis [9-12]. Our primary interest is treating the airway disease in cystic fibrosis using a HIV-1 LV vector to introduce a working copy of the cystic fibrosis transmembrane conductance regulator (CFTR) gene into airway epithelial cells [11].

Producing sufficient quantities of LV vector for use in pre-clinical animal studies and clinical trials has proven challenging. Typically, LVs are produced using either stable packaging cell lines or by the transient transfection of adherent human embryonic kidney (HEK) 293T cells [13]. Ultimately, a stable packaging cell line would be ideal for up-scaling production. However, difficulties in developing a stable cell line capable of high-titer LV production have meant that large-scale, transient transfection-based methods are required.

Current methods for transient LV production are based on the multi-plasmid transfection of adherent HEK 293T cells in single monolayer flasks. However, up-scaling LV production using monolayer flasks is time-consuming, labour intensive, and requires large work spaces for cell cultivation [1,14,15]. Recently, there has been a shift towards developing suspension based cultures using adapted HEK 293T cells to produce high density cultures within a limited work space by using

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stirred-tank and wave-bag bioreactors [16,17]. Despite some early progress, LV production in suspension conditions is still in its infancy. Hence, continued development of adherent-based LV production methods is important until the suspension culture methods are more established. Modest increases in the production capacity of adherent-cell based systems have recently been achieved by employing technologies such as roller bottles, multi-layer flasks, and cell factories [13]. Accordingly, a cell factory method was developed as an interim solution for producing LV vector for pre-clinical studies, with the potential for future large-scale production in a scale-out scenario.

Here, we describe a method to produce VSV-G pseudotyped LV vector using calcium phosphate co-precipitation in 10-layer NUNC™ EasyFill™ cell factories. In this method, downstream processing is performed *via* a Mustang Q XT5 anion exchange step followed by ultracentrifugation (**Fig. 1**). Our results show that concentrating the supernatant up to 3000 times enables us to achieve volumes of 500 µl/cell factory at a titer of 10^8- 10^9 TU/ml (determined by RT-PCR). Although the initial expansion of cells to seed a cell factory remains time consuming, gains are made using multi-layer cell factories, as they avoid the use of multiple monolayer flasks, where one 10-layer cell factory is equivalent to 36 T175 flasks. The use of cell factories increases the surface area available for cell cultivation, thereby improving vector yields, while also decreasing the transfection and post-transfection media-change times, and reducing labor costs. Although this method describes use of a second-generation system, it is applicable to production of first, second or third generation LV vectors by choosing the appropriate plasmid ratios. It can also be used for producing LV vectors with alternative pseudotypes (*e.g.*, HA or GP64 [18,19]) with changes to the ultracentrifugation step, or for use with other transfection agents including polyethylenimine (PEI) [20].

MATERIALS

Cell culture

- HEK 293T cells (American Type Culture Collection, cat.# CRL-3216)
- Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, cat.# 11965-084)
- Fetal calf serum (FCS) (Australian Origin) (Gibco, cat.# 10099-141)
- Penicillin-streptomycin (penicillin 10000 IU/ml, streptomycin 10000 µg/ml) (Gibco, cat.# 15140-122)
- 75 cm^2 cell culture flasks, redtop filter screw cap (T75) (Greiner Bio-one, cat.# 658175)
- Corning tissue-culture treated culture dishes 150 mm × 25 mm (Sigma-Aldrich, cat.# CLS430599-60)
- Phosphate buffered saline (PBS) (Sigma-Aldrich, cat.# D8537)
- TrypLE express cell dissociation reagent (Gibco, cat.# 12604-021)

Plasmid transfection

- NUNC™ EasyFill™ CellFactory™ System (ThermoFisher Scientific cat. # 140400)
- Na_2HPO_4 (Sigma-Aldrich, cat.# S3264)
- HEPES (Sigma-Aldrich, cat.# H3375)
- NaCl (Sigma-Aldrich, cat.# S3014)
- $CaCl_2{\cdot}2H_2O$ (Sigma-Aldrich, cat.# C8106)
- Lentiviral packaging, envelope, and expression plasmids (*e.g.*, for our second generation, 5-plasmid system we use: pTat, pRev, pGag-Pol, pVSV-G and an expression plasmid. See **Table 1** for details). Users should produce the plasmids for their system in the appropriate quantity.
- 225 ml conical tubes (Falcon, cat# 352075)
- L-Glutamine (Gibco, cat.# 21051-024)
- OptiPRO™ SFM (Gibco, cat.# 12309-019)
- Penicillin-streptomycin (penicillin 10000 IU/ml, streptomycin 10000 µg/ml) (Gibco, cat.# 15140-122)

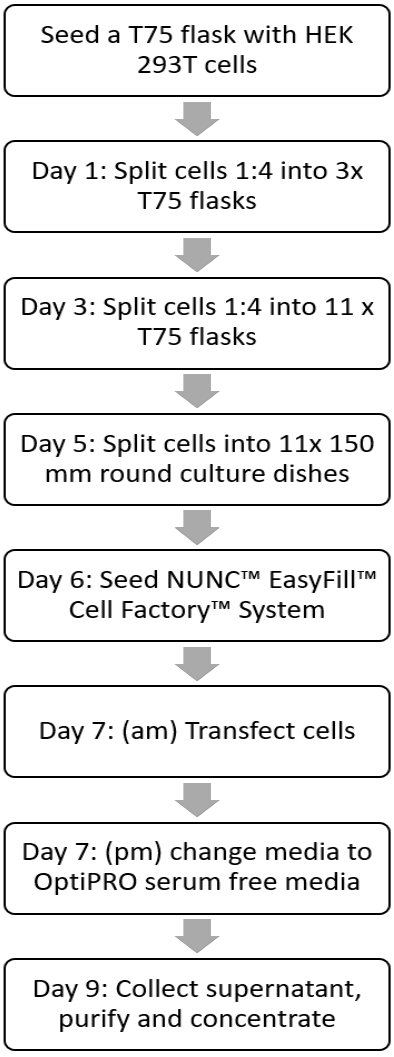

Figure 1. Workflow diagram of the vector production protocol. Flow diagram outlining the main steps at each day of vector production, including culturing HEK 293T cells, transfection of cells with lentiviral plasmids, changing to a serum free media and finally the purification and concentration of the final product.

Vector harvest and purification

- ✓ Fetal calf serum (FCS) (Australian origin) (Gibco, cat. # 10099-141)
- ✓ Bovine serum albumin (BSA) (Sigma-Aldrich, cat. # A7906)
- ✓ NaCl (Sigma-Aldrich, cat. # S3014)
- ✓ 0.9% saline for injection (Fresenius Kabi, cat. # AUST R 197200)
- ✓ Heat-inactivated serum from the species that the vector preparation is intended for (*e.g.*, mouse serum if the vector is to be delivered to mice) to minimize potential immune reaction.
- ✓ 500 ml centrifugation bottles (Beckman Coulter, cat. # 355607)
- ✓ 50 mm Polydisc AS 0.45 µm Whatman filter (GE Healthcare Life Sciences, cat. # 6724-5045)
- ✓ Mustang® Q XT5 capsule (Pall Corporation, cat. # XT5M-STGQPM6)
- ✓ Masterflex peroxide-cured silicone tubing L/S 16 (Masterflex, cat. # 46400-16)
- ✓ Male luer lock with 3.2 mm (1/8") hose barb connector (Cole-Parmer, cat. # EW-45505-04)
- ✓ Female luer lock with 3.2 mm (1/8") hose barb connector (Cole-Parmer, cat. # EW-45500-04)
- ✓ 14 ml thin wall polyallomer ultracentrifuge tubes (Beckman Coulter, cat. # Z604085CA)

Mustang Q XT5 maintenance

- ✓ NaOH (Sigma-Aldrich, cat. # 55881)
- ✓ NaCl (Sigma-Aldrich, cat. # S3014)

Equipment

- ✓ Cole Parmer Masterflex® L/S® pump (Cole Parmer, model # 77200-50)
- ✓ Optima™ L-100 XP Ultracentrifuge with a SW40 Ti rotor (Beckman Coulter, cat. # 392050 and # 331302)
- ✓ Avanti® J-E Centrifuge with a JA-10 rotor (Beckman Coulter, cat. # 369001 and # 369687)

Recipes

- ✓ HEK 293T cell growth media: DMEM, 10% FCS, 10 units/ml Penicillin, 10 µg/ml Streptomycin
- ✓ 2× HeBS buffer: 1.5 mM Na_2HPO_4, 50 mM HEPES, 0.28 M NaCl in water, adjust pH to 7.08 and filter-sterilise
- ✓ 2.5 M $CaCl_2{\cdot}2H_2O$ in water and filter-sterilise
- ✓ Harvest media: OptiPRO™ SFM, 10 units/ml Penicillin, 10 µg/ml Streptomycin, 4 mM L-glutamine
- ✓ 2% heat inactivated serum: use serum from the species that the vector preparation is intended for to minimize potential immune reaction. Dilute serum in H_2O and incubate at 65°C for 30 min
- ✓ 200 mM L-Glutamine in water and filter-sterilise
- ✓ 2.5% (w/v) BSA in PBS
- ✓ 1.5 M NaCl in water and filter sterilise
- ✓ Mustang Q XT5 column equilibration buffer: 1 M NaOH in water
- ✓ Mustang Q XT5 column storage buffer: 0.1 M NaOH and 1 M NaCl in water

PROCEDURE

1. Culture of sufficient HEK 293T cells

   1.1. Pre-warm PBS, DMEM/10%FCS/1%Pen-Strep, and 10% TrypLE-Express in PBS to 37°C.

   1.2. Thaw a vial of frozen HEK 293T cells rapidly in a 37°C water bath and transfer to a 50 ml conical tube and make up to 50 ml with PBS. Centrifuge at 450× *g* for 5 min at 4°C. Aspirate the supernatant, re-suspend the cell pellet in DMEM/10% FCS/1% Pen-Strep and transfer to a T75 flask. Incubate at 37°C in 5% CO_2.

   1.3. When the cells are 90% confluent, split cells (1:4) by aspirating media and washing cells with 4 ml PBS. Aspirate PBS and trypsinize cells using 4 ml of 10% TrypLE-Express in PBS, incubate for 5 min at room temperature, or until cells start to detach. Add 4 ml of media, spritz cells and transfer 2 ml to 3 × T75 flasks containing 12 ml of media.

   NOTE: It takes 20–24 h for HEK 293T cells to double so ensure that splitting the cells (1:2 or 1:4) is performed at the same time each morning.

   1.4. When cells are 90% confluent (approximately 48 h after seeding), repeat step two to achieve a total of 11 × T75 flasks.

   1.5. When the 11 × T75 flasks are 90% confluent, harvest cells as previously and transfer one T75 flask into one 150 mm round culture dish with a final volume of 32 ml.

2. Cell factory seeding

   2.1. Pre-warm PBS, DMEM/10%FCS/1%Pen-Strep, and 10% TrypLE-Express in PBS to 37°C.

   2.2. When the HEK 293T cells in the 150 mm round culture dishes are 90% confluent, harvest by first aspirating the media and washing cells with 6 ml of PBS. Aspirate PBS and detach cells by incubating them with 6 ml of 10% TrypLE-Express for 5 min. Add 6 ml of media to neutralize TrypLE-Express, spritz cells and transfer to a 1 L bottle.

TIP: Using a left-over 1 L media bottle is a useful alternative to using multiple tubes or containers.

2.3. Rinse each round culture dish with fresh media and add to the 1 L bottle containing the cell suspension.

2.4. Perform a viable cell count on the cell suspension and determine the volume of cells required to achieve a final concentration of 3.476×10^5 cells/ml in a total of 1500 ml. Transfer this volume to a sterile 2 L bottle and bring up to a total of 1500 ml with media.

CRITICAL STEP: Using a cell density of 1.65×10^5 cells /cm^2 will ensure that the cells will reach 70%–90% confluency at 24 h post-seeding.

2.5. Pour the cell suspension into a NUNC™ EasyFill™ Cell Factory™ System and equilibrate the chambers as per the manufacturer's instructions.

TIP: Pour the cell suspension into the large port and ensure that media enters all the layers during pouring. This will minimize the formation of bubbles within the large port that can make it difficult to transfer the full 1500 ml.

2.6. Seed a T75 flask at a density of 6.14×10^6 cells/ml to use as a reference of confluency for the cell factory. The reference flask is used to assess cell confluency because the contents of the cell factory cannot be observed under the microscope.

TIP: This number of cells in a T75 ensures that the density is 1.65×10^5 cells/cm^2, which is the same as the cell factory seeding density.

3. Plasmid transfection

3.1. We advise that users optimize the plasmid ratios for their system using small-scale preparations. Here we use example ratios for a second-generation, 5-plasmid LV vector system.

3.2. Prepare plasmid mix (with all reagents at room temperature) in a 50 ml conical tube as per **Table 1**.

3.3. Add the plasmid mix to a 50 ml conical tube containing 4.5 ml of 2.5 M $CaCl_2$ and make up to 45 ml with sterile water.

CRITICAL STEP: Ensure the pH of the 2× HeBS is exactly 7.08 as this is critical for effective DNA calcium-phosphate co-precipitation.

3.4. Aliquot 22.5 ml of room temperature 2× HeBS into two 225 ml conical tubes.

3.5. Begin vortexing the first 225 ml conical tube, and using a 25 ml serological pipette add 22.5 ml of plasmid solution dropwise over 30–40 s. Continue to vortex for a further 20–30 s and set aside at room temperature.

CRITICAL STEP: Smaller tubes/containers will not support vortexing this large volume at a high speed without spillage. Ensure the plasmid mix is added in a dropwise fashion to maximize complex formation between plasmids and calcium phosphate.

Table 1. Plasmid quantities used for transfection of one cell factory (based on a second generation, 5-plasmid system).

	DNA mass (µg)	DNA length (bp)	DNA copy number	Plasmid volume (µl)
Expression vector	2252.58	8986	2.32E + 14	
pcDNATat	39.98	5668	6.53E + 12	
pHCMV-Rev	39.98	5789	6.40E + 12	DNA mass (µg)/plasmid concentration (µg/µl)
pHCMV-gagpol	25.29	9771	2.40E + 12	
pVSV-G	100.08	6363	1.46E + 13	

Note: When using plasmids of different DNA lengths, it is important to maintain the same DNA copy number.

3.6. After a total of 90 s has elapsed from initial vortexing of the first 2× HeBS in the 225 ml conical tube, begin vortexing the second 225 ml conical tube containing 2× HeBS and again add 22.5 ml of plasmid solution dropwise over 30–40 s. Continue to vortex for a further 20–30 s and set aside.

3.7. Retrieve the NUNC™ EasyFill™ Cell Factory™ System from the incubator, remove both caps and gently pour ~400 ml of media into a 1 L bottle *via* the small port, ensuring that cell disturbance is minimized. After four minutes has elapsed from initial vortexing of the first 225 ml conical tube, add the DNA complexes from the first 225 ml conical tube to the ~400 ml of media. After a total of five and a half minutes has elapsed, add the second 225 ml conical tube.

CRITICAL STEP: This incubation period is important to aid in the plasmid-calcium phosphate complex formation.

3.8. With care to minimize cell disruption, gently pour the media containing the plasmid solution back into the 10-layer cell factory *via* the large port, equilibrate again as per the manufacturer's instructions, and place back in the 37°C incubator with 5% CO_2 for eight hours.

3.9. During the eight-hour incubation, pre-warm 1.5 L of OptiPRO SFM supplemented with 4 mM L-glutamine and 1% Pen-Strep to 37°C.

3.10. At eight hours post-transfection, perform a media change by pouring out all of the media from the NUNC™ EasyFill™ Cell Factory™ System *via* the small port (remove as much excess media as possible) and subsequently add 1.5 L of pre-warmed OptiPRO™ SFM into the large port. Pour gently to ensure minimal disturbance to adhered cells and equilibrate media amongst the layers.

TIP: Eliminating FCS from the final vector product is recommended to minimize the risk of a potential immune response to *in vivo*.

3.11. Incubate at 37°C and 5% CO_2 for a further 40 h.

4. LV vector harvest

4.1. Pre-weigh 2 × 1 L bottles.

TIP: Use the OptiPRO™ SFM bottles from the previous day.

4.2. Remove the NUNC™ EasyFill™ Cell Factory™ System from the incubator and carefully pour the viral supernatant into the pre-weighed 1 L bottles.

4.3. Add 200 ml of PBS to the large port of the NUNC™ EasyFill™ Cell Factory™ System and equilibrate to rinse any remaining viral supernatant. Pour the PBS rinse into the pre-weighed 1 L bottles.

4.4. Weigh the bottles again to determine the volume of supernatant collected and add BSA (stored on ice) to achieve a final concentration of 0.1% BSA to the supernatant.

HINT: Divide the volume of the supernatant (assuming 1 g = 1 ml) by 24 and then add that volume of 2.5% BSA (w/v) in PBS to make a final BSA concentration of 0.1%.

4.5. Transfer the supernatant to 500 ml centrifuge bottles. Rinse each 1 L bottle with 20 ml PBS and add to 500 ml centrifuge bottles.

4.6. Centrifuge supernatant at 400× *g* for five minutes at 4°C to remove cellular debris. Pour supernatant back into the 1 L bottles and keep refrigerated at 4°C until required.

5. Lentiviral purification and concentration

5.1. To set up the Mustang Q XT5 column purification system (**Fig. 2**), connect a 70 cm piece of Master-Flex tubing to the Watson Marlow 323 pump. Pump PBS through the tubing at a rate of 10 ml/min, ensuring no bubbles are present within the tubing.

CRITICAL STEP: It is very important to ensure no air bubbles enter the system as the presence of bubbles can cause damage to the lentivirus particles and can significantly reduce the final yield.

5.2. Attach a 50 mm Polydisc AS 0.45 µm Whatman filter at the end of the tubing, secure with a cable-tie, and continue to flush PBS until the filter is saturated.

TIP: Ensure that the inlet side of the 0.45 µm filter is facing downward so that gravity forces the supernatant to

pass through the filter across the entire bed.

5.3. Attach a 10 cm length of MasterFlex tubing with a tubing clamp to the outlet end of the 50 mm Polydisc AS 0.45 µm Whatman filter, secure with a cable-tie, and flush with PBS, ensuring no bubbles are present within the tubing.

5.4. Attach a male M6 thread with a hose barb connector to the Mustang Q XT5 column and connect the inlet port to the 10 cm piece of MasterFlex tubing, securing with a cable-tie. Connect a 40 cm piece of MasterFlex tubing to the outlet port with a male M6 thread with a hose barb connector, securing with a cable-tie, and place the Mustang Q XT5 column in a retort stand with the outlet facing upwards. Place the end of the tubing into a large container to collect the waste.

TIP: Like the filter, ensure that the outlet port of the Mustang Q column is facing up so that gravity forces the supernatant to pass through the entire surface area of the column.

5.5. Continue to pump PBS through the system at 10 ml/min to flush out the buffer that the column is stored in. Once the pH is ~7, the system is ready to use (this can be assessed by using pH indicator strips or a pH meter).

CAUTION: A pH of ~7.20 must be used for all solutions post-harvest as this is the optimal pH environment for the vector.

5.6. Place the 70 cm length of tubing into a 1 L bottle of vector supernatant that has been stored at 4°C. Pump the supernatant through at 10 ml/min until system has been completely flushed with supernatant and then increase the pump rate to 30 ml/min.

5.7. Once all but a few ml of the vector supernatant has been pumped through the system, rinse 1 L bottles with 150 ml of PBS containing 0.1% BSA and pump through the system.

5.8. Turn off the pump before the 10 cm length of tubing has air introduced into it to ensure air bubbles do not enter the Mustang Q XT5 column. Close the tubing clamp and cut off the lower 7 cm of tubing. Attach a female luer fitting with a hose-barb connector to the end of the tubing (that is attached to the column), and secure with a cable-tie.

CAUTION: Clamping of the tubing is important to prevent supernatant leaking from the system due to gravity.

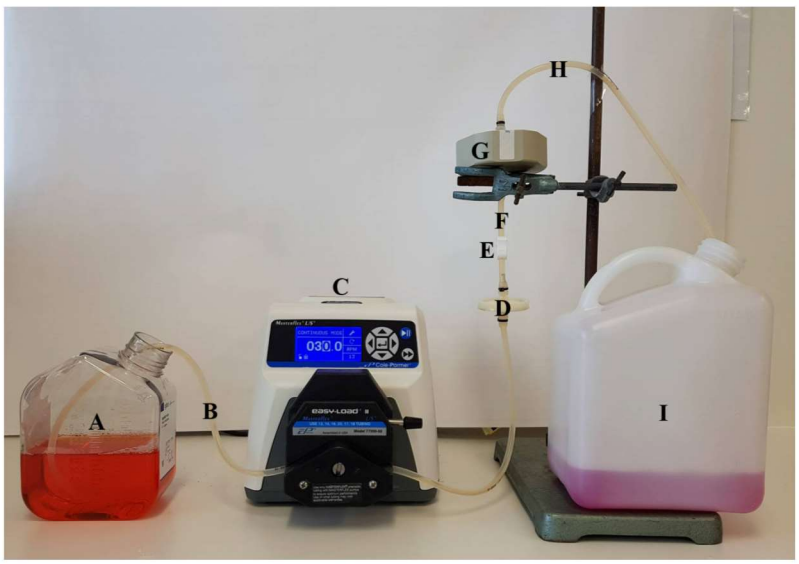

Figure 2. Vector purification setup. A. Vector supernatant obtained from cell factories. **B.** 70 cm length of MasterFlex tubing. **C.** Watson Marlow 323 pump. **D.** 50 mm Polydisc AS 0.45 µm Whatman filter. **E.** Tubing clamp. **F.** 10 cm length of MasterFlex tubing. **G.** Mustang Q XT5 column. **H.** 40 cm length of MasterFlex tubing. **I.** Waste container.

5.9. Using a 50 ml male luer lock syringe, flush the column with 60 ml of PBS.

5.10. Cut off all but the last 10 cm of the 40 cm piece of tubing connected to the Mustang Q XT5 outlet port. Using a 50 ml syringe, elute the vector with 25 ml of 1.5 M NaCl (stored on ice), and capture into 25 ml of 2% heat inactivated animal serum (stored on ice).

5.11. Transfer the elution equally into 4 × 14 ml thin wall polyallomer tubes and place into chilled SW 40 Ti buckets. Centrifuge at 20000 rpm for 90 min at 4°C.

5.12. Following ultracentrifugation, gently pour off the supernatant, ensuring that the vector pellet is not disturbed, and blot tubes on tissue paper to remove as much residual liquid as possible.

5.13. Resuspend the vector pellets in 0.9% saline (stored on ice) by pipetting up and down, while ensuring that no bubbles form and clumps are no longer visible. Rinse tubes to maximize vector recovery. Once resuspended, add 0.1% (v/v) heat inactivated animal serum and keep on ice when not re-suspending.

TIP: To maximize vector recovery, initially resuspend vector pellets in less than a quarter of the desired final volume. Transfer this volume between each ultracentrifuge tube and finally transfer into a screw-cap 1.5 ml microcentrifuge tube. Then rinse the centrifuge tubes with a further quarter of the final resuspension volume, pool into the same 1.5 ml screw-top microcentrifuge tube and repeat with a third wash with a quarter of the final resuspension volume. Top up to the desired final volume.

5.14. Aliquot vector into screw cap tubes and store at −80°C. Avoid freeze-thawing as this can decrease titers up to 10-fold /cycle.

5.15. Test virus functionality using an appropriate method based on the expression plasmid used (**Fig. 3**).

6. Mustang Q XT5 column equilibration and storage

6.1. After elution, equilibrate the column by pumping 25 ml of equilibration buffer at room temperature through the column at 10 ml/min. Stop the pump, close the tubing clamp, and allow to rest for 30 min.

6.2. Pump through 25 ml of Mustang Q XT5 column storage buffer at room temperature at 10 ml/min, stop the pump, disconnect the column, and cap the inlet and outlet ports. Store at room temperature until next use.

NOTE: This allows for long term usage of the column and will limit bacterial growth.

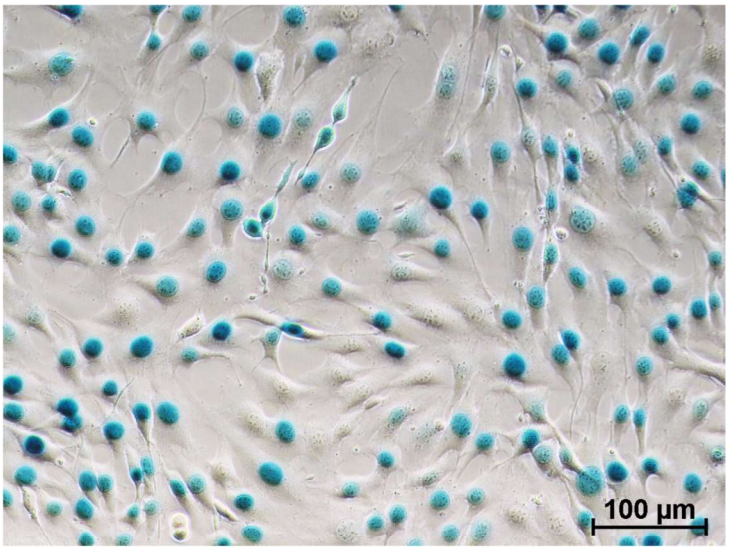

Figure 3. LacZ transgene expression in NIH 3T3 cells following transduction with cell factory produced LV-LacZ vector and subsequent histological X-Gal staining.

TROUBLESHOOTING

Possible problems and their troubleshooting solutions are listed in **Table 2**.

Table 2. Troubleshooting guide.

Step	Problem	Cases	Suggestions
2.3	Low cell yield	• Cells under-confluent • Culture plates not rinsed thoroughly • Poor cell health	• Allow cells to expand longer • Rinse culture plates thoroughly to maximize cell yield • Increase the number of round culture dishes • Thaw a new batch of cells • Check for mycoplasma contamination • Do not overgrow cells (keep culture in logarithmic growth phase)
3.5	HeBS/plasmid complex solution is not cloudy	• Plasmid solution added too quickly • Incorrect pH of 2× HeBS • Not vortexed vigorously	• Plasmid solution needs to be added slowly and dropwise to 2× HeBS • Ensure HeBS has a pH of 7.08 (± 0.01) • Vortex as vigorously as possible
3.5	HeBS/plasmid complex solution has precipitated out	Incorrect calcium chloride used	Make sure to use $CaCl_2 \cdot 2H_2O$ (MW-147.01 g/mol)
5.7	Clogged filter	Too much debris in supernatant	• Replace filter • Centrifuge supernatant twice • Use a filter with a larger binding surface area • Use a series of filters with decreasing pore size
5.14	Low titer	• Poor plasmid quality • Unhealthy cells • Mustang Q XT5 column not equilibrated	• Check quality of plasmids • Ensure only healthy cells are used for production • Re-equilibrate the Mustang Q XT5 column following part 6: Mustang Q XT5 column equilibration and storage • Check the ultracentrifugation tubes are a suitable plastic (*e.g.*, polyallomer)

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Chapter 4: Lentiviral vector production using bioreactor systems

Transient lentiviral vector production using a packed-bed bioreactor system

By Alexandra McCarron, Martin Donnelley, Chantelle McIntyre and David Parsons

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The LV production field is moving towards the use of bioreactor systems to overcome scalability issues and limitations associated with current two-dimensional culture technologies. Bioreactors provide several advantages for producing biomolecules when compared to standard production vessels such as flasks, roller bottles and cell factories. Notable benefits include amenability to automation, availability of online monitoring, the ability to tightly regulate production conditions such as pH and oxygen, ease of scale-up, lower labour input, and reduced risk of batch contamination due to fewer manual manipulations.

Both suspension and adherent cell-based methods are being pursued for LV vector bioreactor production [117-120]. These two approaches have respective advantages and disadvantages that need to be considered when transitioning to bioreactor-based production. Suspension-cell based LV production in stirred-tank reactors has the potential to be scaled-up in a linear fashion by increasing the vessel size to thousand-litre capacities. However, the main difficulty with this approach is the need for compatible downstream processes that can efficiently clarify large batches of LV supernatant. Suspension cells can also be cultured without animal-derived products such as serum, thus reducing dependence on unreliable supply chains and the risk of contaminating the production lot with adventitious agents. While early suspension LV production trials initially revealed lower titres than conventional adherent-cell methods, ongoing optimisation efforts have now bridged this gap making suspension-based approaches feasible [120].

Adherent-cell based bioreactors (e.g. packed- or fixed-beds) contain a substrate for cell-adhesion, which separates the producer cells from the LV supernatant and enables simplified purification. Packed- or fixed-bed bioreactor systems tend to have large surface-area to

volume-ratios, therefore the productivity is high and the volume of supernatant to be processed is low. Unlike stirred-tank bioreactors however, adherent-based systems cannot be scaled-up linearly. Increases in production capacity are typically achieved by scaling-out, which involves the addition of parallel production units. Scaling-out is generally more technically challenging than scaling-up, however, one benefit of a scale-out approach is that contamination of a unit does not result in loss of the whole production lot. Initial expansion of adherent cells with flasks or cell factories is still required for bioreactor seeding, thus increasing the labour associated with this approach.

The aim of this study was to establish a bioreactor-based LV production method to increase LV production capacity in an academic setting. Ideally, the method would also be amenable to use in a clinical-production scenario, with additional modifications to achieve Good Manufacturing Practice (GMP)-grade standards. To develop a bioreactor-based production method, various systems were initially assessed as potential options. This chapter begins by detailing three bioreactor trials that were performed to select a bioreactor production approach to pursue.

4.1. Bioreactor trials

From the locally-available bioreactor options, three production systems were selected for in-house trialling. These systems all employed single-use vessels as unlike stainless steel options their operation is relatively simple for the user, there is no need for labour-intensive vessel cleaning, and on-site sterilisation facilities are not required. Moreover, single-use technology allows for the scale of the operation to be easily altered if production needs change. For simplicity, all systems were run in batch-mode, which consisted of inoculating the media and allowing the cells to grow without medium feeding or removal.

All runs employed polyethylenimine (PEI, 25 KDa linear) mediated transient transfection and a second-generation, 5-plasmid LV system consisting of helper plasmids rev, tat, and gagpol, a vesicular stomatitis virus glycoprotein (VSV-G) envelope and HIV-1 derived transfer vector (HIV-1-MPSV-nlsLacZ) [151]. A plasmid ratio of vector: env: rev: tat: gagpol of 9.1: 0.4: 0.2: 0.2: 0.1 was used and the DNA to PEI mass-ratio was 1:3. Figure 1 illustrates the bioreactor systems employed in the trials. In addition to these bioreactor trials, the commercially available LV-MAX™ Lentiviral Production System (Gibco) was trialled at small-scale in shaker flasks as a potential alternative for the in-house developed suspension system.

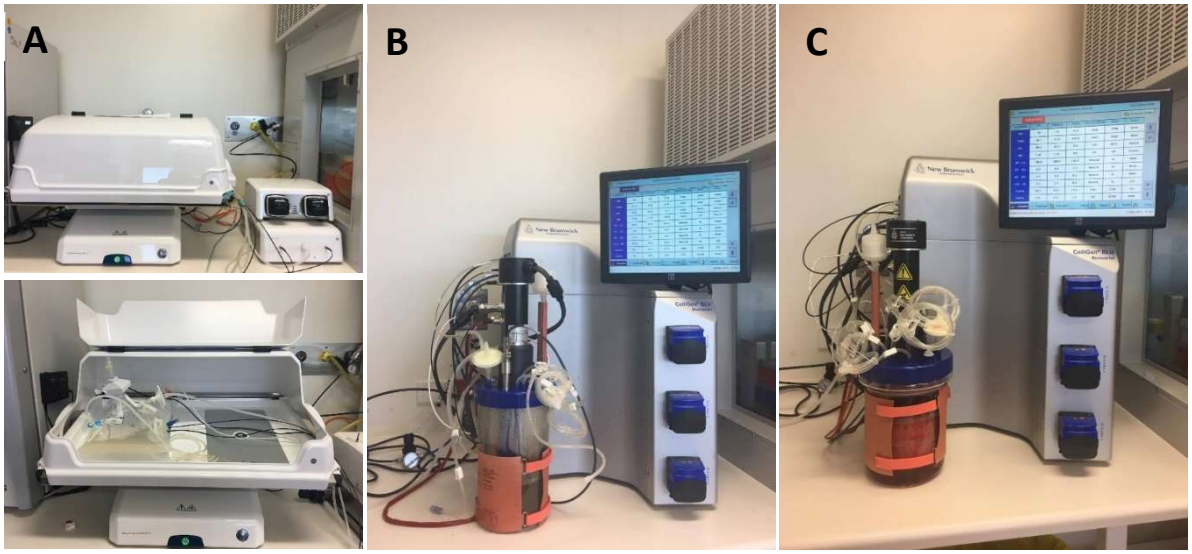

Figure 1: Bioreactor system that was employed for each trial. **(A)** Wave bag vessel operated with the ReadytoProcess WAVE25 system (GE Healthcare). **(B)** Stirred-tank vessel and **(C)** packed-bed vessel both operated with the CelliGen BIOBLU controller (Eppendorf).

4.1.1. Wave bioreactor

A wave (or rocker) bioreactor system consists of an inflated disposable plastic bag that acts as the cell cultivation vessel. The bag sits on a rocking tray that agitates cells in a back and forward wave-like motion to achieve oxygen-transfer and mixing. The wave system has a several benefits. It is simple to operate, maintains a low shear-stress environment for the cells as they are not directly exposed to a fast-moving impeller and gas sparging is not required, and is relatively low-cost due to the elimination of expensive and complex instrumentation. The simplicity and low expense of a wave system makes it particularly well suited to performing lab or pilot-scale trials in academic settings [152]. The wave bioreactor platform has previously been used for the production of gamma-retroviral vectors by employing transient transfection of HEK 293T cells [153], therefore it was reasonable to suggest that this system would also be suited to LV production.

In this trial, the ReadytoProcess WAVE 25 (GE Healthcare) was used. In-house suspension-adapted HEK 293T cells were cultivated in a 3 L wave bag in FreeStyle™ serum-free medium (Life Technologies) with a total working volume of 0.5 L. The HEK 293T cells were expanded in shake flasks and the bioreactor culture was seeded at a density of 5.0 x 10^5 cells/mL. The bioreactor conditions used for this run are outlined in Table 1.

During the trial the cells aggregated into large clumps, most likely due to the low rocking speed used. Extreme cell clumping is known to be detrimental as it can limit transduction

efficiency by preventing DNA complexes from reaching the cells trapped within the interior of the clump [154]. Furthermore, cells trapped in large aggregates can demonstrate reduced viability and growth due to restricted nutrient diffusion [155]. This phenomenon was observed on the final day of culturing (7-days post seeding) where the cell viability dropped to 60% and density plateaued at 9.0 x 10^5 cells/mL. Due to poor growth the target cell density for transfection was not achieved.

It is difficult to draw conclusions from this stand-alone trial of the wave bioreactor. Additionally, the conditions were unoptimised, therefore it is likely that altering parameters such as gas flow rates, rocking speed, seeding density, along with the implementation of media feeding strategies would have likely improved cell growth and viability. However, there is no guarantee that any particular bioreactor system will be suited to the cell line or biomolecule being produced. There are also inherent disadvantages to using a wave system that need to be considered. Unlike stirred-tanks, large production volumes with wave bioreactors have not yet been realised [156]. As such, these systems are better suited to speciality applications, process development stages, seed cultivation steps, or cell bank generation rather than large-scale biomolecule production. Due to these drawbacks and difficulties with the trial run, the wave bioreactor was not pursued further.

Table 1. Wave bioreactor trial conditions.

Parameter	**Set point**
Rocking speed	20 RPM
Rocking angle	7°
Gassing	2-gas mix control (Air and CO_2), 0.1 SLPM
Temperature	37°C
pH	7.2
DO	50%

RPM, revolutions per minute; SLPM, standard litre per minute

4.1.2. Stirred-tank bioreactor

Stirred-tank bioreactors are widely used in industry for producing a range of biomolecules due to their high productivity and amenability to up-scaling. As stirred-tanks have superior oxygen transfer rates, they are particularly suited to the growth of high-density cell cultures in large volume tanks [156]. There are many existing variations on the design of stirred-tank bioreactors, however, the core component of a stirred-tank reactor is the agitator or impeller [157].

In this trial, in-house suspension-adapted HEK 293T cells were cultivated in a rigid single-use vessel with a working volume range of 1.25 – 3.75 L (BioBLU 3c, Eppendorf). The vessel was equipped with a pitched-blade impeller, which is suited to the cultivation of shear-sensitive mammalian cell lines. The production conditions outlined in Table 2 were regulated using the CelliGen® BLU controller (Eppendorf). Cells were initially expanded in shake flasks and the vessel was seeded at a density of 5.0 x 10^5 cells/mL in a total of 1.3 L FreeStyle™ media (Life Technologies). At day three post-seeding the cell density had reached 2.3 x 10^6 cells/mL and transient transfection was performed at using 7.2 mg plasmid DNA and 21.6 mg PEI. Unconcentrated LV vector samples were collected at 24, 48- and 72-hours post-transfection for titering.

The unconcentrated titres from the stirred-tank trial peaked at 48 hours post-transfection, with an average of 1.7 x 10^4 TU/mL. While functional vector was obtained employing this method, the titres were low when compared to standard adherent-based production methods [158]. At these current titres a stirred-tank method would not be considered economical, however, further process optimisation is likely to improve LV yields. While not further pursued here, a stirred-tank bioreactor approach remains a viable option that has great potential for large-scale LV production.

Table 2. Stirred-tank bioreactor trial conditions.

Parameter	**Set point**
Agitation	45-70 RPM
Gassing	2-gas mix control (Air and CO_2), 0.015 - 0.1 SLPM
Temperature	37°C
pH	7.2
DO	50%

RPM, revolutions per minute; SLPM, standard litre per minute

4.1.3. Packed-bed bioreactor

Packed-bed bioreactors consist of a carrier substrate for adherence of cells and a reservoir of media that is recirculated through the bed for distribution of nutrients and oxygen [159]. Packed-bed vessels have a range of advantages for biomolecule production. They have an extremely high surface area to volume ratio, therefore high cell densities can be attained leading to increased productivity. As the cells are separated from the media, downstream clarification is simplified and the system can be readily operated with different modes of media feeding [157]. The packed-bed also protects cells from impeller-induced mechanical shear, turbulence, and bubbles that arise from gas sparing [159, 160].

For this trial, the BioBLU 5p vessel (Eppendorf) was employed with a 3 L working volume. This vessel contains Fibra-Cel® carrier disks, which provides 18 m^2 of surface for cell adherence. The CelliGen® BLU controller was used to regulate the production conditions outlined in Table 3. Adherent HEK 293T cells were cultivated in 10-layer cell factories and the packed-bed was seeded at a density of 7 x 10^3 cells/cm^2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycin (PenStrep). Three days post-seeding the cells were transfected with 18.9 mg DNA and 56.7 mg PEI. Eight hours later a media change was performed into OptiPRO serum-free media (Gibco) supplemented with 1% PenStrep and 4 mM L-glutamine. The viral supernatant was harvested at 48 hours post-transfection for purification and concentration using anion-exchange chromatography and ultracentrifugation as previously described [158].

LV vector supernatant titres achieved 3.0 x 10^5 TU/mL and concentrated titres were 5.4 x 10^8 TU/mL. While further optimisation is necessary, the results from this initial trial were promising and warranted further investigation of this system. Accordingly, the packed-bed was selected for further process development.

Table 3. Packed-bed bioreactor trial conditions.

Parameter	Set point
Agitation	80 RPM
Gassing	2-gas mix control (Air and CO_2), 0.04 – 0.1 SLPM
Temperature	37°C
pH	7.2
DO	50%

RPM, revolutions per minute; SLPM, standard litre per minute

4.2. LV-MAX™ production system

The LV MAX™ production system was briefly explored as an alternative production approach to the in-house developed suspension method. LV MAX™ is a commercial kit that consists of five components: suspension HEK 293 cells, LV MAX™ production media, a novel transfection reagent, a supplement that is added prior to transfection, and an enhancer that is added post-transfection. The LV MAX™ system was trialled in small-scale shaker flasks with a 30 mL production volume. The LV MAX™ production system achieved supernatant titres of 3 x 10^6 TU/mL. These titres were superior to the in-house developed suspension LV production system, where maximum titres of 6 x 10^4 TU/mL were achieved in shake flask optimisation trials. The LV MAX™ kit offers a potential option for achieving high LV vector titres in a suspension, serum-free system that will ultimately be amenable to clinical-grade GMP

production. However, the high expense of the reagents is a major limitation that inhibits uptake of this system, particularly for preclinical research phases.

4.3. Chapter preface

The following chapter consists of an original research article published in the journal of Human Gene Therapy Methods. This paper describes a novel method for LV production that employs the packed-bed bioreactor system. In addition to method development, titres were compared between the packed-bed system and standard 10-layer cell factory to demonstrate whether a bioreactor production approach would be feasible. Finally, the *in vivo* functionality of the packed-bed produced LV vector was validated by performing an airway gene transfer trial in rats. In addition to this manuscript, an application note for this method has been developed in collaboration with Eppendorf.

Statement of Authorship

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Principal Author

Name of Principle Author (Candidate)	Alexandra McCarron		
Contribution to the Paper	Conception and design, data collection, analysis and interpretation, and drafting of manuscript.		
Overall Percentage (%)	90%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	15/10/2020

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Transient Lentiviral Vector Production Using a Packed-Bed Bioreactor System

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Scalable lentiviral vector (LV) manufacturing is vital for successful commercialization of LV-based gene and cell therapy products. Accordingly, efforts are currently focused on developing and adapting technologies to address both upstream and downstream production bottlenecks. To overcome the limitations of current upstream processes, researchers are now favoring the use of bioreactors over traditional two-dimensional culture platforms. Bioreactors provide many advantages for manufacturing biomolecules, including process automation, tight regulation of production conditions, reduced labor input, and higher productivity potential. This study describes a transient LV production strategy employing a single-use, packed-bed bioreactor vessel. Functional LV titers in the 10^6 TU/mL range were achieved, and after concentration yields of up to 10^9 TU/mL were attained. This proof of principle study demonstrates that LV can be successfully produced in a packed-bed system. With further optimization, a packed-bed bioreactor could offer a potential scale-out solution for LV manufacturing.

Keywords: lentiviral vector, packed-bed bioreactor, Fibra-Cel® disks, transient transfection, HEK 293T

INTRODUCTION

LENTIVIRAL (LV) VECTORS are efficient gene delivery tools used in a range of gene and cell therapies designed to treat both inherited and acquired diseases. As candidate LV-based gene and cell therapies move into clinical trial phases,[1–5] a major bottleneck to commercialization is the lack of scalable LV production methods. The high cost of goods associated with standard LV production methods is also a hindrance. To overcome these hurdles, scalable, cost-effective, and reproducible LV production approaches must be established. LVs are most commonly produced by multi-plasmid, transient transfection of adherent human embryonic kidney (HEK) 293T cells that are cultivated in two-dimensional culture systems. At the largest scale, multilayer cell factories can be used in a scale-out system,[6,7] although this approach has disadvantages: initial cell build-up and manipulation of the factories is labor-intensive, there is batch-to-batch variability, and the substantial incubator space requirements are uneconomical.[8] Ultimately, two-dimensional culture technologies do not have the scalability to sustain the growing demand for LV.

Adapting adherent HEK 293T cells to suspension-based production is desirable for moving into traditional large-scale technologies such as stirred-tank bioreactors. However, optimization of these methods is still ongoing, and this type of suspension approach has not yet been used in a clinical setting.[9–11] Compared to suspension processes that are still under development, high titers are already being achieved using adherent systems.[6,12,13] The transition from cell factories to a packed-bed bioreactor system is relatively straightforward, can be easily translated from preclinical to clinical-scale production, allows for process automation and online monitoring in a closed system, and offers advantages for scalable production by using a scale-out strategy. Packed-beds have a large surface-area-to-volume ratio, allowing for exceptionally high cell densities, thereby maximizing the productivity and potency of each batch while minimizing the supernatant volume to be processed. The expan-

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sion of cells is simplified because a reasonably low cell seeding density can be used, with most of the cultivation occurring in the vessel by use of perfusion mode. As the packed-bed separates cells from the medium, perfusion mode, medium exchange, product harvest, and downstream clarification steps are less complicated.[14] Packed-beds are also suited to both transient and stable LV production systems, as well as the use of adherent and suspension cell lines.

Bioreactor manufacturing capacity can be increased by employing either a scale-out (addition of parallel vessels) or scale-up (increasing the size of the vessel) model. Traditional stirred-tank bioreactors tend to be based on the scale-up model, while packed-bed reactors are better suited to a scale-out system. Scale-up biomanufacturing is currently considered to be more cost-effective. However, employing a scale-out model can have several advantages. Maintaining the same bioreactor size throughout all development phases means that a high level of process understanding can be attained in the early stages, thus reducing the time needed to optimize and validate methods for clinical use. A scale-out approach also enables manufacturing decisions to be made when material is needed, thereby allowing for flexibility during clinical and market demand cycles. Compared to large stirred-tank bioreactors, scale-out systems are lower risk, as an adverse event in one bioreactor does not result in loss of the whole production lot. Furthermore, lower upfront capital investment is required to create the facility, and once established it can be validated faster than traditional large-scale bioreactor sites. When employing a scale-out model, packed-bed bioreactors are often run with either single-use or reusable vessels. Single-use vessels allow for faster turnover between manufacturing lots and a reduced chance of contamination events. However, the costs are greater.[15,16]

The aim of this study was to demonstrate that LV can be transiently produced in single-use, BioBLU® packed-bed bioreactor vessels (Eppendorf). These vessels consist of porous Fibra-Cel® disks that provide a solid growth matrix for the attachment and cultivation of cells. The disks are enclosed within two perforated screens, which sit in a reservoir of medium allowing for distribution of nutrients throughout the bed using a hollow-tube impeller. Unlike conventional microcarriers, the packed-bed protects the cells from impeller-induced mechanical shear, turbulence, and bubbles that arise from gas sparing.[14,17] The total packed-bed surface area is 18 m^2, equivalent to approximately 28 standard 10-layer cell factories.

While this study describes the use of Fibra-Cel® disks in a packed-bed configuration, they can also be used as "free-floating" carriers for cultivating adherent and suspension cells in stirred-tank bioreactors, allowing the potential for a conventional scale-up approach. Fibra-Cel® disks have been used in various bioreactor systems for stable and transient retroviral production,[14,18] stable LV production,[19] and recombinant adenovirus production.[20] However, this study demonstrates for the first time that Fibra-Cel® disks can be used for transient LV production in a packed-bed vessel system.

METHODS

Cell culture

HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Gibco) and 1% penicillin/streptomycin (PenStrep; Gibco), in a humidified incubator at 37°C and 5% CO_2. Cells were initially expanded in T-75 and T-175 flasks for bioreactor inoculation. The number of viable cells was counted using a hemocytometer and trypan blue exclusion.

Packed-bed bioreactor culture

The BioBLU® 5p single-use packed-bed vessel with macrosparger and hollow-tube impeller was used (Eppendorf). The vessel was operated in batch mode with a working volume of 3 L (total 3.75 L, including packed-bed volume). A medium hold test was performed overnight to check for presence of contamination, and the following day, the 150 g Fibra-Cel® disks were seeded at a density of approximately 4×10^3 cells/cm^2 (a total of $\sim7\times10^8$ cells). The BioFlo® 320 control station (Eppendorf) was used to monitor and regulate temperature, agitation, pH, and dissolved O_2 (DO). DO and pH were measured throughout the run using a polarographic ISM® DO sensor (Mettler Toledo) and optical pH sensor (Mettler Toledo), respectively. The DO was controlled by sparging O_2 and air, while pH was regulated by addition of CO_2 (acid). Table 1 outlines the operational setpoints used for all runs.

Transient transfection

Three days following seeding, the cells were transiently transfected with a five-plasmid, second-generation LV plasmid system and polyethylenimine (PEI).[21] This system consists of three helper plasmids on separate constructs consisting of Rev, Gag-Pol, and Tat, as well as a vesicular stomatitis virus

Table 1. *Operational setpoints used for the packed-bed bioreactor runs*

Operational parameter	*Setpoint*
Temperature	37°C
Agitation	80 rpm
pH	7.2 (0.1 deadband)
DO	50% air saturation
Gas (supplied by sparger)	Auto 3-gas mix control (Air, O_2 and CO_2)
Total gas flow rate	0.006–0.05 SLPM

DO, dissolved O_2.

glycoprotein (VSV-G) envelope plasmid, and a transfer vector (in this case, a HIV-1 derived bicistronic LNT-EF1α-3XFLAG-fLUC-F2A-eGFP). Plasmid quantities are shown in Table 2. A plasmid DNA-to-PEI mass ratio of 1:3 was used (4.77 mg DNA and 14.31 mg PEI). The plasmids were combined with DMEM (no serum) in 5% of the total production volume. In the same way, 25 kDa linear PEI (Polysciences) was combined with DMEM in 5% of the final production volume. Prior to addition to the bioreactor culture, the PEI was added to the DNA solution, briefly vortexed, and incubated at room temperature for 30 min. At 8 h post transfection, total medium replacement was performed using OptiPRO™ serum-free medium (Gibco) supplemented with 1% PenStrep and 4 mM L-glutamine.

Supernatant harvest and downstream processing

At 48 h post transfection, a complete supernatant harvest was performed, followed by transfer of 3 L of fresh OptiPRO™ (1% PenStrep, 4 mM L-glutamine) into the vessel. The collected supernatant was immediately clarified by pelleting the cellular debris at 5,000 rpm (JA-10 rotor; Beckman Coulter) at 4°C for 10 min. If visible debris was still present, the supernatant was then filtered through 0.45 μM vacuum filter cups (Merck Millipore) for further clarification. Bovine serum albumin was added to the supernatant at a final concentration of 0.1% (w/v), and it was stored at 4°C overnight for processing the following day. At 72 h post transfection, a second harvest was performed in the same manner as the 48 h harvest. The 6 L of vector supernatant was purified using a MustangQ XT5 anion-exchange column (PALL), followed by concentration using ultracentrifugation, as previously described.[7] Each preparation was concentrated to a final volume of 1–1.5 mL in a formulation of saline/0.1% heat-inactivated rat serum.

Table 2. *Plasmid quantities used for one packed-bed run (5-plasmid LV system used)*

Plasmid	*Quantity (μg)*
Transfer vector	4,354
Tat	81
Rev	81
Gag-Pol	51
VSV-G	202

LV, lentiviral vector; VSV-G, vesicular stomatitis virus glycoprotein.

Cell factory vector production

For titer comparisons, LV was also produced using a standard 10-layer cell factory method, as previously described.[7] Briefly, a 10-layer cell factory at a density of $\sim 1.65 \times 10^5$ cells/cm^2 was transfected using calcium phosphate co-precipitation and the same plasmid ratios as the packed-bed bioreactor. At 8 h post transfection, the cell factory medium was changed to OptiPRO™ medium (1% PenStrep, 4 mM L-glutamine). The vector supernatant was harvested 48 h following transfection, and purification and concentrated were performed as outlined above for the packed-bed bioreactor preparation.

Lentivirus titering

Functional titers were determined using flow cytometric detection of green fluorescent protein (GFP)-positive cells. Briefly, HEK 293T cells were plated onto 12-well plates at 5×10^4 cells/well. The vector was serially diluted and applied to cells in the presence of 8 μg/mL polybrene (Sigma–Aldrich). At 72 h post transduction, the cells were harvested and fixed, and flow cytometric analysis was then performed (LSRFortessa™; Becton Dickinson). The proportion of GFP-positive cells was used to calculate functional titers in transducing units per milliliter (TU/mL), as previously described.[22] Physical vector particles per milliliter (VP/mL) were determined by measuring HIV-1 p24 capsid protein by enzyme-linked immunosorbent assay (Abcam). Calculations were based on the previously reported estimation of 10,000 physical LV particles per 1 pg of p24.[23]

Assessment of *in vivo* transduction efficiency

Animal studies were conducted under approval from the University of Adelaide and Women's and Children's Hospital animal ethics committees. To validate the *in vivo* transduction efficiency of the packed-bed-produced LV vector, airway gene transfer was performed in 8-week-old female Sprague Dawley rats. For all procedures, rats were anesthetized with 0.2 mL/100 g body weight of medetomidine (0.2 mg/mL; Orion Corp.) and ketamine (30 mg/mL; Parnell Laboratories) mixture, delivered by intraperitoneal (i.p.) injection. Anesthesia was

reversed with 0.2 mL/100 g i.p. injection of atipamezole (0.5 mg/mL; Orion Corporation).

Anesthetized rats were intubated using an endotracheal tube (16G Terumo intravenous cannula) and fiber-optic light. The airways were conditioned with 200 μL of 0.1% lysophosphatidylcholine (LPC; Sigma–Aldrich), as previously described for mice, but with the modification of a larger 200 μL volume gel tip (Quality Scientific Plastics).[24] At 1 h following LPC delivery, 250 μL of LV vector ($\sim 4 \times 10^7$ TU) was delivered intratracheally. One week following airway gene transfer, *luciferase* gene expression was quantified using bioluminescent imaging (Lumina IVIS; PerkinElmer). Prior to imaging, 150 μL of 15 mg/mL D-luciferin substrate (Cayman Chemical) was delivered to the animal intranasally.

Statistical analysis

GraphPad Prism v7 (GraphPad Software, Inc.) was used for all statistical analysis. Student's *t*-test was used to determine the differences between groups. Results are presented as the mean and standard error of the mean, and $p < 0.05$ was considered statistically significant.

RESULTS

Regulation of DO and pH conditions

The DO steadily decreased over the course of each run, indicating increasing cell biomass (Fig. 1A). A slight DO increase was observed on day 5 post cell seeding for all runs, which corresponded with the 48 h vector harvest and subsequent medium change. The DO was maintained close to the 50% setpoint across all three runs, with an average DO of 47%. However, on days 4 and 6, the DO appeared to drop substantially below the setpoint for runs 1 and 2. As oxygen availability is critical for cell growth and high-titer yields,[25,26] the gassing parameters were altered with each subsequent run to improve maintenance of the 50% DO setpoint. Similar to DO, the pH decreased gradually as the run progressed (Fig. 1B). While the pH of the media was neutral ($\sim$pH 7) for most of the run, by the final day of vector production (day 6), the pH became mildly acidic in all three trials, with an average pH of 6.9. Although CO_2 sparging was used to control pH in the basic range, the pH was not controlled in the acidic range by addition of a base (*e.g.*, sodium bicarbonate).

LV titers achieved using a packed-bed bioreactor system

The packed-bed bioreactor method trialed here exhibited unconcentrated functional titers between 10^5 and 10^6 TU/mL and physical particles in the 10^7–10^9 VP/mL range (Fig. 2A and B). Supernatant samples collected at 24, 48, and 72 h post transfection demonstrated a steady increase in titer over time, with peak production at 72 h, as has been previously observed for LV production.[6] At the peak production time point, an average titer of 1.4×10^6 TU/mL and 4.3×10^8 VP/mL were achieved across the three runs. Following purification and concentration of the 6 L of supernatant, the average functional titer was 1.0×10^9 TU/mL, with an average particle number of 5.3×10^{11} VP/mL (Fig. 3). Figure 3 also demonstrates a slight increase in total LV yield with each bioreactor run, potentially due to optimization of the reactor conditions (*e.g.*, gassing parameters) with each subsequent trial. The highest concentrated yields were obtained in run 3, with 1.9×10^9 TU/mL and 1.1×10^{12} VP/mL achieved.

Comparison of packed-bed bioreactor and cell factory production methods

To evaluate the feasibility of a packed-bed bioreactor approach, the yields from the packed-bed

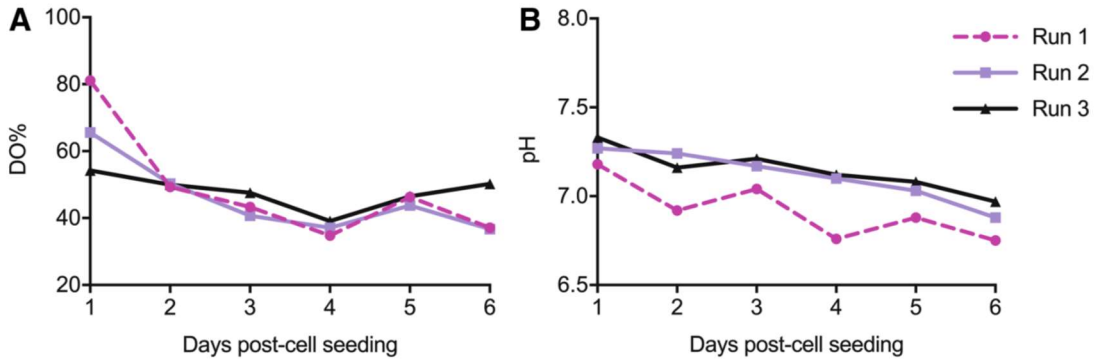

Figure 1. Average daily dissolved O_2 (DO) and pH readings measured over the course of each packed-bed run. **(A)** Average daily DO reading measured over 6 days following cell seeding. **(B)** Average daily optical pH reading measured over 6 days following cell seeding. Color images are available online.

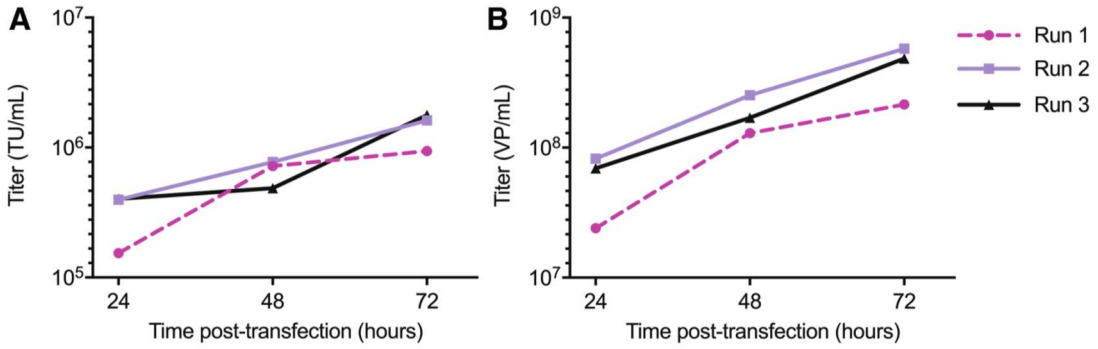

Figure 2. Titers of unconcentrated lentiviral vector (LV) supernatant collected at 24, 48, and 72 h post transfection for each of the three packed-bed runs. **(A)** Functional titers (TU/mL) determined using flow cytometric detection of green fluorescent protein (GFP)-positive cells. **(B)** Physical LV vector particles (VP/mL) determined using an enzyme-linked immunosorbent assay for HIV-1 p24 capsid protein. Color images are available online.

system were compared to a standard 10-layer cell factory production method.[7] When comparing the unconcentrated titers relative to surface area, the packed-bed production method demonstrated lower average functional (3.5×10^4 vs. 4.5×10^5 TU/cm^2) and physical (1.0×10^7 vs. 1.2×10^8 VP/cm^2) titers compared to the 10-layer cell factory (Fig. 4A; $p<0.05$). While the packed-bed approach exhibited lower yields in this study, it is important to note that the packed-bed of Fibra-Cel® disks has a much greater surface area than a 10-layer cell factory (18 m^2 vs. 0.632 m^2), and therefore has a substantially higher productivity potential that was not exploited in this study. The supernatant VP:TU ratio was used to compare the quality of the vector from each production method. A lower VP:TU ratio is desirable, as the proportion of nonfunctional particles in the preparation is lower. Comparing the two methods of production, there was no significant difference in the preparation quality (Fig. 4B; $p>0.05$), with the packed-bed supernatant having an average of 281 VP:TU and the cell factory 266 VP:TU.

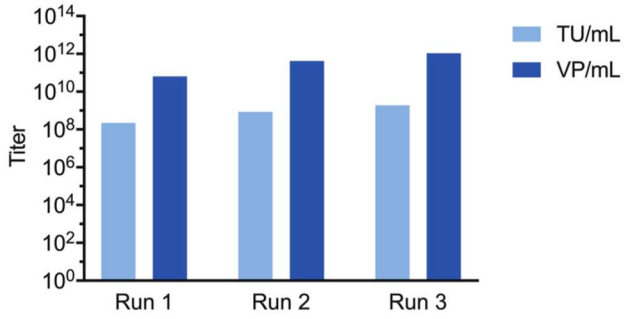

Figure 3. LV yields from each of the three packed-bed runs following purification and concentration. Both functional LV titers (TU/mL) and physical vector particles (VP/mL) are displayed. Color images are available online.

Airway gene transfer using packed-bed-produced LV

To assess the *in vivo* efficacy of the packed-bed-produced LV in a gene therapy setting, airway gene transfer was performed in rats. Airway-directed gene therapy has the potential to treat a range of genetic diseases that affect the lungs, including cystic fibrosis lung disease.[27] The packed-bed-produced LV successfully transduced the airway epithelia and produced functional gene expression (Fig. 5). Both rats showed *luciferase* gene expression in the lungs, while one rat also demonstrated expression in the trachea. On average, radiance measures ranging from 10^5 to 10^6 photons/s/cm^2/sr were achieved.

DISCUSSION

Upscaling LV production is a major hurdle to successful commercialization of LV-based gene and cell therapies. Common platform technologies used to generate LV vectors such as the transient transfection of adherent HEK 293T cells in monolayer flasks and multilayer factories are not inherently scalable and are therefore difficult to transition to commercial use. Although these flask and cell factory-based methods are useful for preclinical studies, there is benefit in adopting a clinical-like production strategy in preclinical phases, as this will significantly reduce the time required to adapt LV production methods to a clinical scale and quality. Moreover, the collated body of preclinical data is likely to be more relevant to clinical studies when the same gene vector production method and formulation are used. Adopting a clinical-like production method also means that a robust supply chain can be identified early on for Good Manufacturing Practice grade materials.

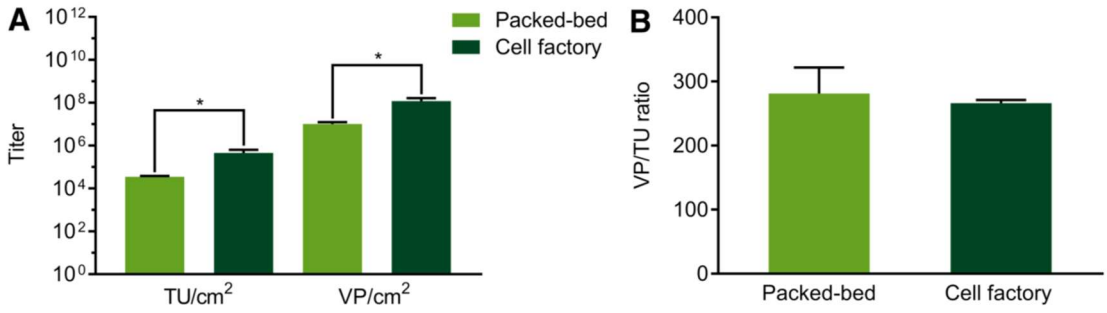

Figure 4. **(A)** Average unconcentrated titers achieved per square centimeter of surface area using a packed-bed bioreactor ($n=3$) or standard 10-layer cell factory method ($n=2$) for production. The packed-bed yielded lower functional and physical titers per square centimeter compared to cell factory production ($^{*}p < 0.05$). **(B)** The VP:TU ratio demonstrates comparable vector preparation quality between the two methods of production ($p > 0.05$). Results are presented as the mean and standard error of the mean. Color images are available online.

In addition to scalability issues, the high costs associated with LV production are an ongoing challenge. Recently marketed LV-based gene and cell therapy products can cost in the realm of U.S.$500,000–U.S.$1 million per patient,[28] with a significant portion of the costs attributed to vector production. Employing technologies that are already used in the biopharmaceutical industry such as bioreactors allows for process automation, which should in turn reduce labor costs. Maximizing LV titers will also be essential in developing a cost-effective method. Higher titers reduce volume requirements, subsequently decreasing the costs associated with consumable use and downstream processing. For example, a 10-fold improvement in titer means that a 100 L LV batch becomes a more manageable 10 L batch.

While this study reports the first use of the Eppendorf BioBLU® 5p single-use packed-bed vessel for transient LV production, a similar system, the PALL iCELLis® Nano, has recently been employed for this application, with yields up to 3.7×10^5 TU/cm^2 achieved. The iCELLis® is a single-use fixed-bed system that is available in two formats, allowing for scale-up biomanufacturing: the iCELLis® Nano (maximum 4 m^2) for bench-scale applications, and

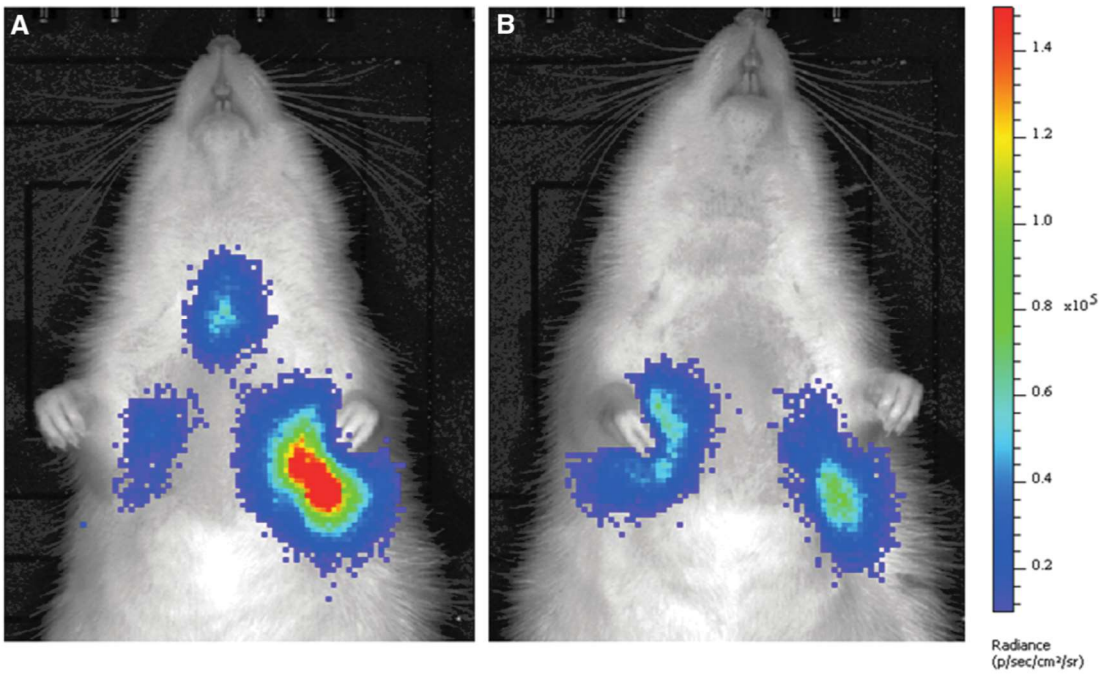

Figure 5. Airway gene transfer performed in rats using packed-bed produced HIV-1 derived, vesicular stomatitis virus glycoprotein pseudotyped EF1α-3XFLAG-fLUC-F2A-eGFP vector. Rat **(A)** shows *luciferase* gene expression in the trachea and lungs, while rat **(B)** only shows expression in the lungs. Color images are available online.

the iCELLis® 500 system (maximum 500 m^2) for industrial production.[29] Even though the iCELLis® offers a large-scale option, there is a considerable leap from small-scale to industrial production, as there are no medium-scale options available.[30] The BioBLU® single-use vessel is currently only available in one format (18 m^2), although the size of the vessel is ideal for employing a scale-out system. Given the similarities between these two systems, the methods developed in this study also have potential to be transferred to the iCELLis®.

This study has presented a novel packed-bed bioreactor LV production method. This approach exhibited unconcentrated functional titers between 10^5 and 10^6 TU/mL and physical vector particles in the 10^7–10^9 VP/mL range. Titers following purification and concentration were in the range of 10^8–10^9 TU/mL, with physical particles between 10^{10} and 10^{12} VP/mL. Compared to cell factory production, the packed-bed bioreactor demonstrated lower functional and physical titers per square centimeter. The reduced productivity of the packed-bed system was most likely due to the lower cell concentration at the time of transfection. While not assessed here, it is also possible that cells within certain areas of the densely packed Fibra-Cel® disks were inaccessible to the DNA complexes, thereby reducing the transfection efficiency. Although the packed-bed yields were lower than the cell factory, the cell densities and plasmid quantities used here did not exploit the full the productivity potential of the packed-bed. Furthermore, the quality of the preparations was comparable between the packed-bed and cell factory production methods, as determined by the VP:TU ratio. When used in a gene therapy scenario, the packed-bed-produced LV generated sufficient concentrated titers to yield detectable gene expression *in vivo*. *Luciferase* gene expression was observed in both the upper and lower airways of the rats, demonstrating that the packed-bed-produced vector is adequate for use in preclinical animal studies.

Although this packed-bed has shown promising preliminary results, there are shortcomings to this approach that should be recognized. Some limitations can be overcome with process optimization, while others are inherent to the system. First, FCS was used to supplement the growth media. Although the harvest media was serum free, there is still potential for contamination of the final gene vector product with foreign serum-derived components. While FCS has been used previously for clinical LV production,[31] its use adds additional regulatory complexity.[12] As such, is it ideal to move toward a completely serum-free system, as is now the direction of the LV production field.[12,32] Some initial labor is required to build-up HEK 293T cells in monolayer flasks to seed the packed-bed. However, a relatively low seeding density can be used, as most of the cultivation can occur in the vessel itself, particularly if perfusion mode is used. Furthermore, as the cells are adhered to the Fibra-Cel® disks during cultivation, it is not possible to assess cell density and viability directly. Although not employed here, this could be overcome by using indirect measures of cell density such as glucose consumption and lactate production.[33] Finally, the 3.75 L (18 m^2) capacity packed-bed vessel used in this study is currently the only size available in single use.

A range of investigations could be performed to optimize this production method further in the future. First, perfusion mode can be used during the run to improve cell viability and density. Employing medium perfusion will allow the full surface area of the packed-bed to be exploited, thereby increasing the overall productivity of the system.[29] The transfection conditions could also be optimized by assessing different plasmid quantities, PEI to DNA ratios, and cell densities. The optimal harvest window could also be further investigated by continuing the run past 72 h post transfection to determine whether titers increase, plateau, or decrease after this time point. To improve regulatory acceptability, the use of serum-free growth medium could also be explored.[32]

It may also be valuable to examine further the effects of mildly acidic conditions on LV production. During all runs the media became slightly acidic on the final day of production, most likely due to cellular lactic acid production and the onset of apoptosis.[25] It has previously been shown that using mildly acidic conditions (pH 6) during VSV-G LV production results in higher functional titers compared to neutral conditions (pH 7.2). Mildly acidic production conditions appear to create an optimal intracellular environment for the HEK 293T cells, leading to increased expression of viral components (*e.g.*, capsid protein) and improvement in titers.[34] Lowering the pH to 7.0 (from 7.2) following PEI-mediated transfection has also proven to increase LV yields and preparation quality in the iCELLis® Nano fixed-bed bioreactor.[29] In the future, it will be informative to assess the use of mildly acidic production conditions in this particular packed-bed system.

In conclusion, this proof-of-principle study shows for the first time that LV can be transiently produced using the BioBLU® packed-bed vessel. With further optimization of bioreactor conditions that

are expected to improve titer, this packed-bed system could offer a potential solution to meet the ever-growing demands for scalable LV production. Compared to conventional methods such as flasks and cell factories, a packed-bed bioreactor allows for higher productivity, process simplification, automation, online monitoring, tight regulation of culture conditions, reduced space requirements, fewer manipulations, and an overall lower risk profile. As the field continues to progress, developing scalable, cost-effective, and clinically acceptable LV manufacturing processes will be imperative in realizing the transformative potential of gene and cell therapies.

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AUTHOR DISCLOSURE

No competing financial interests exist.

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Chapter 5: Airway disease phenotypes in CF animal models

Airway disease phenotypes in animal models of cystic fibrosis

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Animal models are essential for furthering our fundamental understanding of CF pathophysiology and for facilitating the testing of potential therapeutics. CF mouse models have contributed greatly to furthering the CF field, however, they do not develop human-like lung disease, therefore they are limited model for developing airway-targeted therapies. Recent advances in genetic engineering technologies has allowed alternative CF animal models to be generated including rats, pigs, ferrets and sheep. Each animal model recapitulates varying degrees of CF airway phenotypes, which are reviewed in the following article. A comprehensive understanding of airway phenotypes and model characteristics will aid researchers in selecting the most appropriate animal model for lung-based investigations. Ultimately, test models should be thoughtfully selected for each research investigation so that relevant and translatable results are obtained. A follow-up review article on this topic has recently been published in the American Journal of Pathology.

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REVIEW Open Access

Airway disease phenotypes in animal models of cystic fibrosis

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Abstract

In humans, cystic fibrosis (CF) lung disease is characterised by chronic infection, inflammation, airway remodelling, and mucus obstruction. A lack of pulmonary manifestations in CF mouse models has hindered investigations of airway disease pathogenesis, as well as the development and testing of potential therapeutics. However, recently generated CF animal models including rat, ferret and pig models demonstrate a range of well characterised lung disease phenotypes with varying degrees of severity. This review discusses the airway phenotypes of currently available CF animal models and presents potential applications of each model in airway-related CF research.

Keywords: Cystic fibrosis, Animal models, Lung disease, Pathology

Background

Animal models of cystic fibrosis (CF) are crucial for understanding CF pathogenesis and developing therapeutic strategies. Given that CF affects multiple organs including the gastrointestinal tract, lungs, pancreas, liver, and reproductive organs, it desirable to generate animal models that accurately capture all disease facets. For the past 20 years, in vivo CF-related research has predominately used genetically modified murine models [1]. These mouse models have proven invaluable for studying aspects of CF pathophysiology, however, they have important limitations. Obvious disparities between murine and human anatomy and physiology mean that phenotypes observed in humans are not always reproduced in mice [2]. Investigating some features of CF airway disease in mouse models has proven problematic, as CF mice fail to develop hallmark features including mucus obstruction, chronic bacterial infections, and persistent inflammation. Of all clinical manifestations associated with human CF, progressive lung disease is the predominant cause of morbidity and mortality among patients [3]. As such, an accurate animal model of CF lung disease initiation and progression is imperative for investigating pathogenesis, identifying potential treatment targets, and testing experimental therapies.

Following the generation of CF murine models, it became apparent that species characteristics are important to consider when developing an animal model of CF lung disease. Traits that may influence the choice of species include airway cellular architecture, particularly the distribution and abundance of submucosal glands, dominance of alternative chloride secretory pathways, and conservation of the structure and function of the cystic fibrosis transmembrane conductance regulator (CFTR) protein [1]. Since the development of CF mouse models, advances in genetic engineering have facilitated the generation of a number of alternative models including a CFTR knockout rat [4], knockout ferret [5], two knockout pig models [6, 7], and a pig harbouring the common Phe508del CFTR mutation [6]. There are also early reports of CFTR knockout and Phe508del mutant CF rabbit models being developed [8]. Due to the similarities between the ovine and human lung, generation of a CF sheep model has long been proposed [9]. Although a CF sheep model has not yet been established, rapid advances in gene editing could promote its generation in the near-future.

This review details the airway disease phenotypes of existing CF animal models, and examines their strengths and weaknesses for basic and pre-clinical research.

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CF lung disease pathophysiology

Understanding the underlying pathophysiology of CF lung disease is necessary prior to examining the phenotypes observed in animal models. Under normal conditions, the CFTR protein functions as an epithelial anion channel responsible for cyclic-AMP-dependent chloride and bicarbonate secretion, as well as the regulation of epithelial sodium channels (ENaC) [10]. CF arises when mutations occur in the CFTR gene, subsequently disrupting epithelial ion transport. To date, over 2000 disease-causing CFTR mutations have been identified [11]. These mutations are classified into 6 categories based on the mechanism of CFTR dysfunction including defective protein synthesis and processing, dysfunctional channel regulation and conduction, and reduced protein synthesis and stability [12]. The most common CF-causing mutation is the Class II mutation Phe508del, with approximately 90% of CF patients carrying one copy [11].

Although the role of CFTR in transepithelial ion transport is widely accepted, the exact mechanisms underlying CF lung disease development have long been debated. Several hypotheses have been proposed regarding the pathogenesis of CF lung disease [13]. The leading theory with a large body of supporting evidence is known as the "low volume" hypothesis [14–16]. This theory postulates that reduced transepithelial chloride transport due to dysfunctional CFTR, as well as increased sodium absorption caused by a lack of CFTR-dependent inhibition of ENaC, creates an ion imbalance that leads to osmotically-driven water absorption into the tissues, and in turn, reduced airway surface layer (ASL) hydration [3, 10]. The depleted ASL results in dehydration of the mucus layer and consequently, viscous mucus adheres to airway surfaces. Onset of mucus-stasis and impaired mucociliary clearance (MCC) result in ineffective removal of inhaled microorganisms [17]. Bacteria adhere to the airway mucus eventually resulting in the formation of biofilms that effectively evade antimicrobial substances and host neutrophils. Over time, more resistant and atypical organisms colonise the airways including mycobacteria, yeast, and fungus. A state of chronic lung infection incites persistent inflammatory responses that lead to destruction of the airway tissue, bronchiectasis, progressive loss of lung function, and ultimately respiratory failure [18].

Murine models

Shortly after the discovery of the CFTR gene in 1989 the first CF mouse model was generated [19]. Since then, at least 15 CF mouse models have been developed and characterised (reviewed in detail elsewhere [20–22]). Two major groups of CF mouse models exist: null alleles (knockouts) with no detectable mRNA or functional CFTR protein, and mutant alleles with common human CF-causing mutations (e.g. Phe508del or Gly551Asp) introduced into the mouse CFTR sequence [23]. The limited utility of CFTR knockout strains due to early-fatal intestinal obstruction led to the development of a third category; "gut corrected" transgenic CF mice. In this model, human CFTR cDNA is expressed under the control of the rat fatty acid binding protein (FABP) promoter, thereby localising expression to the intestinal epithelium [24], and subsequently increasing the longevity of CF mice [25]. Humanised mouse models that exclusively express human CFTR with common CF-causing mutations are also currently under development [26].

Lung disease phenotype in CF mice

Disease phenotypes and severity among CF mouse models tend to be heterogeneous due to the diversity of genetic backgrounds and variation in gene targeting strategies used to generate the animals [25]. Although early characterisation studies initially reported that CF mice did not demonstrate any lung pathology [19], emerging evidence suggests that they do in fact exhibit some phenotypes reminiscent of human CF lung disease. Similar to the early pulmonary phenotype observed in human CF airways, multiple studies have demonstrated that CF mice display altered respiratory mechanics [27–30]. Furthermore, when a state of hyperinflammation is induced in the lungs of CF and wild-type mice using repeated exposure of an inflammatory stimuli (in this case lipopolysaccharide from *Pseudomonas aeruginosa*), CF mouse lungs undergo remodelling and morphological changes, while the airways of wild-type mice are able to efficiently recover [31]. Recent studies also indicate that CF mice spontaneously acquire *Bordetella* respiratory tract infections [32].

Although CF mice demonstrate features of lung disease, their use is somewhat limited as they do not exhibit the severe pathology characteristic of established human CF lung disease consisting of chronic respiratory infection, inflammation, mucus plugging, and progressive bronchiectasis [23]. Several theories may explain why CF mice fail to develop the overt lung disease that is observed in humans. One is that modifier genes are upregulated in CF mice, leading to partial correction of the underlying ion transport defects. Dominance of the calcium-activated chloride channel (CACC) secretory pathway has been observed in the airway epithelia of CF mice [33]. It has been suggested that this adaptive mechanism compensates for defective CFTR-mediated chloride transport, thereby rectifying the underlying ion imbalance, and protecting the murine airways from disease [33, 34]. In addition to upregulation of the CACC in CF murine airways, the cAMP-mediated CFTR pathway normally has a less dominant role in the respiratory epithelium of mice [25].

The lower airways of adult CF mice do not demonstrate electrical defects that are characteristic of human CF airways, including reduced cAMP-mediated chloride secretion and sodium hyperabsorption. Hyperactivity of ENaC is important in the pathogenesis of CF lung disease as it is thought to contribute to the depletion of the ASL and subsequent cascade of events including mucus accumulation, impaired MCC, infection and inflammation, and lung tissue damage [35]. As such, a lack of sodium hyperabsorption in the lower airways of CF murine models may explain the absence of lung disease development [25]. It is also possible that the structure and function of CFTR evolved differently in mice. Investigations into CFTR homology among species reveal that mouse CFTR is only 88% conserved with human CFTR at the amino acid level, as such, the murine CFTR channel exhibits some different pharmacological and gating properties to human CFTR [36, 37].

CFTR protein in human airways is predominately expressed in the ciliated epithelium and the submucosal glands, therefore histological disparities between murine and human airways may explain the absence of lung disease in CF mice [38, 39]. Mouse and human airways differ in cellular architecture; human distal airways are comprised primarily of ciliated cells, whereas murine lower airways are largely non-ciliated, secretory, club cells [25]. Furthermore, human airways have numerous submucosal glands throughout the trachea and bronchi, while murine airways only have a small proportion of these glands in the larynx and proximal trachea [40]. As submucosal glands are implicated in human CF airway disease, their scarcity in the murine distal airways could contribute to the lack of lung pathophysiology [41].

Environmental factors and host-pathogen interactions may also play a role [20]. It has been hypothesised that CF lung disease is not effectively modelled without the presence of pathogens, therefore the conventional housing of CF mice in semi-sterile, and often specific pathogen free (SPF) conditions may prevent development of the lung disease that is typically observed in humans with CF [22]. However, this theory does not appear to be substantiated, as CF mice reared in a non-sterile environment still fail to develop lung disease [25].

Upper airway and tracheal phenotype in CF mice

Some CF mouse strains display a mild phenotype in the upper airways and trachea. Characteristics observed include distended submucosal glands in the nasal mucosa [42], atrophy of serous gland tissue in the sinuses [19], hyperplasia of goblet cells in the nasal septa, and a reduced ASL in the nasal epithelium [15]. $CFTR^{-/-}$ mouse models also show notable tracheal abnormalities such as incomplete cartilage rings [43], and reduced smooth muscle area [44]. Impaired MCC in the trachea of $CFTR^{tm1HGU}$ and $CFTR^{tm1UNC}$ strains has also been reported [45], however, these findings have been contested by others that found MCC was unaffected in $CFTR^{tm1UNC}$ mice [46].

The nasal epithelium of CF mice demonstrates similar bioelectric abnormalities to human CF airways including reduced chloride transport and sodium hyperabsorption. These defects have been well documented using the nasal potential difference (NPD) measurement technique; an assessment that involves placing fluid-filled cannula electrodes (connected to a millivoltmeter) on the nasal lining to measure the electrical potential produced by ion transport across the epithelium in response to different salt solutions [47]. All CF mice appear to exhibit reduced cAMP-mediated chloride secretion in the nasal epithelium, which is characteristic of human CF airways [22, 25]. The hyperactivity of ENaC, a feature present in human CF nasal epithelium [48], is also observed in the nasal epithelium of most CF mouse models including knockout [49], $CFTR^{\Delta F508}$ [49, 50] and $CFTR^{G551D}$ strains [51]. When amiloride (a drug that blocks ENaC-mediated sodium absorption) is perfused through the nasal epithelium of a CF mouse, a significant depolarisation response occurs, which is consistent with the presence of sodium hyperabsorption [21].

As the nasal epithelium of CF mice accurately recapitulates the electrophysiological profile of human CF airways, it has proven valuable for testing therapeutic agents that replace dysfunctional CFTR (i.e. gene therapies) [52], and those that restore normal ion transport [53, 54]. Mouse strains bearing the Phe508del and Gly551Asp mutations could be particularly useful for testing compounds that correct CFTR processing and trafficking (CFTR correctors), and defective channel gating (CFTR potentiators). However, some CFTR potentiators that strongly augment human CFTR do not have any effect on murine CFTR, most likely due to differences in the murine CFTR channel properties [20]. Once developed, hCFTR CF mouse models will be valuable for trialling small molecule drugs. Although the nasal epithelium of CF mice has provided a useful platform for trialling therapeutics [52–54], it should be noted that murine nasal mucosa is composed of 40% olfactory epithelium and 60% respiratory epithelium (depending on location), thus differing substantially from the cellular composition of human lungs, the intended target organ of these therapies [12].

Bacterial challenge rodent models

The absence of overt lung disease in CF mouse models, even under non-sterile conditions, led some investigators to trial more radical bacterial-challenge approaches to simulate a chronically infected and inflamed airway [20]. In these studies, a range of knockout and mutant

CF strains were inoculated with *P. aeruginosa*, an important pathogen in the development and progression of human CF lung disease [55]. Bacterial challenge with *P. aeruginosa* has previously yielded variable results, most likely due to differences in dose, delivery method, dosing frequency, and the clinical isolate used. Despite this, many of the challenged CF mouse strains demonstrated susceptibility to acute *P. aeruginosa*, characterised by increased mortality and reduced ability to clear the bacteria when compared to non-CF control animals [55–58].

In one example, *P. aeruginosa* laden agarose beads were intratracheally instilled into CFTR^{tm1UNC} mice. In addition to increased mortality (when compared to non-CF controls), challenged mice also demonstrated a significant pulmonary inflammatory response following inoculation, indicated by elevated levels of inflammatory markers in bronchoalveolar lavage (BAL) [55]. Studies have also attempted to deliver *P. aeruginosa* to CF mice in drinking water, resulting in low levels of chronic colonisation within CF airways, while wild-type animals effectively cleared the bacteria [59]. Other CF-related bacterial strains have also been used, in one study CFTR^{tm1UNC} mice were intranasally challenged with the bacterium *Burkholderia cepacia* (*B. cepacia*). Following instillation, bacteria persisted in the airways of CF mice and caused severe bronchopneumonia while wild-type mice remained healthy [60].

These bacterial challenge studies indicate that the absence of CFTR in mouse airways is enough to produce heightened susceptibility to CF-related pathogens [58]. Accordingly, challenged CF mice elicit a sustained inflammatory response that could allow for investigations into the relationship between inflammation and infection in a CFTR-deficient lung [57], as well as trialling of novel anti-inflammatory and antibiotic therapies [60, 61]. However, these bacterial challenge mouse models are limited, as they do not reflect the conditions in which CF patients acquire and respond to infection. Furthermore, instilling CF mice with human pathogens is a somewhat naïve approach as host-pathogen interactions are complex, differ in mice and humans, and do not solely depend on CFTR activity [20].

In addition to bacterial challenge being performed in CF mice, models of respiratory infection have also been established in normal rodents by using key CF-related bacterial species. Mouse models of chronic *Burkholderia cenocepacia* [62] and *P. aeruginosa* [63] infection have recently proven useful for investigating host-pathogen interactions of bacterial species that are pertinent to CF lung disease. Similarly, a rat model of chronic *P. aeruginosa* infection that exhibits pathologic features of human *P. aeruginosa* pulmonary infection has also been developed [64].

Congenic CFTR knockout mouse model

It has been hypothesised that generation of CF mouse models on mixed genetic backgrounds results in upregulation of modifier genes that prevent the development of lung disease. To test this theory, the CFTR^{tm1UNC} CF mouse strain was redeveloped on a single genetic background and was termed B6-CFTR^{tm1UNC}. Interestingly, B6-CFTR^{tm1UNC} mice develop lung disease manifestations including bronchiolar mucus retention, tissue fibrosis, hyperinflated alveoli, and alveolar wall thickening [65]. B6-CFTR^{tm1UNC} mouse lungs also show inflammatory cell recruitment with an influx of neutrophils observed. This inflammatory disease phenotype appears spontaneous, as analysed mice were housed in SPF conditions with no airway pathogens detected prior to, or during the onset of inflammation [66].

In the nose, PD measures indicate that the congenic CF mouse demonstrates reduced chloride secretion, which is consistent with an absence of CFTR. Unlike mixed-background CF mouse strains however, the response to amiloride does not appear to differ between congenic CF mice and wild-type, suggesting that congenic CF mice do not exhibit sodium hyperabsorption in the nasal epithelium [65]. Furthermore, B6-CFTR^{tm1UNC} mice challenged with *P. aeruginosa* demonstrate reduced capacity to control infection [67]. It has been speculated that congenic B6-CFTR^{tm1UNC} mice develop aspects of lung disease as they lack, or fail to activate alternate chloride conductance pathways normally present in mixed background CF mouse models [65].

The B6-CFTR^{tm1UNC} mouse is one of the few models that exhibits inflammatory lung disease, as such it has proven useful for exploring the long-debated question of whether inflammation in CF airways is spontaneous, or induced by infection [66]. Even though the B6-CFTR^{tm1UNC} mouse demonstrates aspects of CF lung disease, the original mixed-background CF mouse strains continued to be used. Unexpectedly, generation of another congenic mouse model based on the CFTR^{tm1HGU} strain resulted in amelioration of the CF phenotype, rather than an increase in severity [68]. Given the potential undesired effects of developing models on mixed-backgrounds, along with the rapid uptake of new gene editing technologies such as CRISPR/Cas9, newly generated CF mouse models are likely to be congenic.

β-ENaC mouse model

Difficulties recapitulating the pathophysiology of CF lung disease in existing mouse models led to the development of the β-ENaC mouse (also known as the *Scnn1b* mouse). The β-ENaC mouse is a transgenic mouse model that overexpresses the β-subunit of ENaC in the lungs, thereby mimicking the sodium ion transport abnormalities observed in CF human airways [69,

70]. The airways of β-ENaC mice recapitulate the key processes of CF lung disease initiation, whereby increased airway sodium absorption results in depletion of the ASL and deficient mucus clearance. Accordingly, β-ENaC mice exhibit CF-like lung disease with features including mucus hypersecretion, mucus obstruction in the conducting airways, MCC impairment (as measured directly by microdialysis), goblet cell metaplasia, and neutrophilic airway inflammation [69–71].

Initial studies performed using adult β-ENaC mice failed to detect spontaneous bacterial infection in the airways, however, when intratracheally challenged with *Haemophilus influenzae* and *P. aeruginosa,* adult β-ENaC mice exhibit impaired pathogen clearance when compared to wild-type mice [69]. More recent studies involving longitudinal analysis of BAL indicate the presence of spontaneous lung infection in neonatal β-ENaC mice, while wild-type neonates had no culturable bacteria. The same study also revealed that the lung bacterial burden and proportion of infected β-ENaC mice appears to decrease with age, attributable to maturation of the immune system that occurs during the postnatal period [72].

The β-ENaC mouse provides a relevant model for investigating CF lung disease pathogenesis, particularly the interactions between ion transport, the ASL, and MCC. The lung disease modelled by the β-ENaC mouse has already proven useful for developing novel respiratory diagnostic tools to localise and measure heterogeneity of lung disease [73]. Furthermore, β-ENaC mice could be used to evaluate a range of treatments including those that target ASL depletion, mucus obstruction, and inflammation [70]. For instance, the β-ENaC mouse could be used to trial ENaC inhibition strategies that may restore normal sodium transport. Such approaches include use of ENaC antagonists, silencing ENaC expression using short-interfering RNAs (siRNAs), and inhibiting proteases that activate ENaC [35]. However, as CFTR expression and function are not altered in the β-ENaC model, it is not suitable for testing therapeutics that replace, correct or potentiate CFTR [70].

CF rat model

Limited usability of CF mouse models attracted researchers to other species to use as platforms for developing a more accurate CF model. Compared to larger animals, rats have the advantages of a short gestation (21–23 days) and early sexual maturity (8 weeks), thereby allowing for rapid breeding. Rats also have fewer animal husbandry expenses compared to large animals, and have been used widely for research purposes meaning they are well characterised in terms of physiology, pharmacology, and toxicology [74]. Moreover, there is a vast array of rat-specific molecular tools available that would be difficult to source or develop for other species. Unlike mice, rats have extensive submucosal glands present throughout the cartilaginous airways (trachea and primary bronchi), similar to humans [75]. As submucosal glands are implicated in the development of CF lung disease in humans [76], rat lungs are an attractive model.

Airway disease in the CFTR knockout rat model

$CFTR^{-/-}$ rats recapitulate important features of lung disease that are observed in humans with CF. Histologically, the respiratory epithelium of the nasal septum in CF rats is normally developed, however, cells tend to exhibit dilation due to increased levels of intracellular mucus, indicative of defective mucus secretion. Morphologically, the trachea of ≤ 6-week-old CF rats appears to develop abnormally, with evidence of diminished tracheal cartilage and gland area when compared to wild-type rats. CF rats also display significant depletion of the ASL with a notable reduction in periciliary liquid (PCL) depth [4]. Furthermore, preliminary findings indicate that young CF rats demonstrate a hyperacidic airway surface pH [77], similar to that observed in CF pigs [78]. Interestingly, a recent study has revealed that ASL pH is not reduced in children with CF [79].

Despite depletion of the ASL, mucociliary transport (MCT) appears to be unaffected in young CF rats (< 3 months) [4]. However, early evidence suggests that as the animal ages and the airway submucosal glands develop, MCT rates reduce significantly when compared to wild-type. Preliminary investigations also suggest that by 6 months of age, CF rats exhibit mucus plugging of submucosal glands in the large airways [77, 80]. Spontaneous infection and inflammation is not present in CF rat airways, with no differences observed in BAL profiles between wild-type and $CFTR^{-/-}$ genotypes [4]. Although spontaneous infection is absent, emerging evidence indicates that similar to CF mice, CF rats have a diminished ability to clear induced *P. aeruginosa* infection [81].

CF-like electrophysiological defects are present in the both the nasal and tracheal epithelium of CF rats as measured by NPD and short circuit current (I_{sc}), respectively. In the nasal epithelium, CF rats demonstrate reduced chloride transport and show no evidence of cAMP-mediated chloride secretion following attempts to stimulate CFTR with forskolin (a cAMP agonist), both features consistent with an absence of CFTR. I_{sc} measures of transepithelial ion transport performed on freshly excised CF rat tracheal tissue using Ussing chambers demonstrates a markedly lower baseline I_{sc} in CF rats when compared to wild-type, typical of a CF bioelectric profile. Furthermore, administration of an inhibitor of CFTR-dependent chloride transport to the tracheal tissue has minimal effect on I_{sc}, suggesting low

basal CFTR activity [4]. $CFTR^{-/-}$ and wild-type rats demonstrate similar responses to amiloride in both the nasal and tracheal epithelium, suggesting that unlike human CF airways and the nose of CF mice, CF rat airways do not demonstrate sodium hyperabsorption.

Recapitulation of key CF airway features in the $CFTR^{-/-}$ rat model including reduced ASL and MCT will provide opportunities to further investigate the mechanisms involved in lung disease development. Electrophysiological, MCT and ASL measures may also be useful in assessing the efficacy of airway-targeted treatments including genetic therapies. Despite these and many other potential applications, the advantages of CF rats such as their small size, rapid breeding, ease of husbandry, and display of key airway disease phenotypes, are yet to be exploited [4]. Additional in-depth investigations of the airway phenotype are required to reinforce initial characterisation reports, and longitudinal studies are necessary to follow lung disease development over the lifetime of the animals. Furthermore, carrying out bacterial challenge studies in CF rat airways like those performed in CF mice [57], may reveal disease characteristics that are otherwise undetected in a non-infected lung. Recent advances in gene editing will also enable the future development of CF rat strains carrying human-specific CFTR mutations.

CF ferret model

Due to similarities between ferret and human lung cell biology and anatomy, normal ferret lungs have been used to model human pulmonary infections including severe acute respiratory syndrome [82], and influenza virus [83]. Ferret airways also have important features that make them attractive for developing a model of CF lung disease. Like humans, ferret airways have submucosal glands throughout the trachea and primary bronchi that express high levels of CFTR [38, 84]. Additionally, the predominant secretory cell type in ferret and human proximal cartilaginous airways is the goblet cell, unlike in mice, where the equivalent is the club cell [85]. Ferret CFTR also has a high degree of amino acid sequence conservation with human CFTR (95%), and accordingly, the bioelectric and pharmacologic properties of CFTR are similar in ferrets and humans [36].

Airway disease in the CFTR knockout ferret model

The CF ferret demonstrates a severe lung phenotype that is heterogeneous between individual animals. Mucus plugging is observed in the small and large airways, and in some cases, causes complete blockage. In addition to the presence of mucus, pockets of trapped air and deflated alveoli (atelectasis) are also present. Dilation of the submucosal glands and ducts, goblet cell hyperplasia, and presence of inflammatory cells and bacterial colonies within the mucus are all observed in CF ferret airways. In instances where bronchopneumonia is present, purulent inflammation, necrosis, and pulmonary consolidation can also occur. CF ferrets also occasionally present with mucus accumulation and inflammation in the sinuses. When MCC is measured using fluorescent bead migration in ex vivo tracheas, CF ferrets exhibit a significantly reduced (by seven-fold) MCC rate in comparison to their non-CF counterparts [86].

Bioelectric I_{sc} measures performed on CF ferret tracheal tissue demonstrate electrophysiological abnormalities that are typical of CF [5]. CF ferret tracheal epithelia exhibits a large reduction in transepithelial current when an inhibitor of non-CFTR epithelial chloride channels is delivered, suggesting decreased CFTR activity. Furthermore, delivery of various cAMP agonists (e.g. forskolin) to the tracheal tissue does not appear to stimulate significant responses, and subsequent addition of a CFTR inhibitor also results in little change in ion transport, features that are all consistent with a lack of CFTR function and subsequent defective cAMP-dependent chloride transport [87]. Fluid secretion from the submucosal glands is also substantially reduced in CF ferret tracheal tissue, a feature that is also present in human CF proximal airways [5]. Interestingly, there is no difference in ENaC activity in tracheal tissue taken from CF and wild-type animals >3 months of age (as measured by Ussing chamber I_{sc} analysis). However, there is some evidence that CF ferrets display an age-dependent increase in ENaC activity that is not observed in wild-type controls, suggesting a possible link between ENaC activity and airway disease progression [86].

In striking contrast to CF rodent models, CF ferrets are highly susceptible to lung infection, and antibiotic treatment is required from birth to ensure survival. Bacteriologic studies have identified a diverse range of bacteria in the lungs of CF ferrets with the most common species from the genera *Streptococcus, Staphylococcus,* and *Enterococcus.* Interestingly, enteric bacterial species from the *Enterococcus* and *Escherichia* genera are abundant in the lungs of CF ferrets. The significant overlap in lung and intestinal flora suggests that the intestines provide a primary source of lung-colonising bacteria [5].

The CF ferret is one of the few models that exhibits spontaneous lung infection, a crucial factor implicated in CF lung disease. However, the onset of lung infection in the CF ferret is rapid and severe, differing from the slow progressing chronic infection observed in humans [88]. As CF ferrets are susceptible to lung infections continuous antibiotic treatment and ongoing high levels of care are required from birth to improve survival [5]. Along with infection, the ferret lungs demonstrate mucus plugging, inflammation, and reduced MCC, all important

manifestations of a CF airway. Given this, the ferret could be useful for testing the efficacy of potential therapeutics under conditions of infection and inflammation, which has not been possible with previous CF models. The presence of spontaneous lung infection will also assist in understanding the complex changes in the CF lung microbiome with disease progression [86]. One disadvantage of the CF ferret model is the lack of ENaC dysregulation in the airway tissues, a process thought to be significant in the pathogenesis of lung disease [36].

Along with lung disease, CF ferrets also develop a range of gastrointestinal pathologies, similar to those observed in CF patients. Notably, CF ferrets demonstrate a high prevalence of meconium ileus at birth (75% of kits) that is often fatal, as well as pancreatic dysfunction, malnutrition, and liver disease [5, 89]. To alleviate this severe gastrointestinal phenotype, a $CFTR^{-/-}$ model expressing ferret CFTR cDNA under direction of the FABP promoter has been developed. This gut-corrected model will enable more CF ferrets to survive to maturity thereby allowing lung disease progression to be investigated longitudinally [5].

CF pig models

Parallels between humans and pigs in regards to anatomy, physiology, biochemistry, size, life span, and genetics make pigs a suitable candidate for modelling a range of human diseases. Porcine lungs share several anatomical and histological features with human lungs, including similar tracheobronchial tree structure, and abundance of airway submucosal glands [90]. Pigs have previously been used to model pulmonary diseases involving inflammation and infection such as chronic bronchitis [91]. Furthermore, as human CF lung disease progresses over the lifetime of an individual, the longevity of pigs allows for long-term investigations of lung disease pathogenesis and assessment of therapeutics, which is not possible in rodent or ferret models due to their relatively short lifespan or disease severity, respectively [90]. The pig CFTR amino acid sequence is also 95% conserved with human, and the electrophysiological properties of porcine airway epithelium and submucosal glands resemble those observed in humans [36, 92].

Adeno-associated virus-mediated gene targeting of CFTR was used to generate the first CF pig models containing either a null allele ($CFTR^{-/-}$) or the common Phe508del mutation ($CFTR^{\Delta F508/\Delta F508}$) [6, 93]. Cross-breeding of $CFTR^{+/-}$ and $CFTR^{+/\Delta F508}$ heterozygotes was also performed to produce a third model, denoted $CFTR^{-/\Delta F508}$ [94]. Shortly thereafter, Klymiuk et al. generated another knockout CF pig using an alternative approach that involved sequential targeting of CFTR using bacterial artificial chromosome (BAC) vectors [7]. Following breeding of the first CF pigs, it was realised that maintenance of the animals requires intensive and costly husbandry due to the severe gastrointestinal phenotype. Newborn CF pigs have a 100% penetrance of meconium ileus, with piglets requiring surgery soon after birth to relieve the obstruction. CF pigs also demonstrate pancreatic insufficiency and other gut-related issues requiring treatments including enzyme replacement therapy to aid digestion, oral vitamin supplements to prevent malnutrition, oral proton pump inhibitors or H2 blockers to control gastric acid, and laxatives to prevent bowel obstruction [93]. Using the same approach employed to create the gut-corrected CF mouse and ferret models, a transgenic $CFTR^{-/-}$ pig was generated to express porcine CFTR in the intestines thereby overcoming the need for surgical correction of meconium ileus at birth [95].

Airway disease in CF pig models

$CFTR^{-/-}$, $CFTR^{\Delta F508/\Delta F508}$ and $CFTR^{-/\Delta F508}$ pig strains (hereafter collectively referred to as CF pigs) spontaneously develop key features of human CF lung disease within months of birth. These manifestations include airway inflammation, infection, tissue remodelling, mucus accumulation, and obstruction [94]. As in human CF populations, the severity of the lung phenotype varies between animals, with individual lobes also demonstrating heterogeneity [93]. Examination of neonatal CF pig airways shows no evidence of inflammation, comparable to newborn humans with CF [94, 96]. BAL profiles also indicate no difference in leukocyte and interleukin 8 (IL-8) concentrations between newborn CF and non-CF pigs. Over time however, inflammation ensues, ranging from mild to severe leukocytic infiltration, with severe cases occasionally exhibiting ulceration and abscess formation of the airway wall, and destruction of submucosal glands. Shortly after birth, CF pig lungs demonstrate defective bacteria eradication with newborn CF pigs challenged with *Staphylococcus aureus* failing to eradicate the bacteria as effectively as wild-type pigs [94]. This defective bacterial killing has been attributed to reduced airway surface pH in the CF pig and subsequent diminishing of the ASL antimicrobial function [78].

CF pigs demonstrate heterogeneous airway remodelling, with some cases showing evidence of goblet cell hyperplasia, airway wall thickening, and rarely, distended submucosal glands [94]. As observed clinically in infants with CF [97, 98], CF pigs present with airway obstruction comprising of atelectasis, hyperinflation, air trapping, and pneumonia [99]. Purulent material appears to obstruct the trachea and bronchi, often containing bacteria, neutrophils, and macrophages [94]. Tracheal abnormalities are also observed in CF pigs including a triangular rather than circular shaped trachea [7], smaller lumen area and circumference, irregular cartilage

rings, and altered smooth muscle [93, 100]. Similar tracheal malformations have been noted in CF mouse models [43], CF rats [4], and infants with CF [100]. Interestingly, newborn CF pigs do not demonstrate reduced PCL depth in the trachea [101], but they do exhibit impaired MCT [102].

A range of ion transport measures performed on the nasal, tracheal and bronchial epithelia of CF pigs using tissues, cultures and in vivo approaches reveals electrophysiological defects consistent with loss of CFTR activity [101]. These abnormalities tend to be more pronounced in $CFTR^{-/-}$ airway epithelia when compared to $CFTR^{\Delta F508/\Delta F508}$ pigs, most likely due to the $CFTR^{\Delta F508/\Delta F508}$ epithelia retaining some residual CFTR function [93]. Characteristic of CF, CFTR-mediated chloride transport is substantially reduced in the nasal, tracheal, and bronchial epithelium of CF pigs when compared to wild-type. CF pig nasal epithelia also exhibits a CF-like response to amiloride perfusion, but the trachea does not. Further investigation into this phenomenon revealed that the amiloride response observed in the CF pig nasal epithelia was due to reduced CFTR-mediated chloride conductance rather than sodium hyperabsorption [101]. Excised tracheal tissue from CF pigs also demonstrates diminished bicarbonate conductance, which is consistent with the human CF phenotype [93, 101].

Given that CF pigs capture many features of human CF airway disease, they have been useful for several research applications including investigations of lung disease pathogenesis [94], studying electrolyte transport defects [101], and exploring mechanisms involved in CFTR-Phe508del biosynthesis and misprocessing [92]. CF pigs have also provided a useful platform for exploring the origins of inflammation and infection within CF airways [94], identifying the role of ASL acidification in lung disease development [78], and trialling viral-mediated airway gene therapy approaches [103, 104]. In the future, CF pigs may also be useful for the long-term testing of therapeutics such as CFTR modulators, and for assessing airway disease prevention strategies [105].

Concluding remarks

CF mouse models have proven useful for researching many facets of CF, but their use for airway-related investigations is restricted because they only exhibit mild features of CF lung disease, and induced airway disease using bacterial challenge approaches has limitations. Although the nasal epithelium of CF mice has provided a platform for exploring pathogenesis and assessing therapeutics, the lack of overt lower airway disease manifestations and the high degree of phenotype heterogeneity between strains means that new models are required. The β-ENaC mouse has provided a useful alternative to CF mice as it models lower airway obstruction, although it is not suitable for all applications (e.g. trialling CFTR-directed therapies) as CFTR expression and function are unaffected. Endeavours to overcome deficiencies of the available mouse models has led to development of new CF animal models including CF rat, ferret, and pig models. These models demonstrate an array of CF lung disease phenotypes with varying severity, as outlined in Table 1.

Rats have intrinsic advantages for routine use as a model of CF including low cost and maintenance, rapid reproduction rate, and large litter sizes. The CFTR knockout rat demonstrates many pulmonary phenotypes including airway surface defects, a CF-like electrical profile in nasal and tracheal tissues, abnormal mucus production, and tracheal malformations. Although promising, further in-depth characterisation of the airway disease that is present, or readily inducible, would aid investigators in using this model for research applications.

The lung disease modelled by the CF ferret shares several similarities to that observed in humans. The concurrent presence of airway inflammation, infection, and mucus obstruction provides a highly valuable platform for further elucidating lung disease pathogenesis, as well as trialling therapies in a lung environment that closely replicates that of a human CF patient. However, the severe disease phenotype of the CF ferret model continues to limit its use, as the ferrets often have poor survival and require ongoing antibiotic treatment, therefore additional husbandry needs may constrain investigations.

Pigs share several airway characteristics with humans that make them suitable for developing a model of CF lung disease. Recently generated CFTR knockout and Phe508del mutant pig models exhibit lung disease hallmarks including inflammation, airway obstruction, and infection, traits that will facilitate both basic and preclinical airway-related research. However, CF pig models have drawbacks that may hinder their usability including the need for costly therapies to prolong survival of piglets, and the inherent costs and logistical limitations that arise when using large animal species for research.

Recently, CRISPR/Cas9 has been used to generate CFTR knockout and Phe508del mutant CF rabbit models [8]. Rabbits are considered to be an ideal species for modelling CF lung disease, as their airways are similar to humans in terms of anatomy and inflammatory responses [106]. Initial characterisation reports of the CF rabbit models reveal a CF-like lung phenotype including evidence of sodium hyperabsorption in trachea, mucus plugging, and bacterial infection in the lower airways, with BAL predominately containing bacteria from the *Streptococcus* and *Staphylococcus* genera [8]. Although these findings are preliminary, the CF rabbit models show promise for future use in CF-related research.

Table 1 Airway phenotypes of CF animal models compared to humans

	Human	CFTR knockout/mutant mice	β-ENaC mouse	Rat	Ferret	Pig
ASL HEIGHT IN LOWER AIRWAYS	Reduced [107]	NR in lower airways Reduced in nasal epithelium [15]	Reduced in bronchi [69, 70]	Reduced in trachea [4, 77, 80]	NR	Normal in trachea of newborn pigs [101]
MUCOCILIARY CLEARANCE IN LOWER AIRWAYS	Impaired [108]	Impaired in trachea of some strains [45] (has been contested [46])	Impaired in trachea [70]	Normal in young rats (< 3 months) [4] Impaired in the trachea of older rats (> 3 months) [77, 80]	Impaired in trachea [86]	Impaired in trachea [102]
REDUCED CHLORIDE SECRETION IN AIRWAYS	Present [109, 110]	Present in nasal epithelium [21] Absent in lower airways [25]	Absent [69]	Present in nasal and tracheal epithelium [4]	Present in tracheal epithelium [5, 87]	Present in nasal, tracheal and bronchial epithelium [93, 101]
SODIUM HYPERABSORPTION IN AIRWAYS	Present [48, 110–112] (has been contested [109])	Present in nasal epithelium [49–51] Absent in lower airways [25]	Present in trachea [69]	Absent in nasal and tracheal epithelium [4]	Absent in trachea [86, 87]	Absent in nasal and tracheal epithelium [101]
MUCUS OBSTRUCTION IN LOWER AIRWAYS	Mucus plugging [113]	Absent [15]	Mucus plugging [69]	Increased stored nasal mucus [4] and preliminary evidence of mucus plugging of submucosal glands in large airways [77, 80]	Mucus plugging [86]	Mucus plugging [93]
LUNG INFECTION	Present [114]	Absent [21]	Present in neonates but reduces with age [72]	Absent [4]	Present [86]	Present [94]
AIRWAY INFLAMMATION	Present [115]	Absent [21]	Present [69]	Absent [4]	Present [86]	Absent in newborn pigs but develops overtime [94]

NR = not reported
Data insufficient for CF rabbit models

Animal models will continue to play a fundamental role in furthering our understanding of CF. Along with the development of rapid and precise gene editing technologies such as CRISPR/Cas9 comes the potential for new species to be used as platforms for modelling CF, as well as modification of existing models by introduction of human-specific CFTR mutations. Animal models that accurately recapitulate the hallmark features of human CF lung disease will be crucial in providing researchers with a resource for trialling experimental lung therapies, identifying new treatment targets, and elucidating the complex mechanisms that underlie CF lung disease. Although a perfect model of CF lung disease does not exist, each animal model has unique advantages and can be used in complementary manner to investigate CF-related questions.

Abbreviations

ASL: Airway surface layer; BAL: Bronchoalveolar lavage; CACC: Calcium-activated chloride channel; CF: Cystic fibrosis; CFTR: Cystic fibrosis transmembrane conductance regulator; cAMP: Cyclic adenosine monophosphate; ENaC: Epithelial sodium channel; FABP: Fatty acid binding protein; I_{sc}: Short circuit current; MCC: Mucociliary clearance; MCT: Mucociliary transport; NPD: Nasal potential difference; PCL: Periciliary liquid; siRNAs: Short-interfering RNAs; SPF: Specific pathogen free

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Chapter 6: Phenotype characterisation of two Australian-generated cystic fibrosis rat models

Phenotypic Characterisation and Comparison of Cystic Fibrosis Rat Models Generated Using CRISPR/Cas9 Gene Editing

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Following on directly from Chapter 5, this chapter details the phenotype characterisation and comparison of two new Australian-generated CF rat models. One rat strain harbours the common Phe508del mutation and is the first mutation-specific rat model to be developed worldwide, while the second is a *CFTR* KO model. These CF rat strains were generated by the Australian Phenomics Network (APN) in collaboration with the Cystic Fibrosis Airway Research Group (CFARG) and are currently being bred in Adelaide, South Australia. Both rat models recapitulate a number of key features of CF, as will be described in detail in the manuscript presented below. Availability of these new CF rats in the Southern hemisphere provides an alternative model for trialling airway-gene addition therapy, as well as other novel therapeutic strategies.

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Contribution to the Paper	Assisted in the design of the characterisation studies; produced Figures 3 A-I & J, 7, 8, 9, 10, 11 and 12; was involved in the interpretation of the results from all figures; and drafted a substantial portion of the manuscript.		
Overall Percentage (%)	65%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

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ii. permission is granted for the candidate to include the publication in the thesis; and
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ANIMAL MODELS

Phenotypic Characterization and Comparison of Cystic Fibrosis Rat Models Generated Using CRISPR/Cas9 Gene Editing

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Animal models of cystic fibrosis (CF) are essential for investigating disease mechanisms and trialing potential therapeutics. This study generated two CF rat models using clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeats—associated protein 9 gene editing. One rat model carries the common human Phe508del (ΔF508) CF transmembrane conductance regulator (*CFTR*) mutation, whereas the second is a CFTR knockout model. Phenotype was characterized using a range of functional and histologic assessments, including nasal potential difference to measure electrophysiological function in the upper airways, RNAscope *in situ* hybridization and quantitative PCR to assess *CFTR* mRNA expression in the lungs, immunohistochemistry to localize CFTR protein in the airways, and histopathologic assessments in a range of tissues. Both rat models revealed a range of CF manifestations, including reduced survival, intestinal obstruction, bioelectric defects in the nasal epithelium, histopathologic changes in the trachea, large intestine, and pancreas, and abnormalities in the development of the male reproductive tract. The CF rat models presented herein will prove useful for longitudinal assessments of pathophysiology and therapeutics. *(Am J Pathol 2020, 190: 977—993; https://doi.org/10.1016/j.ajpath.2020.01.009)*

Cystic fibrosis (CF) is a chronic and life-shortening autosomal recessive disorder caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (*CFTR*) anion channel. In epithelial cells, the CFTR channel is responsible for chloride and bicarbonate transport, as well as the regulation of epithelial sodium channels.[1] CFTR dysfunction leads to disease manifestations in a diverse range of organs, including the lungs, gastrointestinal tract, liver, pancreas, and reproductive tract.[2] Pulmonary disease is currently the greatest contributor to morbidity and mortality among CF patients, and is therefore the primary focus for therapy development.

Animal models of CF that can accurately recapitulate the human disease are critical for elucidating the mechanisms that underlie disease pathogenesis and for trials of potential therapeutics. In the United States, CF models now include several mouse models,[3,4] pigs,[5] ferrets,[6] and rats.[7] Sheep[8] and rabbit[9] models have also recently been reported.

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Characterization of these animal models reveals varying presence and severity of CF phenotypes, with no single animal model recapitulating all features of human CF disease.[10–12] Nonetheless, these models are proving useful for investigating CF pathophysiology[13,14] and evaluating therapeutics, such as airway gene therapies,[15–20] CFTR modulators,[21] and novel pharmacotherapeutics.[22]

Because of quarantine laws and licensing restrictions, US-generated CF animal models can be difficult to establish in Australia. As such, this study sought to generate an alternate CF animal model for Australasian researchers. Unlike nonlaboratory animals, rodents do not require specialized housing facilities, have relatively low husbandry costs, and are easy to breed. Rat airways are attractive candidates for CF modeling as they have similar cellular architecture to humans, including an abundance of submucosal glands throughout the trachea and bronchi, which are known to be implicated in the development of CF airway disease.[23,24] Furthermore, a previously developed CFTR knockout (KO) rat has already shown encouraging results, with the rats developing a range of CF manifestations, including aspects of lung disease.[7,14] US-generated KO rats also appear to exhibit milder disease phenotypes when compared with pig and ferret models, thus enabling longitudinal assessment of therapeutics.

Using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing, this study generated the first known mutation-specific CF rat model bearing the Phe508del CFTR mutation (alias ΔF508). Phe508del is a class II *CFTR* mutation, and is the most common, with approximately 90% of patients carrying at least one copy.[25] The Phe508del mutation results in an absence of phenylalanine at position 508, which leads to protein misfolding, retention of the mutant CFTR in the endoplasmic reticulum, and its subsequent degradation by the cellular quality control mechanisms. Although a small amount of CFTR protein may be trafficked to the plasma membrane, the channel gating is also defective and the protein has a short half-life.[26,27] In the process of generating a founder animal carrying the Phe508del mutation, an animal with an 8-bp deletion in exon 11 of *CFTR* was generated as a result of nonhomologous end joining DNA repair. This mutation results in the introduction of a premature termination codon at position 510, and as such, the animal was used as a founder to breed KO rats.

This article describes the generation, phenotype characterization, and a comparative assessment of the Phe508del and KO rat strains.

Materials and Methods

Generation of Founder Animals

CRISPR/Cas9 Gene Editing and Microinjection

Design of the CRISPR/Cas9 strategy, microinjections into rat embryos, and identification of founder animals were performed by the Australian Phenomics Network at Monash University (*http://www.australianphenomics.org.au*, last accessed October 28, 2019). Generation of the founders was conducted under approval from the Monash University animal ethics committee. The Phe508del model was generated using CRISPR/Cas9 and a homology-directed repair DNA template containing a TTT deletion corresponding to position 508 in the rat CFTR sequence (NM_031506.1). The guide RNA was generated using the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, Ipswich, MA). Table 1 contains the sequences for the homology-directed repair oligonucleotide and guide RNA used. Microinjection was performed into Sprague-Dawley one-cell embryos (C076 line) using 30 ng/mL of Cas9 mRNA, 15 ng/mL of guide RNA, and 30 ng/mL of homology-directed repair template. Embryos were subsequently transferred to pseudopregnant females, resulting in generation of the founder animals.

Identification of Founder Animals

DNA extraction was performed on tail biopsies using the Qiagen (Hilden, Germany) DNeasy blood and tissue kit, as per the manufacturer's instructions. PCR was performed using forward 5′-CTGGAAGCGTCAGAGGGAAT-3′ and reverse 5′-ACACAAGTAGCTAACACGGATGT-3′ primers flanking the mutation target site. The subsequent PCR product was purified and cloned into pGEM-T Easy vectors. The clone DNA was isolated and sequenced by the Micromon Sequencing Facility (Monash University) to identify mutants. The selected founders were mated with wild-type Sprague-Dawley rats (sourced from the South Australian Health and Medical Research Institute) to produce heterozygous animals. Heterozygous siblings were subsequently paired to generate homozygous Phe508del and KO animals.

Characterization of the Models

Animal Husbandry

Animal studies were conducted under approval from the University of Adelaide Animal Ethics Committee. All animals were maintained in conventional rat cages with a 12-hour light/dark cycle. Food and water were provided ad libitum, with all rats receiving a 50:50 mix of normal and high-fat (10%) rodent chow. Once identified, wild-type and heterozygous animals were provided with normal drinking water, whereas CF animals received 4.5% ColonLYTELY (Dendy Pharmaceuticals, Moorabbin, VIC, Australia) in the water to reduce the incidence of fatal gastrointestinal obstructions. CF rats experience incisor overgrowth; therefore, their teeth were trimmed as required using a small rotary-saw (Dremel 4300-5/50) while anesthetized with inhaled isoflurane (2%). Moistened rodent chow was provided daily to animals that had ongoing tooth issues. KO rat production was maintained by breeding heterozygous

Table 1 CRISPR/Cas9 gRNA and HDR Template Sequences

Template	Sequence
gRNA	5′-ATCAAAGAAAATATCATCTT(TGG)-3′
HDR oligonucleotide	5′-GTTTCATTCTCCTCTCAAATT-TCTTGGATTATGCCGGGTACT-ATCAAAGAAAATATCATCGGT-GTTTCCTATGATGAGTACAGA-TATAAGAGTGTTGTCAAAGCT-TGCCAACTACAGGAG-3′

PAM shown in parentheses.

Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; gRNA, guide RNA; HDR, homology-directed repair; PAM, protospacer adjacent motif.

pairs, whereas Phe508del animals were maintained using homozygous Phe508del females and heterozygous males.

Genotyping

Genomic DNA was extracted from ear notch tissue using the Wizard SV Genomic DNA Purification System (Promega), as per the manufacturer's instructions. Genomic DNA was amplified using a T100 thermal cycler (Bio-Rad, Hercules, CA) with Extract-N-Amp PCR reaction mix (Sigma, St. Louis, MO) and 0.4 μmol/L of each primer (as stated above for genotype identification). PCR conditions were an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 72°C for 5 minutes. This PCR results in amplification of a 189-bp DNA fragment for the wild-type *CFTR* allele, a 186-bp (3 bp deleted) DNA fragment for the *Phe508del* allele, and a 181-bp (8 bp deleted) fragment for the knockout allele. PCR products were purified (PureLink Quick PCR Purification Kit; Invitrogen, Carlsbad, CA) and directly sequenced using the Big Dye Terminator Cycle sequencing version 3.1 (Applied Biosytems, Foster City, CA) and run on a 3730/3730xl DNA Analyzer with 50-cm capillaries, running POP-7 polymer (Applied Biosystems). Sequence analysis was performed using SnapGene (GSL Biotech LLC, San Diego, CA).

Quantitative Real-Time PCR for CFTR mRNA Quantification

Frozen lung samples stored at −80°C in RNA*later* (Sigma-Aldrich, St. Louis, MO) were used for *CFTR* mRNA quantification. Total RNA was isolated from lung samples using a Qiagen RNeasy PowerLyzer tissue and cell kit, and cDNA was subsequently synthesized using a Qiagen QuantiTect Reverse Transcription Kit. For measuring total *CFTR* mRNA, primers for rat CFTR (forward, 5′-AAGCTGAAAGCAGGTGGGAT-3′; and reverse, 5′-TGCTCCGACCACAATGAACA-3′) and housekeeping gene cyclophilin A (forward, 5′-GGTTGGATGGCAAGCATGTG-3′; and reverse, 5′-TGCTGGTCTTGCCATTCCTG-3′) were used in separate reactions. Cyclophilin A was selected as the housekeeping gene because of its stable expression in bronchial epithelial cells.[28] *CFTR* mRNA abundance was quantified by quantitative PCR using SYBR Green (Bio-Rad CFX Connect Real-Time PCR system). The thermocycler conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 58°C for 60 seconds. *CFTR* mRNA expression was normalized to cyclophilin A mRNA expression.

Histology

Histologic analyses were performed on tissues from 1-month−old animals, except for the testes, where tissues from sexually mature males (aged 8 to 12 weeks) were used. Lungs were cannulated, and the left and top right lung lobes were inflation fixed with 10% neutral-buffered formalin. Heads were collected (for examination of the nasal passages), immersion fixed in 10% neutral-buffered formalin, and transferred to 25% EDTA for 7 to 14 days (at 37°C) to decalcify the tissue. The nasal cavity was then cut into four sections at specific palatal sites, as previously described.[29] Liver, pancreas, and gastrointestinal tract tissues were immersion fixed in 10% neutral-buffered formalin for 24 to 48 hours and stored in 70% ethanol until processing. Testes were preserved in Davidson's fixative, followed by daily changes with 70% ethanol for 3 days. All fixed tissues were paraffin embedded, divided into sections (6 μm thick), and stained with hematoxylin and eosin or Alcian blue periodic acid−Schiff for blinded evaluation by a veterinary pathologist (J.F.). Wild-type age-matched rats were used as controls, and a minimum of eight animals (four females and four males) were used for analysis of each organ. Histologic images were examined and captured on a Nikon Eclipse E400 microscope (Tokyo, Japan) with DS-Fi2-U3 camera and NIS-elements D software version 4.20.02 (Nikon).

Morphometric Analysis of Tracheal Tissue

Alcian blue periodic acid−Schiff stained longitudinal tracheal sections were imaged at either ×100 or ×200 magnification. Using the NIS-elements D image analysis software, the submucosal gland mucus components were identified and traced, and their areas were quantified. The length of the trachea section was also measured in millimeters to normalize submucosal gland mucus area.

Table 2 Genotype Distribution Observed for the Phe508del and Knockout Rat Strains

Genotype	Wild-type	Heterozygous	CF
Phe508del*	0	47.2	52.8
Knockout†	26.7	51.9	21.4

Data are given as percentage.

*Heterozygous male to homozygous female breeding.

†Heterozygous male to heterozygous female breeding.

CF, cystic fibrosis.

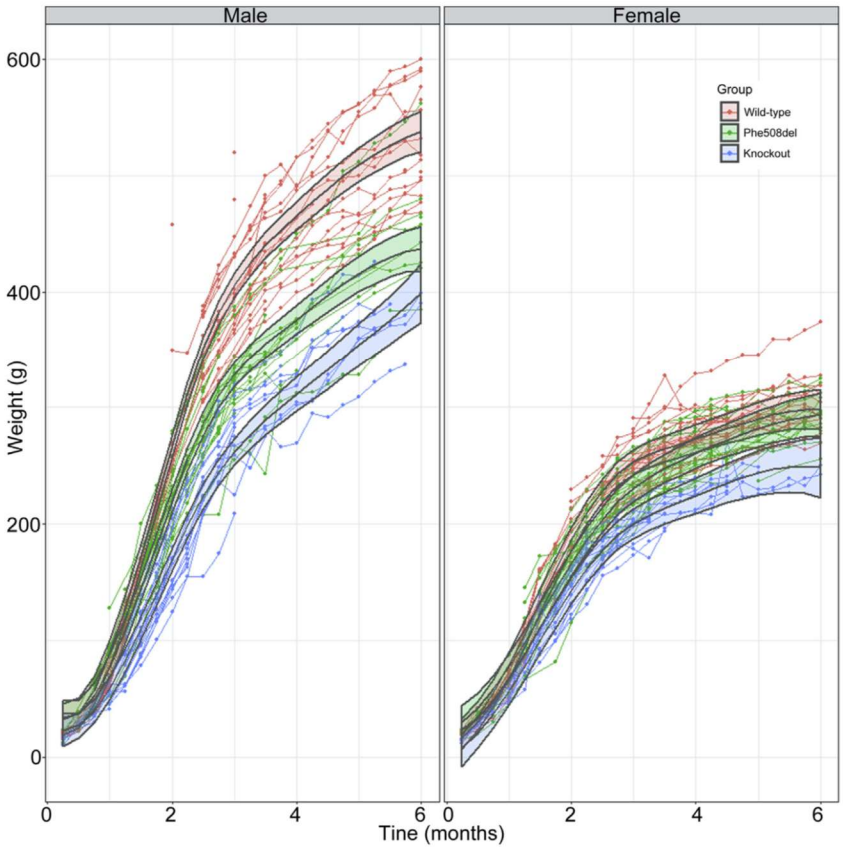

Figure 1 Body weight for male and female wild-type, Phe508del, and knockout rats from day 1 postnatal to 6 months of age. Data presented as the estimated mean weight at each time point from the linear mixed model and 95% CI. $n = 23$ to 36 animals per group.

RNA *in Situ* Hybridization (RNAscope)

Inflation-fixed, paraffin-embedded lung tissue sections from 1-month–old rats were used for analyses. *In situ* RNA detection was performed using the RNAscope 2.5 Red HD Detection kit from Advanced Cell Diagnostics Inc. (Hayward, CA). The probes used were rat *CFTR* (cystic fibrosis transmembrane conductance regulator from *Rattus norvegicus*) Rn-Cftr-No-XHs, positive control rat probe (peptidylprolyl isomerase B), and negative control probe DapB (4-hydroxy-tetrahydrodipicolinate reductase from *Bacillus subtilis*). Slides were hybridized with the probes in a HybEZ oven (Advanced Cell Diagnostics Inc.), at 40°C for 2 hours. After hybridization, slides were subjected to signal amplification, according to the manufacturer's instructions. Hybridization signals were detected using a mixture of solutions A and B (1:60) in RNAscope 2.5 HD Red Assay. After counterstaining with 50% hematoxylin, slides were dried in a 60°C dry oven for 30 minutes and mounted with VectaMount (Advanced Cell Diagnostics Inc.). The integrated probe design with signal amplification and detection was used to

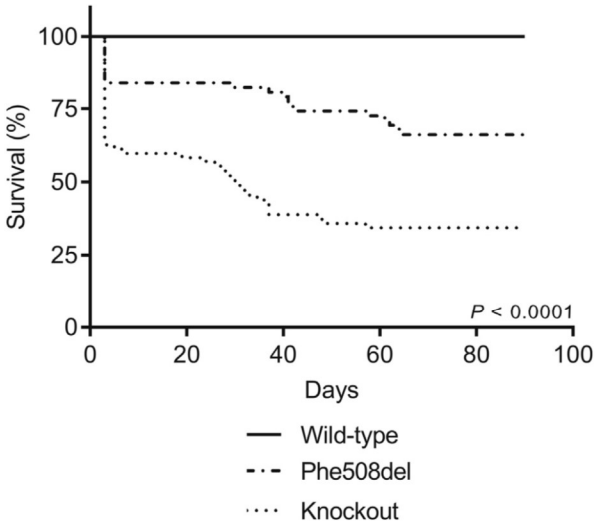

Figure 2 Survival curve for Phe508del and knockout rats from postnatal day 1 to 90. All animals included in the survival analyses were reared on high-fat rodent chow and ColonLYTELY in the drinking water. Individuals with unknown genotypes were not included. $n = 38$ to 67 animals per group. $P < 0.0001$ for all groups (pairwise comparisons).

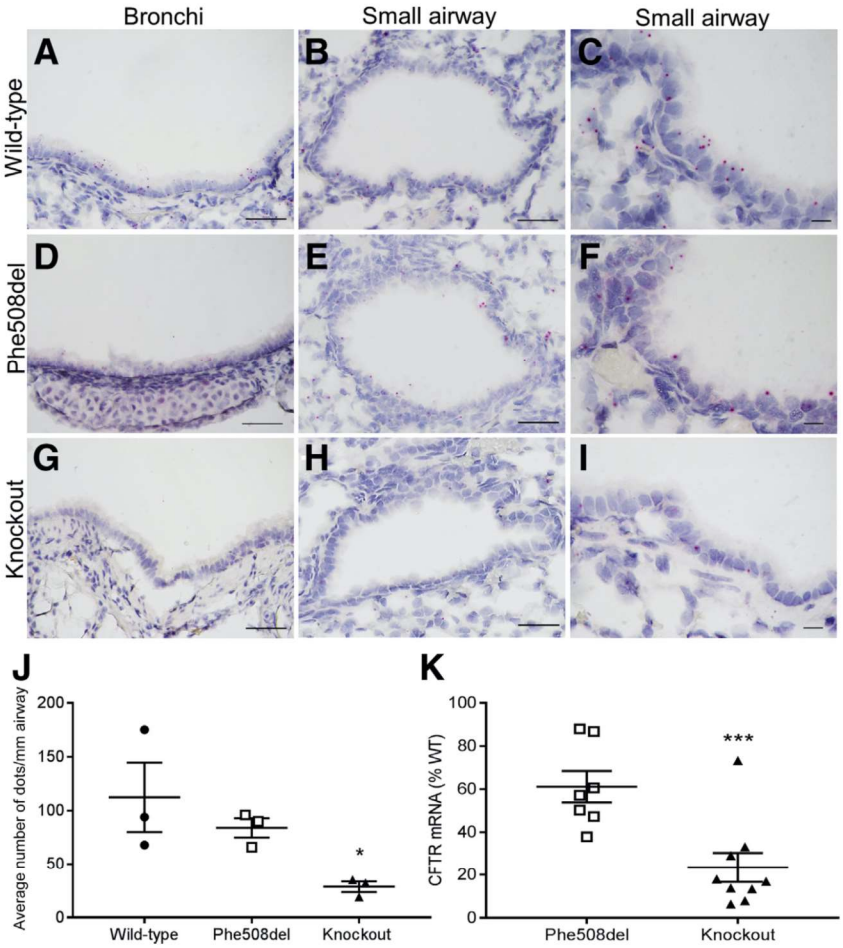

Figure 3 Cystic fibrosis transmembrane conductance regulator (*CFTR*) mRNA expression in the lungs of Phe508del and knockout (KO) rats. **A**—**K:** Representative images of RNAscope *in situ* hybridization for the detection of *CFTR* mRNA in the lungs. **A**—**C:** Bronchi and small airways of wild-type (WT) animals show robust *CFTR* mRNA expression, indicated by the magenta dots. **D**—**F:** Phe508del airways show similar mRNA expression levels and localization to wild-type rats. **G**—**I:** KO rats show a significant reduction in *CFTR* mRNA levels in both large and small airways. **J:** Quantification of airway epithelial *CFTR* transcripts from RNAscope *in situ* hybridization. Graph presents average counts of five airways per animal. **K:** *CFTR* mRNA levels evaluated using quantitative PCR. *CFTR* expression is displayed as a percentage of wild-type CFTR expression. Data are expressed as means ± SEM (**J** and **K**). $n = 3$ animals per group (**A**—**J**); $n = 7$ to 9 animals per group (**K**). $^{*}P < 0.05$, $^{***}P < 0.001$ versus WT (one-way analysis of variance). Scale bars: 50 μm (**A**—**I**, **left** and **middle columns**); 10 μm (**A**—**I**, **right column**).

achieve single-molecule detection of *CFTR* mRNA (labeled magenta).

RNAscope CFTR Transcript Quantification

Hybridized lung sections were imaged at ×400 magnification. Quantification of CFTR transcripts was performed using the NIS-elements D image analysis software. Five airways were randomly selected per animal, each airway was measured in length (millimeters), and the magenta-labeled dots were manually counted within the airway epithelial layer.

Immunohistochemistry

Lung samples from rats and a University of North Carolina CFTR-knockout mouse (used as a CFTR-negative control) were fixed in 10% neutral-buffered formalin, paraffin embedded, divided into sections (5 μm thick), and deparaffinized using standard histologic procedures. Antigen retrieval was performed using 10 mmol/L sodium citrate (pH 6.0) for 20 minutes, followed by permeabilization in Tris-buffered saline/Triton X-100 (0.3%) for 10 minutes. Sections were blocked for 1 hour at room temperature in a solution of serum-free protein block (Dako, Santa Clara, CA), 10% normal goat serum, and 0.1% Tween-20. Primary antibodies rabbit α-CFTR (1:300 dilution; Abcam, Cambridge, UK; ab181782) and mouse α-lysosomal-associated membrane protein 1 (1:200 dilution; Santa-Cruz Biotechnology, Dallas, TX; sc-17768) were resuspended in blocking buffer and incubated with samples overnight at 4°C. A secondary antibody-only control was incubated in blocking buffer without primary antibody overnight at 4°C. Slides were washed three times in Tris-buffered saline/Tween-20 (0.1%) for 5 minutes, followed by incubation with α-rabbit Alexa Fluor 488 (Invitrogen; ab150073) and α-mouse Alexa Fluor 568 (Invitrogen; A11004) in blocking buffer at room temperature for 1 hour. Samples were washed three times in Tris-buffered saline/Tween-20 (0.1%), followed by counterstaining and mounting with ProLong Diamond Antifade Mountant with DAPI (Molecular Probes, Eugene, OR). Images were generated using a Nikon Ts2 or Olympus FV3000 confocal microscope (Toyko, Japan). The rabbit α-CFTR antibody used had an epitope specificity to amino acids 100 to 200 of the CFTR protein.

Nasal Potential Difference Measures

Rats were anesthetized using a mixture of medetomidine (0.4 mg/kg) and ketamine (60 mg/kg) by i.p. injection and

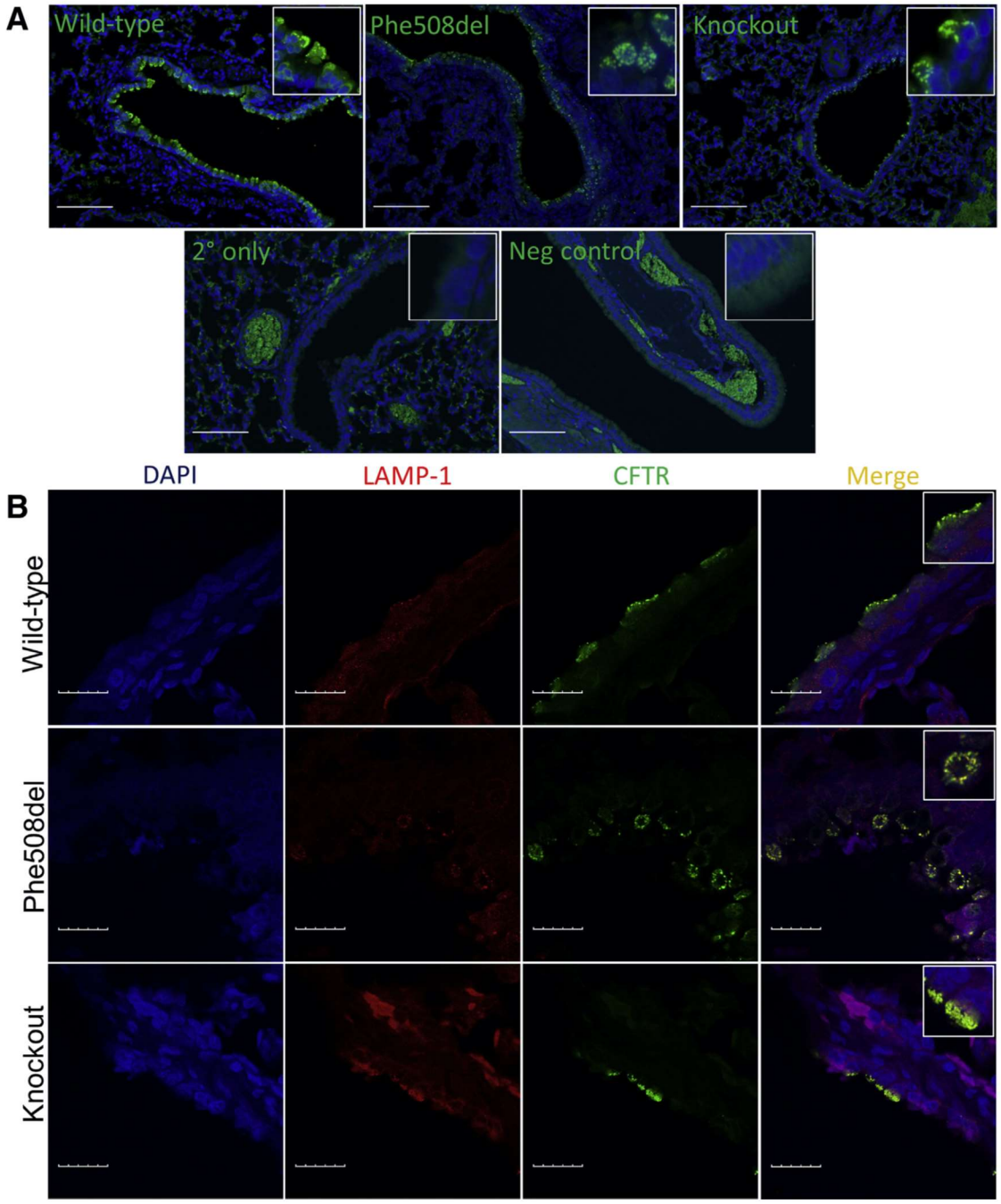

Figure 4 Immunohistochemical localization of cystic fibrosis transmembrane conductance regulator (CFTR) in Phe508del and knockout (KO) rats. **A:** Bronchioles exhibit varying levels of CFTR protein expression between the genotypes. Lung tissue from University of North Carolina CFTR-knockout mice was used as a negative control for CFTR. **Insets:** Enlarged images of CTFR stained airway epithelium. **B:** Colocalization of CFTR with the lysosomal marker, lysosomal-associated membrane protein 1 (LAMP-1), demonstrates degradation of misfolded protein and differential localization of CFTR in Phe508del and KO rats when compared with wild-type. **Insets:** Enlarged images of colocalized staining. $n = 3$ animals per genotype (**A** and **B**). Scale bars: 100 μm (**A**); 20 μm (**B**). Neg, negative.

were nonsurgically intubated with a 16G i.v. cannula (Terumo, Tokyo, Japan) to permit normal breathing during nasal perfusions. Rats were suspended by their incisors on a frame that held them upright, and the exploring electrode was inserted approximately 7 mm into the nasal cavity, with a reference electrode inserted subcutaneously into the abdomen. Nasal cavities were perfused at a rate of 10 to 25 μL/minute with the following solutions: i) normal Krebs-Ringer buffer (basal Krebs-Ringer buffer), ii) Krebs-Ringer buffer containing 100 μmol/L amiloride, iii) low-chloride

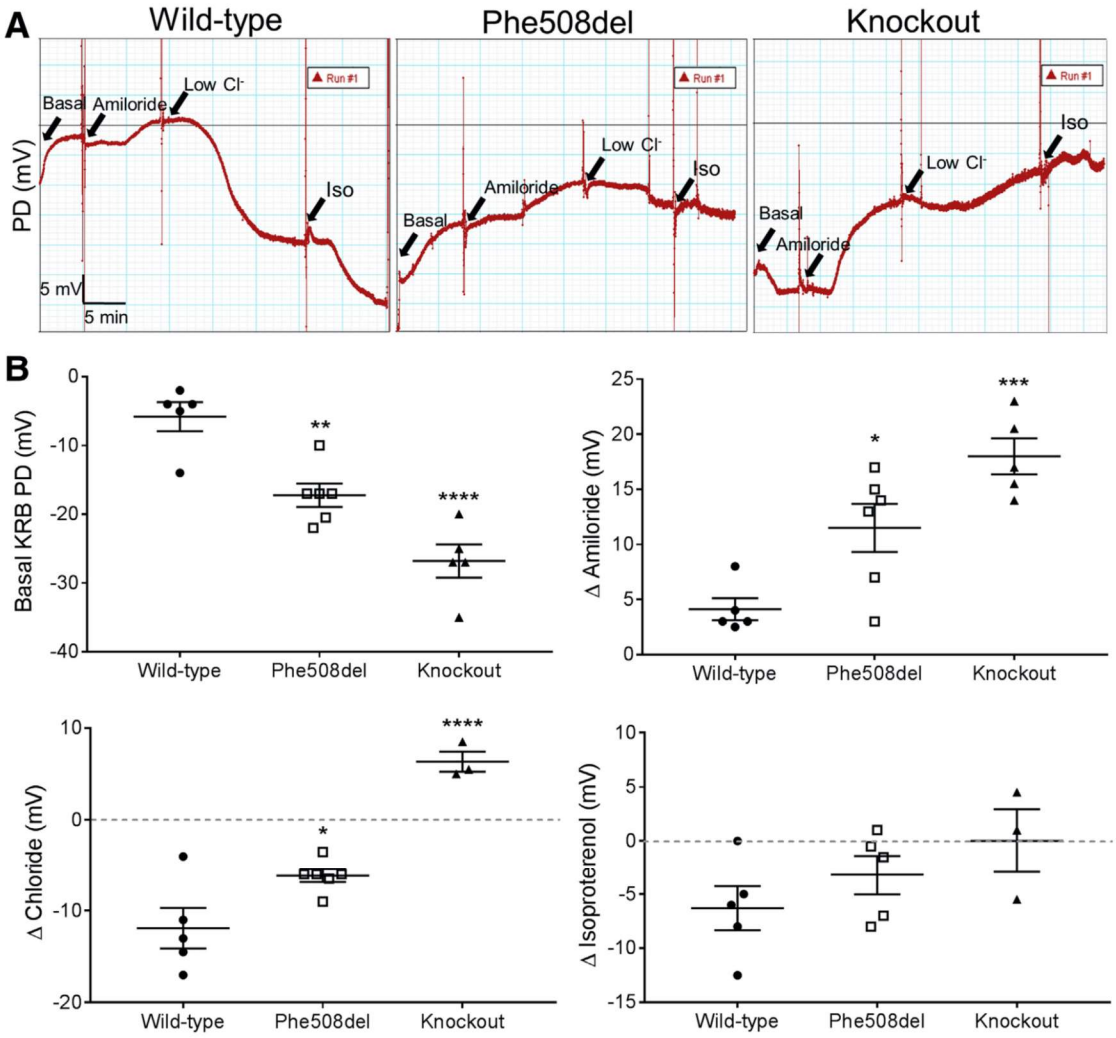

Figure 5 Nasal potential difference (NPD) measurements. **A:** Representative NPD traces from wild-type (WT), Phe508del, and knockout rats. **B:** Summary data for NPD measurements for baseline PD response to basal Krebs-Ringer buffer (KRB), ΔPD amiloride, ΔPD chloride, and ΔPD isoproterenol (Iso). **Dashed lines** indicate 0 mV. Data are expressed as means ± SEM (**B**). $n = 3$ to 6 animals per genotype (**B**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ versus WT (one-way analysis of variance).

Krebs-Ringer buffer with 100 μmol/L amiloride, and iv) low-chloride Krebs-Ringer buffer containing 100 μmol/L amiloride plus 100 μmol/L isoproterenol. Each solution was perfused for at least 5 to 10 minutes until a plateau of 1 to 2 minutes was maintained. All nasal potential difference tracings were interpreted by an experienced assessor blinded to the animal genotype (D.P.).

Blood Counts and Serum Chemistry

Blood was collected from adult rats by terminal cardiac puncture and stored in lithium heparin tubes. Whole blood was processed for complete blood cell counts and serum chemistry by Gribbles Veterinary Pathology (Clayton, VIC, Australia). Serum samples were analyzed on a Siemens Advia 1800 (Tarrytown, NY). The results were compared with a reference range for wild-type Sprague-Dawley rats.[30]

Bronchoalveolar Lavage Fluid Cell Differentials

Tracheas were cannulated and lungs were lavaged with 3.0 mL phosphate-buffered saline. Lavage was centrifuged onto slides (Cytospin), and cells were fixed and stained using Giemsa. A minimum of 300 cells per animal were counted.

Statistical Analysis

Statistical analyses were performed using either GraphPad Prism version 7 (GraphPad Software, Inc., San Diego, CA) or R version 3.6.1.[31] To investigate the differences in body weight trajectories over time between genotypes and sexes, a linear mixed model was fitted using the lme function from the nlme statistical package (*https://cran.r-project.org/package=nlme*) in R. A B-spline (with 5 Df) for time was fitted as a fixed effect using the bs function from the

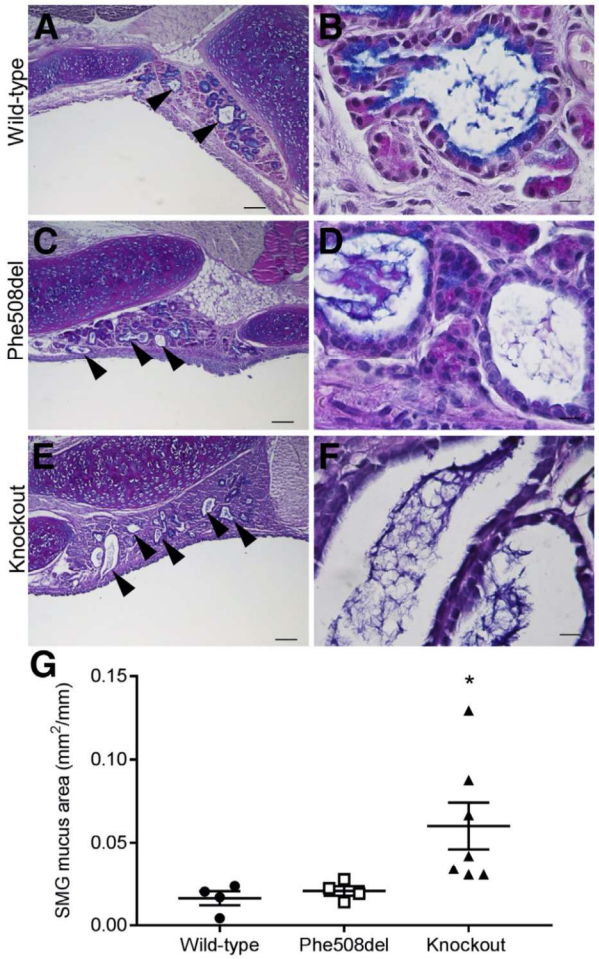

Figure 6 Trachea histology and submucosal gland area quantification in Phe508del and knockout (KO) rats. **A**–**D:** Histology (Alcian blue periodic acid–Schiff stained) of the trachea from 1-month–old rats. **A** and **B:** Normally developed trachea tissue in wild-type (WT) rats with occasional dilated submucosal glands (SMGs). **C** and **D:** Phe508del rats demonstrate submucosal gland dilation at a similar frequency to the wild-type rats. **E** and **F:** KO rats show abundant dilated submucosal glands filled with mucus material. **Arrowheads** indicate dilated submucosal glands. . **G:** Morphometric quantification of the submucosal gland mucus area. Data are expressed as means ± SEM (**G**). $n = 4$ to 7 animals per group (**A**–**G**). *$P < 0.05$ versus WT (one-way analysis of variance). Scale bars: 100 μm (**A**, **C**, and **E**); 10 μm (**B**, **D**, and **F**).

splines package (R Core Team 2019) to model the nonlinear relationship of weight over time. Genotype (wild-type, Phe508del, or knockout) and sex (female or male) were also fitted as fixed effects, as well as all possible interactions between fixed effects. Time nested within rat identification number was fitted as a random effect. The within-subject variance was estimated separately for each sex to allow for heterogeneity in weight across the sexes. The fixed effects were examined using likelihood ratio tests, including assessment of interactions. Tukey's honestly significant difference pairwise multiple comparisons of genotypes at each time point for each sex were performed using the emmeans statistical package (*https://cran.r-project.org/web/packages/emmeans*) in R. Results are shown as the estimated mean weight at each time point and associated 95% CIs.

For survival analysis, Kaplan-Meier survival curves were plotted in GraphPad. The survdiff function from the survival statistical package (*https://cran.r-project.org/package=survival*) in R software version 3.6.1[31] was used to compute the log-rank test to compare the survival curves. Pairwise comparisons between groups were performed using the pairwise_survdiff function from the survminer statistical package (*https://cran.r-project.org/web/packages/survminer*) in R. Other normally distributed outcome variables were analyzed using one-way analysis of variance with Dunnett's multiple comparisons test, where $P \leq 0.05$ was considered statistically significant.

Results

Phe508del and KO Founders Were Generated Using CRISPR/Cas9

Of the 30 pups born (from 90 transplanted embryos), six animals (20%) were identified to be carrying at least one mutant allele within a 187-bp region of exon 11 (surrounding the TTT deletion site). Two (6.7%) of the screened animals were identified as having the TTT deletion. One rat had an allele that contained the desired homology-directed repair edited TTT deletion and was designated the Phe508del founder (c.1522_1524delTTT). The second rat also harbored an allele with the TTT deletion. However, this rat was a mosaic, containing two alleles with undesired mutations and, therefore, was not used for breeding. A rat with an 8-bp deletion upstream of the TTT site (c.1514_1521delATATCATC) was used to establish the KO strain. This mutation causes a frameshift after amino acid 505, resulting in the introduction of a premature termination codon at position 510. The remaining three mutant pups harbored various nonhomologous end joining–repaired alleles and were not used for breeding. Selected founders were mated with wild-type Sprague-Dawley rats to produce heterozygous breeding pairs that were subsequently used to breed CF rats.

Breeder Rats Have Normal Reproduction Rates

For Phe508del breeding, three heterozygous Phe508del × homozygous Phe508del breeding pairs gave birth to 158 pups in 16 litters, with a mean litter size of 9.8 pups. For KO breeding, seven heterozygous breeding trios gave birth to 306 pups in 26 litters, with a mean of 11.7 pups per litter. The litter sizes were comparable to wild-type Sprague-Dawley breeding (mean litter size of 10.2 pups). When using homozygous Phe508del females and heterozygous males for breeding, the ratio of CF/heterozygous pups was close to the expected 1:1 ratio. Similarly, when using heterozygous female and male pairings for the

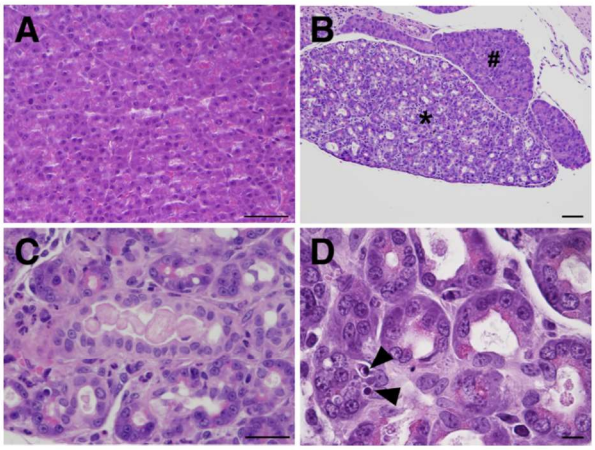

Figure 7 Pancreas histopathology in knockout (KO) rats. Hematoxylin and eosin–stained pancreas sections from 1-month–old rats. **A:** Normal pancreas histology in wild-type rat. **B:** Pancreas from KO rat with focal degeneration of the exocrine pancreas (**asterisk**) adjacent to normally developed pancreatic tissue (**hash mark**). **C:** Pancreatic duct filled with eosinophilic secretions. **D:** Apoptotic bodies within acinar cells indicated by **arrowheads**. $n = 8$ animals per genotype (**A**–**D**). Scale bars: 50 μm (**A** and **C**); 10 μm (**B** and **D**).

KO breeding, the ratio was approximately 1:2:1 (wild type/heterozygous/CF). The genotype distribution for both CF rat strains is outlined in Table 2.

Growth Trajectories Are Reduced in Phe508del and KO Rats

The linear mixed model analysis of body weight data showed that the growth trajectories differ significantly between wild-type, Phe508del, and KO rats for both males and females ($F_{10,1449} = 12.60$; $P < 0.0001$). The fitted splines for each genotype and sex are shown in Figure 1. Pairwise comparisons revealed that from 1.25 months of age onwards, the estimated mean body weights for Phe508del and KO were significantly lower than wild-type, with the exception of female Phe508del rats, where the estimated mean body weight did not become significantly lower than wild-type until 2 months of age. In addition to this, KO rats of both sexes had a significantly reduced estimated mean body weight when compared with Phe508del rats. These differences in body weight were maintained for all genotypes and sexes until 6 months of age, apart from female Phe508del rats, where the difference was no longer statistically significant from wild-type after 3.5 months.

Survival Is Impaired in Phe508del and KO Rats

The survival of both Phe508del and KO rats is significantly reduced when compared with wild-type animals ($P < 0.0001$) (Figure 2). KO rats are more severely affected, demonstrating a significantly lower survival when compared with Phe508del rats. By 1 week of age, the proportion of surviving Phe508del and KO pups is decreased compared with wild-type (84% versus 60% survival, respectively). Early death is attributed to meconium ileus (MI) and failure to thrive. Around the time of weaning (21 days) until approximately 2 months of age, both Phe508del and KO rats demonstrated further reductions in survival, typically due to gastrointestinal obstructions and poor weight gain. By approximately 2 months of age, 66% of Phe508del rats and 34% of KO rats remained. After this stage, survival plateaued for both genotypes, with no deaths observed from this point onwards.

CFTR mRNA Is Reduced in the Lungs of KO Rats while Phe508del Levels Are Unaffected

RNAscope *in situ* hybridization was performed to localize *CFTR* mRNA in the lungs of wild-type and CF rats. RNAscope analyses demonstrated that *CFTR* mRNA was robustly expressed in the bronchi and small airway epithelium of wild-type rats, as well as in the alveolar regions, where it localized to type II pneumocytes. Phe508del rats showed similar *CFTR* mRNA levels and localization patterns when compared with wild-type, whereas KO rats exhibited lower *CFTR* mRNA expression in the airways and alveolar tissue (Figure 3, A–I). Quantification of *CFTR* mRNA molecules revealed that Phe508del levels did not differ from wild-type, whereas KO transcripts were significantly reduced ($P < 0.05$) (Figure 3J).

Quantitative real-time PCR was performed to measure *CFTR* mRNA expression in the lung tissue. Consistent with the RNAscope findings, *CFTR* mRNA levels in Phe508del rat lungs were approximately 61.2% of wild-type levels, with no statistically significant difference observed. This is comparable to humans with the Phe508del mutation, where the level of *CFTR* transcripts is similar to that of non-CF.[32] *CFTR* mRNA expression was significantly reduced in KO rats, with levels present at 23.6% of wild-type ($P < 0.001$) (Figure 3K).

Immunostaining Reveals Reduced CFTR and Differential Localization in Phe508del and KO Rats

CFTR was highly expressed in the bronchioles of wild-type rats, whereas Phe508del and KO rats had reduced CFTR expression. Enlarged images show that in wild-type animals, most cells lining the bronchiole express CFTR on their apical surface. In contrast, CFTR expression in Phe508del and KO rats displayed cytoplasmic localization with intense punctate staining. KO expression of CFTR was also found to have a lower proportion of CFTR-positive cells lining the bronchioles. The University of North Carolina CFTR-knockout mouse negative control tissue exhibited no CFTR staining. The secondary antibody-only control was also negative for CFTR staining (Figure 4A). Dual staining using lysosomal-associated membrane protein 1 demonstrates that CFTR colocalizes

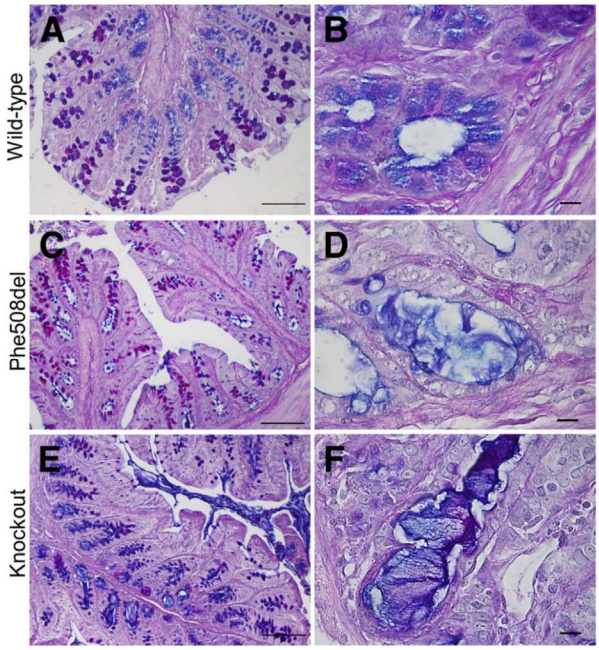

Figure 8 Large intestine histology in Phe508del and knockout (KO) rats. Alcian blue periodic acid—Schiff—stained large intestine sections from 1-month—old rats. **A** and **B:** Normally developed large intestine in wild-type rat with absence of mucus in the crypts. **C** and **D:** Phe508del rats show mild crypt dilation with small amounts of mucus. **E** and **F:** KO rats exhibit abundant mucus in markedly dilated crypts, with mucus extrusion into the lumen frequently observed. $n = 8$ animals per genotype (**A**—**F**). Scale bars: 100 μm (**A**, **C**, and **E**); 10 μm (**B**, **D**, and **F**).

to the lysosome in both Phe508del and KO rats (Figure 4B).

Hematology and Serum Chemistry Parameters Are Unaffected in CF Rats

Total white blood cell count and differentials were not different between wild-type, Phe508del, and KO animals (Supplemental Table S1). Red blood cell, hemoglobin, and hematocrit levels were also unchanged. Mean corpuscular hemoglobin levels were significantly higher in KO animals ($P < 0.05$) but were still within normal range. There were no significant changes in most of the serum biochemistry analytes. Interestingly, the levels of liver enzyme alkaline transferase were significantly increased in the KO animals compared with wild-type ($P < 0.05$); however, all alkaline transferase levels were above the reference range for Sprague-Dawley rats, and there was no histologic evidence of hepatocellular injury.

Bioelectric Defects Are Present in the Nasal Airways of Phe508del and KO Rats

The transepithelial voltage across the nasal airway surface was measured to characterize the airway bioelectric profile. KO rats demonstrated classic CF electrophysiological defects in the nasal respiratory epithelium, whereas Phe508del rats showed an intermediate phenotype (Figure 5). Under basal conditions, KO and Phe508del rats demonstrated a significantly more negative baseline potential difference when compared with wild-type animals, with this parameter more pronounced in KO rats. Both Phe508del and KO rats exhibited an enhanced depolarization response to perfusion of the epithelial sodium channel inhibitor amiloride, indicating the presence of increased sodium absorption in the nasal airways. Wild-type animals, on the other hand, showed an expected small depolarization response.

Perfusion of a low-chloride solution produced marked hyperpolarization in wild-type rats, indicating robust CFTR function in the nasal airways. Comparatively, the hyperpolarization response in Phe508del rats was blunted and depolarization was observed in KO rats. KO rats also exhibited a minimal ΔPD to perfusion of cAMP agonist isoproterenol when compared with WT rats, suggesting an absence of CFTR-mediated chloride transport; however, this effect did not reach statistical significance. Phe508del rats displayed a small hyperpolarization response to CFTR stimulation with isoproterenol, suggesting that although residual CFTR function is present, it is considerably impaired.

Lung, Nasal, and Liver Tissues Are Histologically Normal in Phe508del and KO Rats

The lung tissues of Phe508del and KO rats showed no evidence of histopathology at 1 month of age (Supplemental Figure S1). Furthermore, bronchoalveolar lavage cell profiles did not exhibit any differences between Phe508del, KO, and wild-type rats, indicating a lack of inflammation in the lungs of both CF rat models (data not shown). Microscopically, nasal and liver tissues from Phe508del and KO rats at 1 month of age also appeared normally developed (Supplemental Figures S2 and S3).

Tracheal Tissue Is Histologically Abnormal in KO Rats

Macroscopically, the trachea of both CF rat models appeared normally developed. Histologically, KO rats frequently showed dilated submucosal gland lumina containing abundant mucus secretion, the identity of which was confirmed by Alcian blue periodic acid—Schiff staining. In addition, KO rats exhibited increased numbers of dilated submucosal glands. Phe508del and wild-type rats also demonstrated occasional dilation of the submucosal glands with eosinophilic, protein-like material, a previously reported normal finding in rats (Figure 6, A—F).[33] Morphometric measurements of trachea section images confirmed these observations, with KO rats exhibiting a significantly larger submucosal gland mucus area when compared with wild-type and Phe508del rats ($P < 0.05$) (Figure 6G).

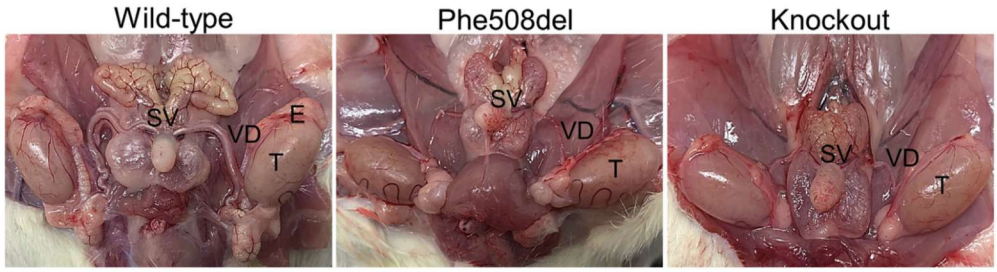

Figure 9 Male reproductive tract anatomy in Phe508del and knockout (KO) rats. Phe508del and KO rats lack properly formed vas deferens (VD) and seminal vesicles (SVs), and epididymides (E) are not observable in either cystic fibrosis rat model. $n = 3$ animals per group. T, testis.

KO Rats Exhibit Disease of the Exocrine Pancreas

Macroscopically, the pancreas of both Phe508del and KO rats was indistinguishable from wild-type rats. Histologically, the pancreas of Phe508del rats was normally developed, whereas approximately 25% of KO rats exhibited multifocal degeneration of the exocrine pancreas. The exocrine pancreatic histopathology observed in a proportion of KO rats was characterized by apoptosis of acinar cells, forming apoptotic bodies and necrosis, desquamation of cellular debris into dilated lumina, and occasional ducts distended by eosinophilic, proteinaceous secretion (Figure 7). These lesions are similar to those observed in early human CF pancreatic disease.[34–36] The endocrine tissue (islets of Langerhans) did not appear to be affected in either Phe508del or KO rats.

Phe508del and KO Rats Demonstrate Lethal Intestinal Obstruction

Gastrointestinal obstruction is common in all reported CF animal models, including mice,[37] rats,[7] ferrets,[38] pigs,[39] and sheep.[8] During the early neonatal period, both Phe508del and KO rats experienced mortality due to meconium ileus. KO rats had a higher occurrence of MI, with 40% affected compared with 16% of Phe508del rats. Rat pups with MI were substantially smaller than their littermates and presented with dark and distended abdomens, had dehydration, as indicated by skin tenting, and often lacked a milk band, suggesting that they had not fed. At post-mortem, obstruction was observed in the small intestine, which consisted of tenacious meconium. After weaning to solid food and up until 2 months of age, CF rats exhibited another wave of mortality that was attributed to intestinal obstruction.

Phe508del and KO Rats Exhibit Colonic Crypt Dilation and Mucus Accumulation

The small intestine (duodenum and ileum) appeared histologically normal in both rat models, whereas the large intestine demonstrated some histopathologic features. KO rats exhibited markedly dilated crypts that were often distended with mucus. Mucus extrusion into the lumen of the large intestine was frequently observed and was often overlying the mucosal surface. Although Phe508del rats also demonstrated this pathology, it was typically milder in nature, with fewer and smaller dilated crypts and only occasional mucus extrusion into the lumen. In both CF rat models, the intestinal crypts were found to be dilated with Alcian blue periodic acid–Schiff positive mucin (Figure 8).

Male Phe508del and KO Rats Exhibit Reproductive Tract Malformations

Analysis of the male reproductive tract showed significant morphologic differences in Phe508del and KO rats when compared with their wild-type counterparts. Phe508del and KO rats exhibited a marked reduction in the size of the vas deferens and seminal vesicles, whereas the epididymides were unobservable (Figure 9). Isolation of cauda epididymal sperm from the vas deferens indicated that Phe508del rats had normal to moderate sperm levels; however, a proportion of rats exhibited sperm abnormalities, with separation of the tail and head observed. KO rats, on the other hand, had a

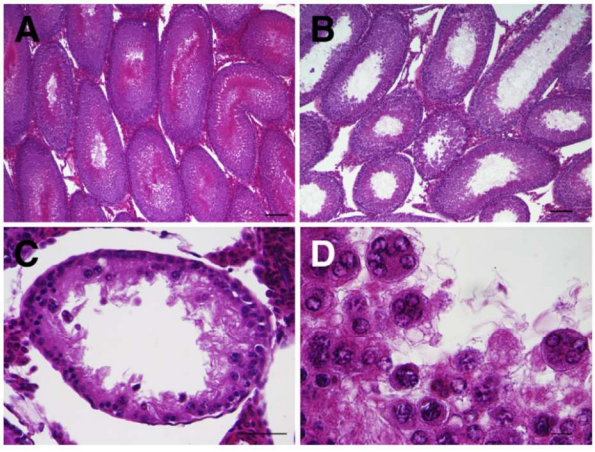

Figure 10 Testicular histology in Phe508del and knockout (KO) rats. Hematoxylin and eosin–stained testicular sections from 8- to 12-week–old rats. **A:** Normally developed testis tissue in wild-type rat. **B–D:** Phe508del and KO rats demonstrate testicular degeneration and reduced sperm abundance (**B**), diminished germinal epithelium in the seminiferous tubules (**C**), and multinucleated giant cells in the lumina of some tubules (**D**). The extent of tubule degeneration was variable between animals of the same genotype. $n = 3$ animals per group (**A–D**). Scale bars: 100 μm (**A** and **B**); 50 μm (**C**); 10 μm (**D**).

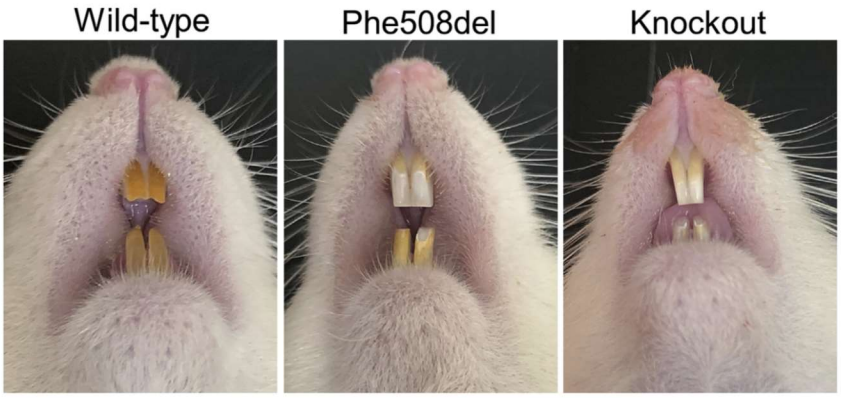

Figure 11 Dentition in Phe508del and knockout (KO) rats. A lack of incisor enamel pigmentation is observed in both Phe508del and KO rat strains. Wild-type rats exhibit characteristic yellow-brown enamel, whereas Phe508del rats demonstrate yellow-white enamel and KO rats demonstrate bright white incisors. $n = 3$ animals per group.

complete absence of cauda epididymal sperm (data not shown).

Although the Phe508del and KO rat testes appeared macroscopically normal, the tone of the testis on palpation was soft as opposed to the firm testis from the wild-type rats. Microscopically, in both Phe508del and KO rats, seminiferous tubules were reduced in size and there was disorganization, degeneration, and exfoliation of germinal epithelial cells, which were often diminished in number. Multinucleated giant cells, derived from spermatid coalescence, were abundant in the lumen of some tubules. This testicular degeneration resulted in disrupted spermatogenesis and a marked paucity of spermatozoa. In a few seminiferous tubules, germ cells were markedly depleted, with only basal Sertoli cells and occasional spermatogonia remaining (Figure 10).

Phe508del and KO Rats Demonstrate Abnormal Dentition

Both CF rat strains demonstrated abnormal dentition, as has been previously observed in CF mouse models[40] and the US-generated KO rat model.[7] Compared with wild-type animals that demonstrate normal yellow-brown incisor enamel, both CF rat strains lack enamel pigmentation. KO rats exhibit bright white enamel, and Phe508del rats appear to show an intermediate phenotype of yellow-white teeth (Figure 11). Rats from both genotypes have a tendency to develop grossly malformed teeth that become curved and overgrown, requiring regular incisor trimming.

Discussion

Advances in gene modification technologies have enabled the development of a range of CF animal models, including mice, ferrets, pigs, sheep, and rabbits. Although these animals are proving useful for a range of mechanistic and therapeutic studies,[21,41–43] species such as ferrets, pigs, and sheep have severe disease phenotypes, require costly and labor-intensive husbandry, and cannot be imported into Australia because of quarantine and licensing restrictions. Rats were selected because of the logistical advantages of breeding and rearing, and evidence of a mild lung disease phenotype in US-generated KO rats[7,14] that permits longitudinal assessments of therapies. In addition to the existing CFTR KO paradigm, this study generated the first CF rat model bearing the common Phe508del mutation to provide an *in vivo* platform for investigating Phe508del-specific disease mechanisms and treatments, including pharmacologic agents and genetic therapies.

The characterization from this investigation found that Phe508del rats consistently exhibited milder disease phenotypes when compared with KO rats. In the airways, the CFTR-Phe508del protein showed predominant cytoplasmic staining that was localized with the lysosome, indicating protein degradation. This finding is consistent with the human equivalent, where peripheral protein quality control systems remove CFTR-Phe508del from the cell surface for lysosomal degradation.[44] The nasal potential difference profile of the Phe508del rat indicated low-level CFTR-mediated chloride transport in the nasal airways. It is, therefore, likely that a fraction of rat CFTR-Phe508del mRNA is partially processed, allowing mutant CFTR to reach the apical membrane and retain partial function as a chloride channel. Although this level of CFTR function is not sufficient to prevent disease altogether, it may explain the milder disease phenotype observed in Phe508del rats when compared with KO. The Phe508del mutation is known to result in severe disease in humans, differing from the mild phenotype observed herein in the rat. This disparity could partly be explained by species differences in the processing and function of CFTR-Phe508del protein. In a previous study,[45] the CFTR-Phe508del processing defect was found to be more severe in humans when compared with pigs or mice. Unlike with human CFTR-Phe508del protein, mutant pig and mouse CFTR was found to be partially processed, resulting in residual transepithelial chloride transport, which is also likely the case for the Phe508del rat.

KO rats demonstrated greater disease severity across all described phenotypes, which aligns with quantitative PCR, RNAscope, immunohistochemical, and nasal potential

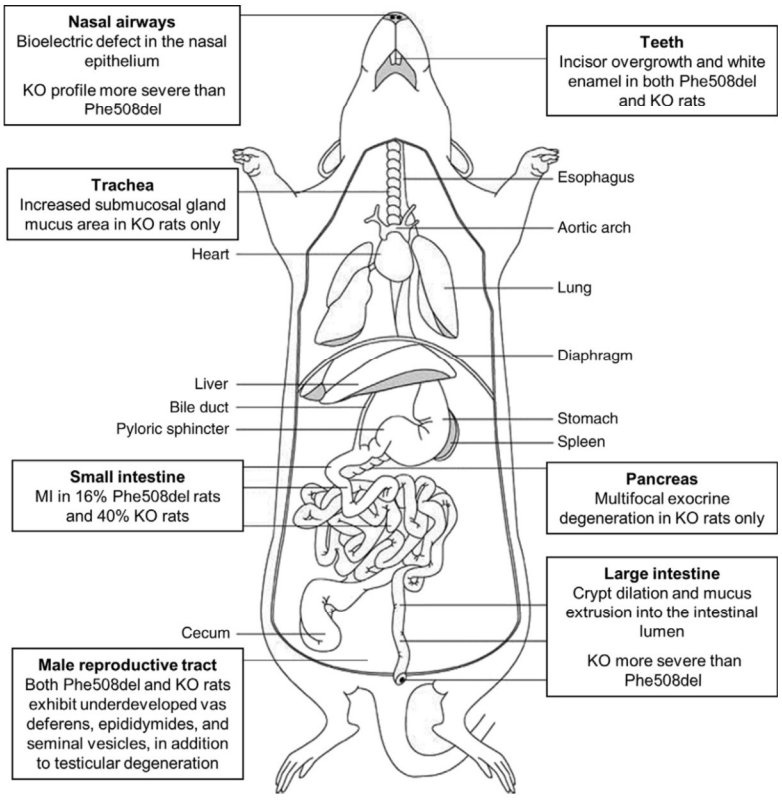

Figure 12 Schematic diagram indicating the cystic fibrosis phenotypes observed in Phe508del and knockout (KO) rats.

difference findings. KO rats had significantly reduced lung *CFTR* mRNA levels, likely because of the triggering of nonsense-mediated mRNA decay. Accordingly, CFTR protein levels were also substantially decreased in the airways, and the protein that was produced was truncated because of the presence of a premature stop codon, thus leading to a loss of CFTR function. The cytoplasmic presence of CFTR in KO airways suggests that some transcripts escape nonsense-mediated decay, a phenomenon that is present in human *CFTR* mutations, such as 3905insT.[46] As in Phe508del rats, KO CFTR was also colocalized with the lysosome, indicating degradation of the truncated protein. The lack of critical functional domains of the CFTR protein, in conjunction with the nasal potential difference profile indicating loss of CFTR function, suggests that although the CFTR protein is present, the function is lost, leading to generation of a more severe CF phenotype.

To the knowledge of the authors, this is the first study to assess *CFTR* mRNA localization in rat lungs using RNAscope *in situ* hybridization. These results revealed that *CFTR* mRNA is expressed robustly in cartilaginous and intrapulmonary airways of wild-type rats and localizes to type II pneumocytes, which has been observed in human adult lungs.[47] Comparatively, wild-type mice have low endogenous *CFTR* mRNA expression in the airways.[48] The high level of *CFTR* mRNA observed herein in wild-type rats provides an advantage over the use of mouse models for *CFTR*-related investigations.

Rats exhibit naturally high numbers of submucosal glands throughout the trachea and bronchi, resembling those of humans.[23,33] This is an advantage for a CF rat model given that serous cells of the submucosal glands express high levels of CFTR and the glands themselves are implicated in the development of human CF airway disease.[24,49] In this study, KO rats exhibited excess submucosal gland mucus production and accumulation, whereas Phe508del rats did not. This phenomenon in KO rats may be explained by a complete loss of CFTR-mediated anion transport in serous cells and a subsequent reduction in secretion of fluid, which, in turn, leads to viscous mucus in the lumen of the glands.[49–51] Mucus-filled submucosal glands are considered an early sign of CF lung disease, with this histology observed in the tracheae of human fetuses.[49,52] As human CF lung disease progresses, the submucosal glands become hypertrophied along with mucus plugging of the airways.[24] To date, overt signs of lung disease, such as mucus plugging, inflammation, and bronchiectasis, have not been observed in young Phe508del or KO rats, although it is currently unknown how the submucosal gland defect present in KO rats influences airway disease progression with age. Interestingly, US-generated KO rats also demonstrate defects in the tracheal submucosal glands; however, this pathology was not apparent until 6 months of age.[14]

Phe508del and KO rats demonstrate lethal gut obstructions during two developmental stages: the first week of the neonatal period and from post-weaning up until approximately 2 months of age. Interestingly, US-generated KO rats have not been reported to demonstrate MI during the neonatal phase; however, they do appear to develop post-weaning intestinal obstruction,[7] as was observed herein. As reported in humans and other CF animal models[3,38,39]—except for CFTR KO pigs, where 100% of animals are affected—variable penetrance of MI is observed in both CF rat models. In this study, neonatal MI affected 40% of KO and 16% of Phe508del rats, similar to the 15% incidence observed in humans.[53] Studies in humans and mice indicate that modifier genes are likely to be responsible for incomplete penetrance of MI.[54–56] In addition to MI, both rat models demonstrated colonic crypt obstruction with mucoproteinaceous secretions. This intestinal histopathology is frequently observed in humans as well as CF animal models, including the US-generated KO rat and CF mouse models.[7,57,58]

At the histologic level, the exocrine pancreas of 1-month–old KO rats demonstrated multifocal degeneration of acinar cells, whereas the tissue of Phe508del rats appeared normally developed. It is currently unknown whether this pathology in 1-month–old KO rats advances with age, as is observed in human disease, where the pancreas undergoes acinar destruction, inflammation, fibrosis, and fatty infiltration.[59] Pancreatic phenotypes are highly variable between CF animal models. Mouse models were initially thought to lack pancreatic disease; however, some reports have described mild age-related exocrine pancreas manifestations in congenic KO mice.[60] CF sheep and pigs both possess severe pancreatic disease, with advanced atrophy of the exocrine pancreas present at birth.[8,39,57] CF ferrets display a pancreatic phenotype most similar to the human course of disease, with a mild pancreatic disease at birth characterized by multifocal areas of dilated acinar cells and subsequent age-related pancreatic destruction.[38] Interestingly, pancreatic histopathology has not been reported in the US-generated KO model.[7] Similar to the findings from this study, Phe508del pigs and Phe508del mice demonstrate less severe pancreatic destruction or no pancreatic pathology, respectively, when compared with their KO counterparts.[41,60,61]

Only 25% of assessed 1-month–old KO rats exhibited multifocal degeneration of the exocrine pancreas. The incomplete penetrance of pancreatic pathology could be explained by the influence of modifier genes that protect some animals from loss of CFTR function in the exocrine pancreas.[38] In human CF disease, MI is almost exclusively observed in individuals with exocrine pancreatic insufficiency.[54,62] Accordingly, the low prevalence of pancreatic pathology at 1 month of age may be attributed to premature death of rats with MI that would otherwise go on to develop pancreatic disease. Future studies assessing the presence of pancreatic histopathology in pups that die neonatally from MI are warranted to further examine this phenomenon. Furthermore, longitudinal studies are required to determine whether pancreatic pathology appears in older Phe508del rats. The pancreas disease modeled in KO rats could provide a platform for investigating early disease pathogenesis.

Both CF rat models demonstrate malformations of the male reproductive tract, including hypoplasia of the vas deferens and seminal vesicles and an absence of the epididymides. These observations are similar to the CF human phenotype of congenital bilateral absence of the vas deferens. The hallmarks of congenital bilateral absence of the vas deferens include atresia of the vas deferens and atrophy or absence of the epididymides and seminal vesicles, which results in obstructive azoospermia and renders the patient infertile.[63] In addition to anatomic abnormalities, this study also reports that testicular development and spermatogenesis are impaired in Phe508del and KO rats. *CFTR* is known to be expressed in human testes, yet histologic examinations from males with CF and congenital bilateral absence of the vas deferens have yielded variable results, ranging from normal spermatogenesis to significantly reduced sperm counts and sperm abnormalities.[64,65] Although progress has been made in understanding CF male infertility, the underlying mechanisms of congenital bilateral absence of the vas deferens pathogenesis and the role of CFTR in sperm maturation are yet to be fully understood.[66] Accordingly, Phe508del and KO CF rat models could provide a novel platform for elucidating the physiological mechanisms of CF male infertility.

In summary, both Phe508del and KO rat models recapitulate a range of CF phenotypes (Figure 12). These characteristics include impaired survival, reduced weight gain, intestinal obstruction, bioelectric defects in the nasal epithelium, tooth malformations, and male reproductive tract abnormalities. Histopathologically, Phe508del and KO rats exhibited colonic crypt dilation due to excess extruded mucus, whereas KO rats also demonstrated topographically increased submucosal gland mucus secretion in the trachea and multifocal degeneration of the exocrine pancreas. CF lung pathology was not observed in either rat model at 1 month of age, and longitudinal studies assessing disease progression over time are in progress. Given that these rats are housed under clean conditions, future studies will investigate lung pathogen challenges with CF-relevant bacterial, viral, and fungal pathogens as these may be necessary to generate CF lung pathology.

These rat models will provide novel platforms for trialing therapeutics and elucidating the complexities of CF pathogenesis. In particular, the Phe508del model could be used for screening pharmacologic agents, such as CFTR modulator drugs, whereas KO rats may be employed in trialing mutation-agnostic treatments, such as gene therapies. In addition, access to two CF rat models with differing *CFTR* mutations on the same genetic background

will allow for direct comparisons and investigations into the role of genotype on disease progression and treatment response. Finally, the CRISPR/Cas9 generation of a Phe508del model has provided precedence for the future development of rat models with other human *CFTR* mutations.

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2020.01.009*.

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Chapter 7: Increasing the efficacy of lentiviral-mediated airway gene delivery

Breaching the delivery barrier: Chemical and physical airway epithelium disruption strategies for enhancing lentiviral-mediated gene therapies

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Effective delivery of genetic therapies to the airway cells remains one of the greatest challenges to achieving successful translation to the clinic. One way to overcome resistance of the airway barrier is to transiently disrupt the integrity of the epithelium prior to gene vector delivery, thus opening airway tight-junctions and allowing the vector access to appropriate basolateral receptors and underlying cell types including basal cells. Chemical conditioning and physical airway epithelial perturbation approaches have previously been trialled in preclinical models and show evidence of enhanced transduction levels. In this study, LPC conditioning and physical perturbation strategies were assessed in rat airways for the first time to determine whether LV-mediated gene transfer efficacy could be improved.

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Breaching the delivery barrier: Chemical and physical airway epithelium disruption strategies for enhancing lentiviral-mediated gene therapy

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Short title

Strategies for improving airway gene transfer

Key words

Lentiviral vector, airway, epithelium, perturbation, rat

ABSTRACT

The lungs have evolved complex physical, biological and immunological defences to prevent foreign material from entering the airway epithelial cells. These mechanisms can also affect viral and non-viral gene transfer agents, and significantly diminish the effectiveness of airway gene-addition therapies. One strategy to overcome the physical barrier properties of the airway is to transiently disturb the integrity of the airway epithelium prior to delivery of the gene transfer vector. In this study, chemical (lysophosphatidylcholine, LPC) and physical epithelium disruption approaches were assessed for their ability to improve airway-based lentiviral (LV) vector mediated transduction and reporter gene expression in rats. When assessed at 1-week post LV delivery, LPC airway conditioning significantly enhanced gene expression levels in rat lungs, while a long-term assessment in a separate cohort of rats at 12 months revealed that LPC conditioning did not improve longevity of expression. In rats receiving physical perturbation to the trachea prior to gene delivery, significantly higher gene transduction levels were found when compared to LPC-conditioned or LV-only control rats when evaluated 1-week post gene transfer. While this work is proof-of-principle, airway epithelial disruption strategies based on physical perturbation demonstrate significant benefit for improving LV-mediated airway gene transfer in the trachea and warrant further exploration.

INTRODUCTION

Airway gene-addition therapy is currently under development for treatment of a range of hereditary and acquired pulmonary diseases. Of particular interest is the development of a gene therapy to treat airway disease caused by the genetic disorder cystic fibrosis. Gene-addition therapy employs a vector (non-viral or viral) to deliver wild-type gene copies to the relevant airway cells, with the ultimate goal of correcting the disease pathophysiology that results from the defective gene. While vectors can be readily delivered into the lung airways, physical, anatomical, and immune barriers have evolved to protect the airway host cells against airborne pathogens. These natural defences are also directed towards gene vectors, thus limiting gene transfer to the airway epithelium.

Major hurdles to efficient viral-vector mediated gene transfer include a polarised epithelium, paucity of viral receptors on the apical membrane, and the presence of airway tight-junctions that prevent vectors from accessing receptors located on the basolateral side [137]. One way to overcome these barriers is to perform epithelial perturbation prior to, or in conjunction with, gene vector delivery. Perturbation acts to disrupt tight-junction integrity, allowing vector particles access to basolateral receptors, thus enhancing vector-mediated gene transfer [131, 136, 139]. Epithelial perturbation can also potentially expose cells for transduction that are not in direct contact with the airway lumen, particularly the basal cells, which function as tissue-specific stem cells [101].

To achieve basolateral access, airway conditioning with a 'tight-junction opener' can be employed to increase paracellular permeability. Airway conditioning with compounds including ethylene glycol tetraacetic acid (EGTA) [161], perfluorochemical [144], sodium caprate [145], and sulphur dioxide inhalation [139] have proven effective for disrupting tight-junctions and increasing viral-vector mediated gene transfer in proof-of-principle animal studies [162]. Another compound that has been investigated extensively for this purpose is lysophosphatidylcholine (LPC) [100, 108, 163-165]. LPC is a natural component of lung surfactant that can be used to transiently open cellular tight-junctions when applied to the airway surface. A two-step dosing regimen employing LPC conditioning prior to the delivery of a HIV-1-derived lentiviral (LV) vector pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) has proven effective at increasing airway gene transfer in mice [100, 108].

Chemical conditioning approaches have disadvantages when it comes to performing LV-mediated gene transfer. LV vectors are highly fragile due to the presence of an outer lipid envelope [166] and can be inactivated upon contact with conditioning agents such as LPC. Although two separate administrations – one to deliver the conditioning compound, and then a second to deliver the vector after the chemical effect occurs – can be used to enable LV vectors to take advantage of this delivery enhancement effect, this two-step process increases procedure time and complexity. Additionally, transduction may be variable as there is no guarantee that the conditioning compound and vector will localise to the same regions [167].

An alternative strategy to disrupt the integrity of the airway epithelium is via physical perturbation. Early airway gene therapy studies employing adenoviruses demonstrated that externally applied mechanical injury to the airway epithelium via use of fine forceps is a simple and effective way to produce strong gene expression in the trachea of mice [146, 147]. Mechanical perturbation also resulted in high levels of basal cell transduction as this technique removed the superficial transduction-resistant columnar cells and exposed underlying basal cells to the vector [147]. While those initial studies validated the potential efficacy of a mechanical perturbation strategy, it has not been investigated further for preclinical development, likely due to the perceived impracticalities of performing controlled airway-surface perturbation in animal models. To our knowledge there are no reports of physical airway injury being used in combination with LV vectors for *in vivo* gene delivery.

In this study we provide evidence of the effectiveness of LPC conditioning over both short-term (1-week) and long-term (12-month) durations for the first time in rat airways. The efficacy of physical airway epithelium perturbation was explored as an alternative strategy to improve *in vivo* gene transfer levels and this method was compared to standard LPC-conditioning and a perturbation-free LV-only control group.

METHODS

Multiple treatment groups were employed in this project. The first study assessed the efficacy of LPC conditioning in rat airways to determine whether gene expression levels could be improved over short- and long-term durations. In this study, rats received LPC conditioning or a PBS (phosphate buffer saline) sham treatment, followed by a HIV-1 derived EF1α-3XFLAG-fLuc-F2A-eGFP vector (LV-FLAG-Luc-GFP) vector. In one cohort (n=6 per group) *luciferase* gene expression was measured 1-week following gene delivery, and in a separate cohort (n=12 per group) expression was measured 12-months after LV delivery.

To investigate and compare airway epithelial disruption strategies rats received either no epithelial disruption (control group), physical perturbation, or standard LPC conditioning, followed by delivery of a HIV-1-MPSV-nlsLacZ (LV-LacZ) vector (n=6 per group). Gene expression levels were assessed 1-week post gene transfer in this cohort of animals, as it was expected that the damaged airway epithelium would be regenerated and that gene expression would be readily apparent by this stage [147, 168].

Lentiviral vector production

LV vector was produced using previously described methods [158, 169]. The bicistronic LV-FLAG-Luc-GFP vector expressed firefly *luciferase* and enhanced *green fluorescent protein* (eGFP) genes under control of the EF1α promoter, together with three epitope FLAG tags. The LV-LacZ vector construct expressed a nuclear-localised *β-galactosidase* gene directed by the MPSV promoter. LV-FLAG-Luc-GFP vector was titered by quantifying the proportion of GFP positive cells using flow cytometry [169]. The titre of the LV-LacZ vector was determined using real-time quantitative PCR as previously described [170].

Animals

All animal procedures were approved by the University of Adelaide Animal Ethics Committee under applications M-2016-086 and M-2019-061. Female and male Sprague Dawley rats >8 weeks old were used. For all LV-dosing procedures, rats were anaesthetised with medetomidine (0.4 mg/kg) and ketamine (60 mg/kg) by intraperitoneal (IP) injection. Following anaesthesia, the rats were non-surgically intubated with a 16-gauge intravenous cannula (Terumo, SR-FF1651) acting as an endotracheal (ET) tube, which was positioned with the tip just past the vocal cords so that the majority of the tissue was exposed to the conditioning or perturbation and LV-vector. After procedure completion anaesthesia was

reversed with atipamezole (1 mg/kg). Rats that received physical airway perturbation were given subcutaneous administration of meloxicam (1 mg/kg) for precautionary pain relief.

Intratracheal administration of LPC and LV-vector

Animals were placed in a supine position and received either 25 µL of 0.1% LPC (Sigma-Aldrich) or PBS. Fluid was administered to the trachea via the endotracheal (ET) tube using a gel-loading pipette tip that was lengthened with fine polyethylene tubing to reach the end of the ET tube. LV vector delivery was performed one hour following LPC conditioning (or PBS-sham) as this is the time when LPC epithelial tight-junction opening effects are apparent [100]. LV-FLAG-Luc-GFP (1 x 10^9 TU/mL) or LV-LacZ (2 x 10^9 TU/mL) vectors were delivered to the trachea in two aliquots of 25 µL (50 µL total per animal).

Physical epithelial perturbation

With the intubated animal in the supine position, a pliable wire-basket (NCircle® Nitinol Tipless Stone Extractor NTSE-022115-UDH, Cook Medical; Figure 1) was fed in the retracted position down the ET tube to approximately the carina (based on an average trachea length measurement). The basket was extended, fully expanded, and drawn up and down the trachea to the carina (approximately 10 times) for 30 seconds followed by withdrawal from the lung. Two 25 µL aliquots of LV-LacZ (2 x 10^9 TU/mL) were delivered via the ET tube ten minutes after the perturbation event to allow time for the animals' respiration to return to normal following the procedure.

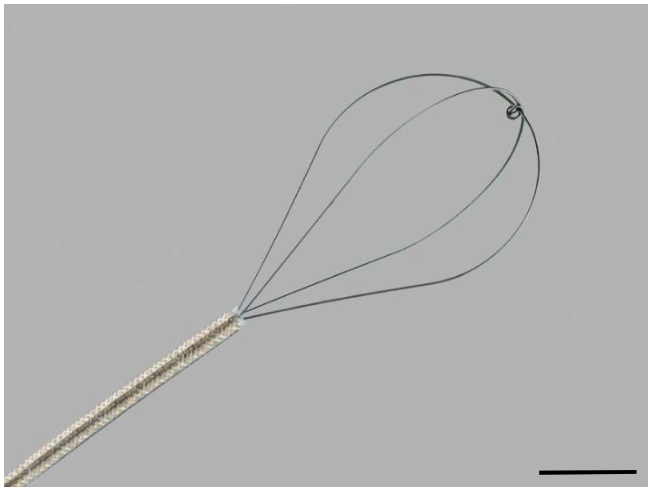

Figure 1: Flexible wire basket used to physically perturb the tracheal airway epithelium. The basket is 1 cm in diameter at full expansion and 1.5 cm in length. Scale bar = 5 mm.

Bioluminescence imaging (BLI)

Either 1-week or 12-months following LV-FLAG-Luc-GFP delivery rats were anaesthetised with 2.5% inhaled isoflurane. Once unconscious, 200 µL of 15 mg/mL D-luciferin (Cayman Chemicals, USA) was delivered to the nostrils as a bolus. The animal was imaged in the supine position (IVIS Lumina XRMS *in vivo* imaging system, PerkinElmer, USA) while maintained with 1.5 - 2% isoflurane. Images were acquired with an automatic exposure at 5-minute intervals for up to 15 minutes post D-luciferin delivery. The animal was then humanely killed with IP delivery of sodium pentobarbital (150 mg/kg). The trachea and lungs were immediately excised and imaged separately in a petri dish containing PBS. The resulting bioluminescence flux (photons/second) was measured using the contour tool (Living Image, version 4.7.2, PerkinElmer, USA).

Assessment of LacZ gene expression

Animals were humanely killed, and the airway tissues harvested for LacZ staining. Tissues were fixed in 4% PFA and stained for β-galactosidase (LacZ) activity using X-gal as previously described [171]. Following X-gal staining, the trachea was cut open longitudinally, and separated into two halves. *En face* images of the tissue were acquired with a Nikon SMZ1500 stereo microscope, DigiLite 3.0 MP Camera and TC capture software (Tucsen Photonics, China). Illumination intensity was not altered between samples. Tracheas were subsequently paraffin-embedded, sectioned at 5 µM and counterstained with nuclear fast red. Histology images were captured with a Nikon Eclipse E400 microscope, DS-Fi2-U3 camera and NIS-elements D software (v5.20.00).

Digital quantification of LacZ staining in en face trachea images

The amount of LacZ staining in the *en face* images of the trachea was quantified using a custom-written script in Matlab (R2010a, MathWorks) that calculated the area of the trachea that was the specific shade of blue/green that matched the LacZ-positive cells. Briefly, images were converted from the RGB to HSV colour space, and separate thresholds were applied to the hue ($0.45 < H < 0.6$) and saturation ($s > 0.4$) channels. The value channel was not used because the illumination across the sample was uneven and did not help discriminate the transduced cells. A binary mask was created from the regions of the image that satisfied both criteria. The mask area was converted from pixels into mm^2.

Statistical analyses

Statistical analyses were performed using either R v4.0.0 [172] or GraphPad Prism v8 (GraphPad Software, Inc.). Statistical significance was set at $p \leq 0.05$. For the short-term study, comparisons of flux between PBS and LPC groups were performed using Welch two sample t-tests on log-transformed data. To compare flux between the PBS and LPC treatments for the long-term study, a hurdle regression model was fitted using the *hurdle* function in the *pscl* package in R [173]. The hurdle regression model is a two-part model; a binary logit model to distinguish between the positive counts and those below the detection limit (assigned the value 100), and a zero-truncated negative binomial model for the positive counts. Treatment (PBS, LPC) was fitted as the regressor in both parts of the model. Differences between the groups were assessed using a Wald Chi-Squared test. The LacZ staining area measurements from the *en face* images were analysed using a one-way ANOVA with Tukey's multiple comparisons test.

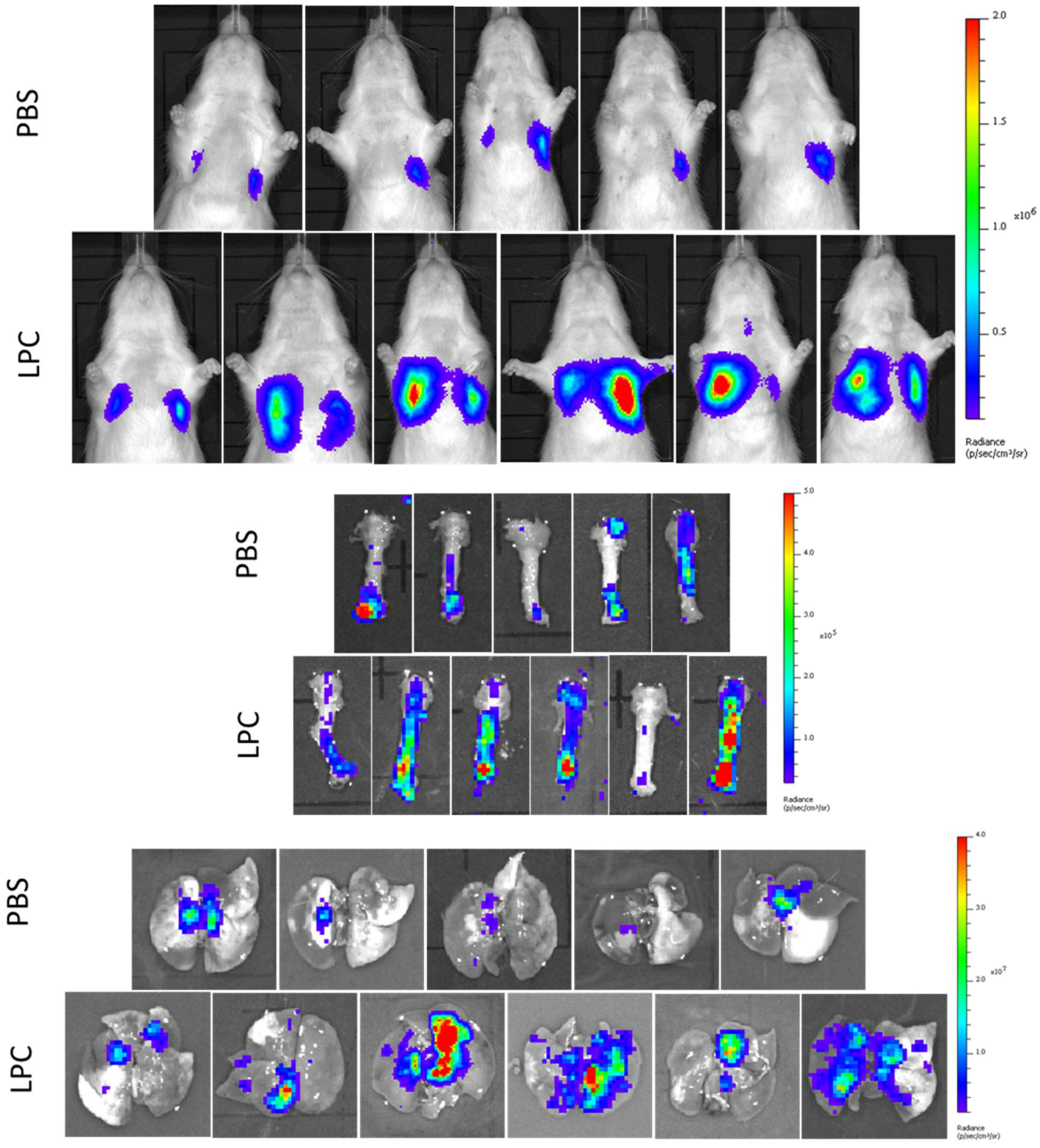

Figure 2: Bioluminescence images acquired 1-week following LV-FLAG-Luc-GFP delivery in PBS-sham and LPC conditioned rats. Top panel: *In vivo* images indicate the localisation of bioluminescence predominantly to the lung region. **Middle panel:** *Ex vivo* images of the trachea. **Bottom panel:** *Ex vivo* images of the lungs following removal of the trachea.

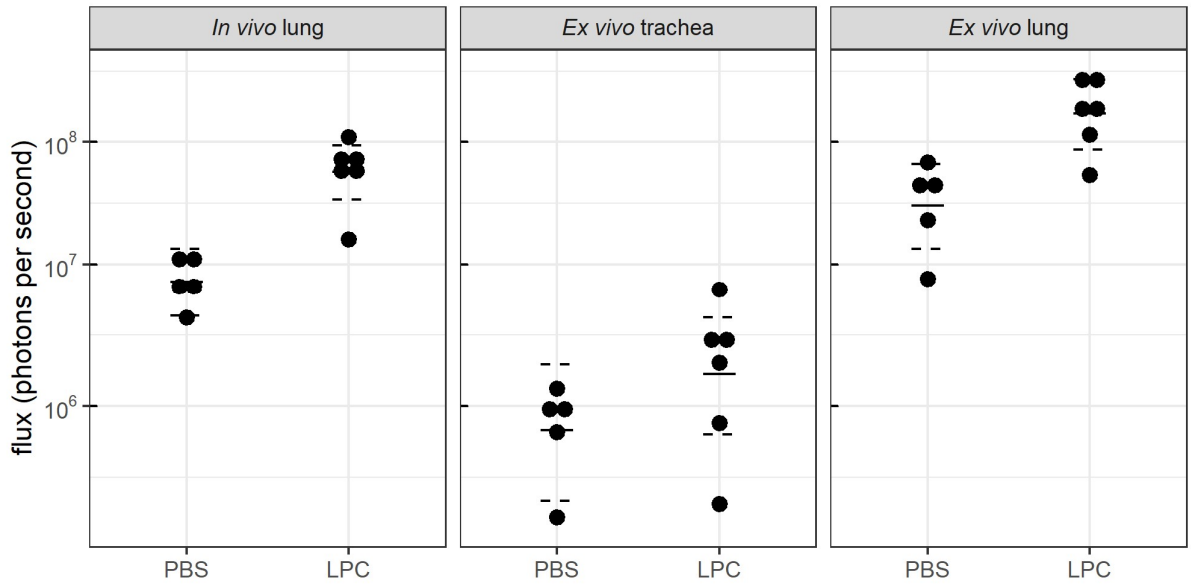

Figure 3: *In vivo* and *ex vivo* bioluminescence flux acquired 1-week following LV-FLAG-Luc-GFP delivery in PBS-sham and LPC conditioned rats. Statistical analyses of the *in vivo and ex vivo* lung data revealed significant differences in flux between PBS and LPC conditioned groups, with p=0.0002 and p=0.005, respectively. Statistical analysis of the *ex vivo* trachea indicated there was no significant difference in flux between LPC and PBS groups, p=0.2. The plot indicates the estimated median and 95% confidence intervals. n = 5-6 animals per group, Welch two sample t-test.

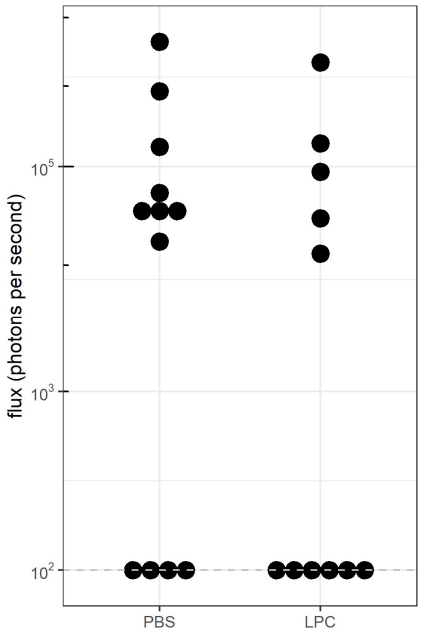

Figure 4: *Ex vivo* lung bioluminescence flux acquired 12 months following LV-FLAG-Luc-GFP delivery in PBS-sham and LPC conditioned rats. The dashed line indicates the limit of detection of the IVIS machine (10^2). No significant difference in flux was observed between animals in the PBS and LPC groups, p=0.6. n = 11-12 animals per group, Wald Chi-squared test.

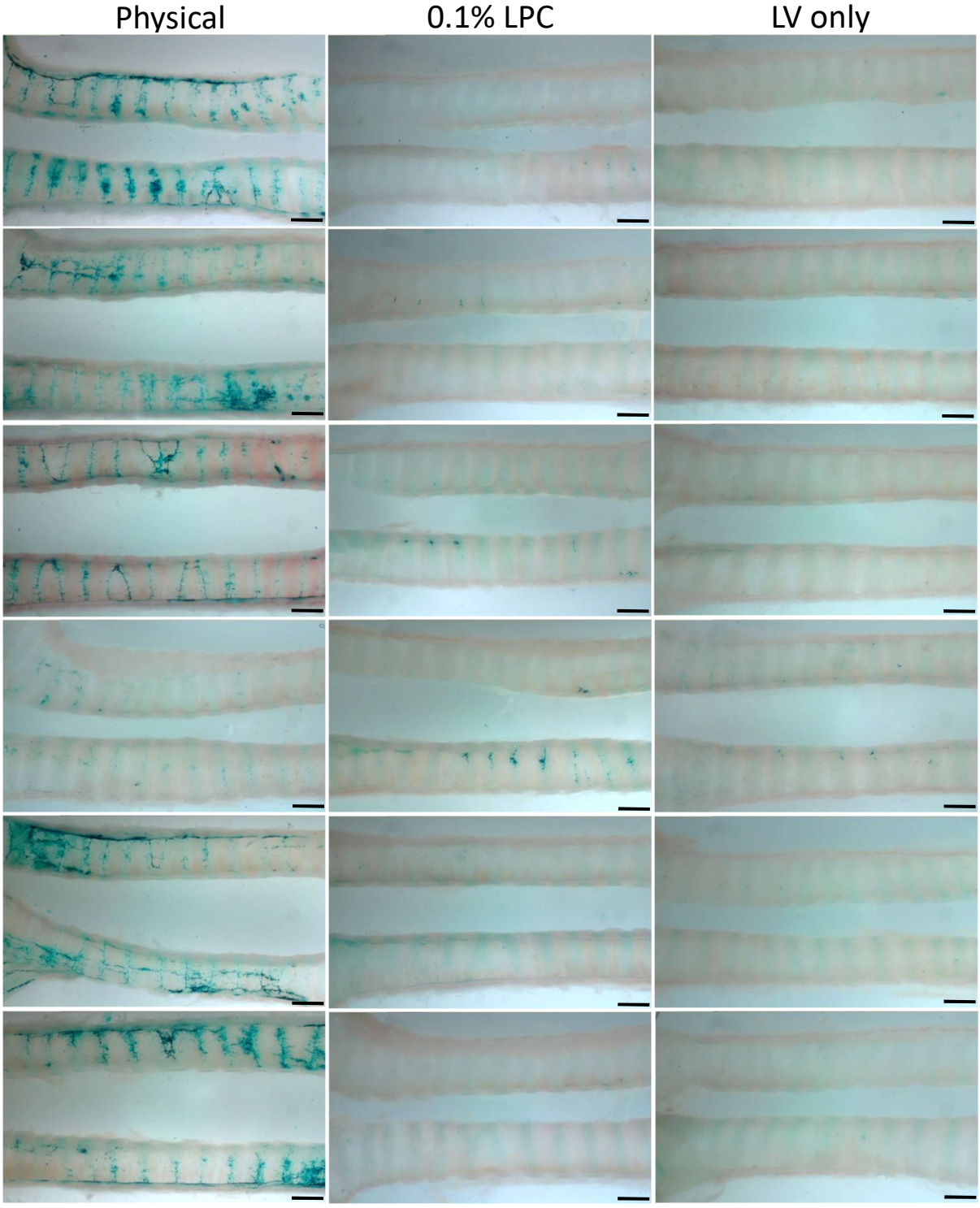

Figure 5: Comparison of *en face* LacZ staining using physical perturbation, chemical conditioning with 0.1% LPC or LV vector only assessed 1-week post gene transfer. Images show the trachea from each individual animal longitudinally cut into two sections to reveal the LacZ staining present within the lumen. The images display the middle portion of the trachea and are oriented to show the proximal trachea on the right. *n* = 6 rats per group. Scale bar = 1.5 mm.

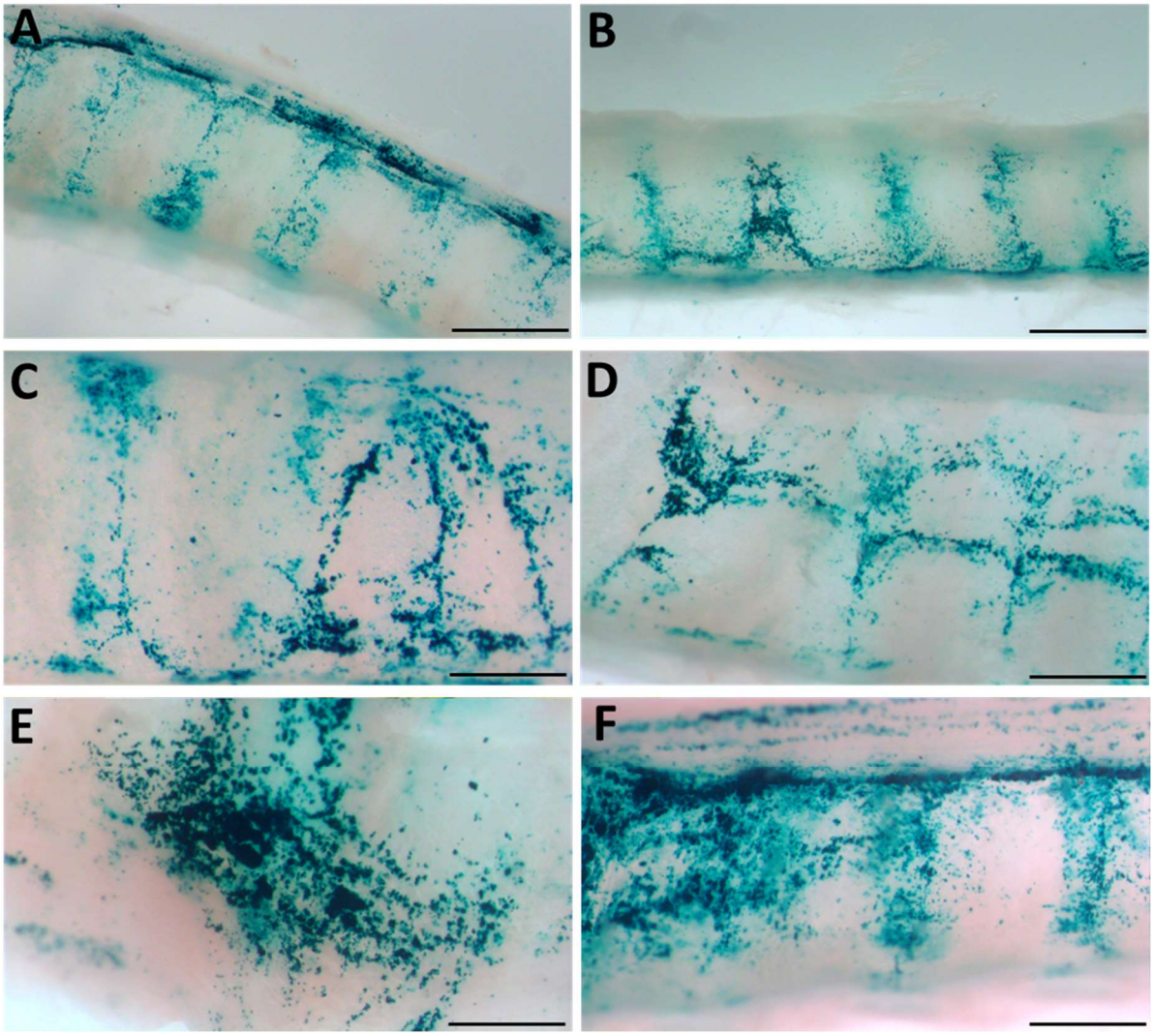

Figure 6: Physical perturbation reveals varying patterns of LacZ staining. (A, B) LacZ staining is increased in the areas overlying the inter-cartilage segments. **(C-F)** Basket-induced perturbation created focal regions of strong LacZ staining. High magnification images are acquired from the samples shown in Figure 5 and indicate staining patterns present in multiple animals. Scale bars A, B = 1.5 mm; C-F = 0.75 mm.

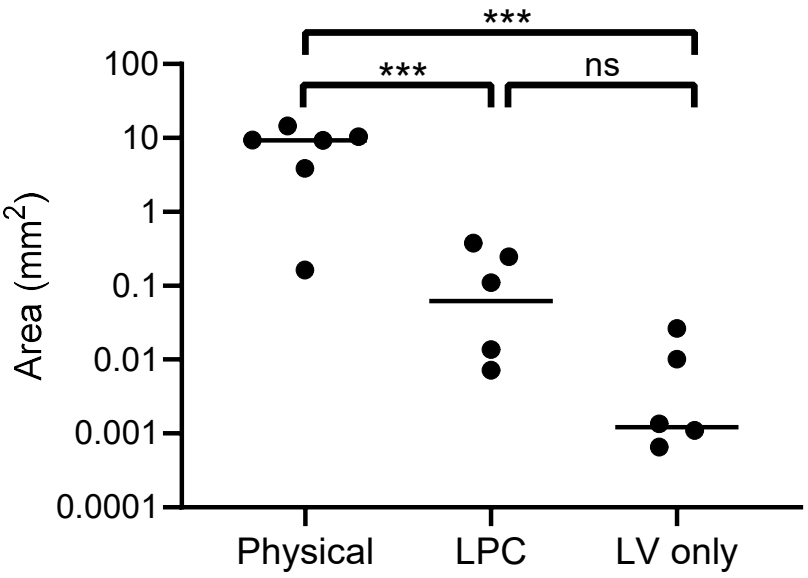

Figure 7: Quantification of LacZ staining area from *en face* images shown in Figure 5. Physical perturbation had a significantly greater area of tracheal LacZ staining after 1-week when compared to LPC conditioned (p=0.001) and LV vector only control animals (p=0.0008). There was no significant difference in the area of LacZ staining between LPC conditioned animals and those that received only LV vector (p=0.1). The plot indicates the median. n = 6 animals per group, one-way ANOVA with Tukey's post-hoc test. Note that two animals are not observed on the plot as the quantified area was zero.

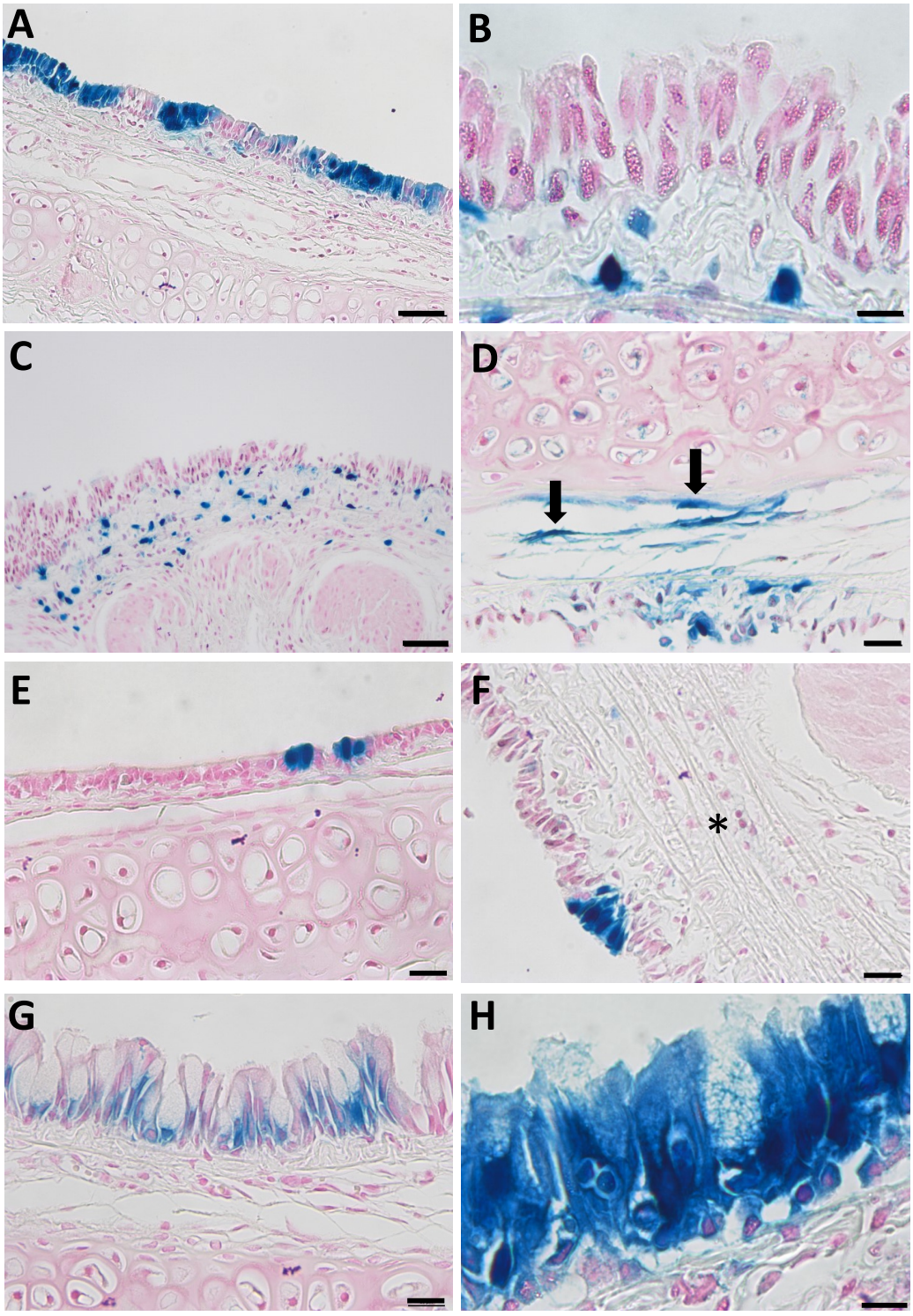

Figure 8: Histological observations in the tracheal epithelium of rats receiving physical perturbation prior to LV vector delivery assessed 1-week post gene transfer. (A) LacZ-positive surface epithelial cell types including ciliated and non-ciliated cells, and **(B)** transduced basal cells identified by their distinct triangular shape and contact with the basal lamina. Transduced cells within the lamina propria including suspected **(C)** macrophages and **(D)** possible fibroblasts (indicated by black arrows). While most of the tissue demonstrated full epithelial regeneration, some regions showed **(E)** a flattened epithelial layer lacking pseudostratification. **(F)** Connective tissue proliferation (fibrosis) in response to healing (denoted by asterisk). **(G, H)** Goblet cell hyperplasia observed at the carina. Some hyperplastic goblet cells exhibited LacZ staining. Images are shown from selected animals. Nuclear fast red counterstain. Scale bars A, C = 50 µM, D-G = 20 µM B, H = 10 µM.

Luciferase gene expression assessed 1-week post gene transfer

In the short-term assessment, rats received either PBS or LPC conditioning and were imaged 1-week following a single LV gene delivery (Figure 2). *In vivo* BLI revealed that LPC conditioning resulted in significantly higher flux when compared to animals that received the PBS sham treatment (Figure 3). Bioluminescence was predominantly observed in the lungs, with only one animal exhibiting signal within the trachea region. Upon excision of the tissues and subsequent imaging, bioluminescence was observed in the trachea of all animals. *Ex vivo* data revealed that there was no difference in flux between the LPC and PBS conditioned groups for the trachea, while the excised lungs revealed that LPC conditioned rats had significantly greater flux levels, consistent with the *in vivo* data. Adjusting for sex in the modelling did not result in differences from the unadjusted models.

Luciferase gene expression assessed 12-months post gene transfer

In the long-term study assessing chemical conditioning, few animals from either the LPC or PBS group had detectable levels of *in vivo* bioluminescence at the 12-month time point, therefore statistical comparisons could not be performed on this data. *Ex vivo* imaging of the trachea also revealed insufficient animals with bioluminescence present to perform statistical analysis. Imaging of the *ex vivo* lungs showed that close to half the animals from both LPC and PBS groups had values recorded at the limit of detection. Additionally, the flux levels measured in the *ex vivo* lungs at 12 months were substantially lower compared to the animals imaged at 1-week post-delivery. There was no significant difference in *ex vivo* lung flux between LPC conditioned and PBS animals 12-months following LV-delivery (Figure 4).

Tracheal LacZ staining following chemical conditioning and physical perturbation

En face observations of the trachea revealed varying levels of LacZ staining as a result of the perturbation method used (Figure 5). Visually, rats that received physical perturbation prior to LV-delivery demonstrated greatly enhanced LacZ staining in the trachea when compared to rats that received LPC conditioning or LV vector only. Interestingly, one animal in the physical perturbation group exhibited much lower staining levels compared to the others, indicative of potential variability with this technique. The transduction patterns observed as a result of physical perturbation were not uniform in appearance. Striated regions of increased staining over the intercartilaginous ligaments were apparent in all specimens, possibly reflecting the presence of cilia-rich zones that overlay the ligament segments [174] and preferential transduction of ciliated cells by the LV gene transfer vector. Varying LacZ

staining patterns and strong expression were observed in animals that received physical perturbation (Figure 6). Notably, rats in the LPC and LV only groups demonstrated the highest levels of staining in the proximal trachea (supplementary Figure 1), likely due to unintentional ET tube-induced epithelial damage in this region, providing further support for the effectiveness of physical perturbation in boosting airway gene transfer. *En face* LacZ staining was also observed in the lungs of all specimens, however, this tissue was not analysed because the physical perturbation was only applied to the trachea, thus comparisons of lung transduction could not be performed between groups.

Digital quantification of the blue-green coloured regions in the *en face* trachea images demonstrated significantly greater levels of LacZ staining in the physical perturbation group, when compared to LPC conditioned and LV vector only control rats (Figure 7). The physical damage group demonstrated more than a 1000-fold increase in the area of LacZ staining when compared to the control group. There was no statistically significant difference in staining area between LPC conditioned rats and controls that received only LV vector with no epithelial perturbation prior to delivery.

Histological observations arising from physical perturbation

A range of different cell types were transduced as a result of performing physical perturbation prior to LV vector delivery (Figure 8). Based on morphological appearance and location within the tissue, LacZ-positive ciliated, non-ciliated, and basal cells were observed. Cells within the lamina propria were also transduced, including suspected fibroblasts and possible macrophages. The tracheal tissue demonstrated evidence of repair processes caused by damage to the epithelium and surrounding regions. Within the lamina propria there were regions of connective tissue proliferation, consisting of collagen formation and fibroblasts. Most regions of the tracheal epithelium had fully regenerated by 1-week post treatment, consistent with the timing observed in previous studies [147, 168]. However, occasionally areas of epithelium were found to still be undergoing repair, as indicated by an attenuated appearance, lack of cilia, and rounding of the columnar cells. Goblet cell hyperplasia was also apparent at the carina and upper bronchi of two animals. Interestingly, many of these hyperplastic goblet cells appeared to be LacZ-positive.

DISCUSSION

Gene-addition therapy is a promising option for treatment of certain pulmonary diseases, but there are still many challenges to successful translation to the clinic. Delivery of therapeutic agents to cells of the airway epithelium remains one of the greatest challenges to achieving effective lung-based genetic therapies, including gene-addition and gene editing strategies. The lung has evolved mechanisms to resist invasion by foreign bodies, with the airway epithelium acting as a physical barrier that significantly reduces the efficacy of airway gene transfer. Many viral vectors used for airway gene therapies have a natural tropism toward receptors located on the basolateral membrane and thus produce limited transduction via the apical surface.

To overcome inefficient transduction, strategies have been employed to disrupt the integrity of the airway epithelium prior to gene delivery, allowing the vector access to basolateral membrane-located receptors and to cells that are not in direct contact with the airway lumen such as basal cells [131]. Given our recent generation of two CF rat models [175], the work reported here employed normal rats to develop and assess chemical and physical airway perturbation methods. In these studies, the trachea was used as surrogate for lower airways, as it is easily accessible for physical perturbation techniques and the cellular architecture is similar to human airways [176, 177].

In this work, LPC conditioning was assessed for the first time in rat airways. While mouse studies have indicated enhanced gene expression when using LPC conditioning prior to LV gene transfer [108, 171], its effects in rat airways were unknown. In this study, gene expression levels were found to be significantly increased in the lungs of rats that received LPC conditioning when assessed 1-week post vector delivery. Upon *in vivo* imaging, only one animal exhibited bioluminescence signal within the trachea, but following excision of the tissues and subsequent imaging, bioluminescence was observed in the trachea of all animals, albeit at varying levels. When imaged *ex vivo* the flux emitted from the trachea was substantially lower than the *ex vivo* lung, therefore the low bioluminescence in combination with the need to detect the signal through the overlying skin and tissue may explain the lack of tracheal bioluminescence upon *in vivo* imaging. Notably, when the trachea was excised and imaged, LPC did not appear to enhance bioluminescence flux when compared to the PBS-sham group.

Long-term gene expression was assessed in a separate cohort of rats, which revealed that *luciferase* gene expression persisted in the lungs of some animals for up to 12 months following a single LV-delivery, irrespective of whether LPC airway conditioning was performed. These two studies indicate that while LPC conditioning initially improved gene transduction levels in the lungs, it did not increase the longevity of gene expression, suggesting that stem cell transduction was not substantially enhanced with use of LPC. However, it is suspected that stem cells were transduced in animals from both LPC and PBS-sham groups, as the duration of gene expression exceeds the four month lifespan of terminally differentiated tracheal-bronchial epithelium in adult rodents [142].

While LV-mediated gene transfer demonstrated persistent *luciferase* gene expression in a proportion of rats, many individuals from both the LPC conditioning and PBS-sham groups had undetectable flux by 12 months. We do not have data to explain why some individuals responded better than others, though this phenomenon is consistently observed in airway gene therapy animal studies. Possible factors may include but are not limited to, the proportion of stem cells transduced, varying host immune responses to the vector and formulation, size of the animal (which dictates the surface area to vector volume ratio), variable distribution of LPC and vector within the airways, animal respiration rate/depth, which can result in variable gene vector distribution within the airways, and the amount of time that the vector is in contact with the airway surface. An improved understanding of the basis of these variable individual responses to airway gene-addition therapy would provide significant value for progressing to clinical trials.

In the separate study here employing LV-LacZ vector, physical perturbation of the airway epithelium demonstrated very high levels of gene transduction in the trachea when compared to animals receiving LPC chemical conditioning or LV vector only. Moreover, LPC conditioning did not significantly enhance tracheal LacZ expression above the control (LV only) group, consistent with the short-term bioluminescence study where LPC use did not result in a significant increase in tracheal flux when compared with the PBS-sham group. However, it is important to note that the LPC conditioning protocol used in these studies had been previously established for mice, therefore the concentration or volume employed may not be optimal for rats.

As with many viral receptors, the VSV-G receptor (low-density lipoprotein) is located on the basolateral surface, and transduction via the apical surface is typically low level [178]. While the mechanisms here are not fully understood, it is likely that physical perturbation increases gene transfer via two potential routes: (1) disruption of the integrity of the epithelial tight-junctions allowing the gene transfer vector entry to epithelial cells via basolateral receptors, or (2) removal of transduction-resistant columnar cells and exposure of more susceptible cell types on the basement membrane (e.g. basal cells). In this study, physical damage was found to produce basal cell transduction. Both *en face* and histological examination revealed distinct clusters of LacZ-positive cells (e.g. Figure 6E), reminiscent of basal cell clonal expansion observed in previous studies of induced regeneration following polidocanol epithelial stripping [95, 115]. These clusters suggest that basal cells have been transduced and subsequently differentiated into a LacZ expressing pseudostratified epithelium, consistent with previous studies showing the timing of epithelial regeneration in rat airways following mechanical injury [168]. However, further work including lineage tracing studies would be necessary to confirm these hypotheses. Alternatively, clusters of LacZ-expressing cells may be due to strong focal damage or repetitive contact with the basket, making these areas more susceptible to LV-transduction.

Physical perturbation may provide a newly-applied method to boost access to basal cells. An airway gene-addition therapy that transduces only the terminally differentiated surface cells could result in an inevitable waning of transgene expression with normal cell turnover, and the need for vector readministration. While it is unlikely to be completely avoided, repeated administration of viral-based gene therapies is undesirable as immune responses may be elicited upon subsequent deliveries, reducing the efficacy with each dose [95, 179]. Basal cells are multipotent stem cells of the conducting airways and have the capacity for self-renewal, clonal expansion, and the ability to differentiate into epithelial cells types to maintain homeostasis and repair following injury [101, 141, 180, 181]. Successful transduction of basal cells has the ability to produce enduring gene expression, as the gene-corrected progeny will repopulate the surface epithelium following natural cell turnover. It is therefore desirable to develop an airway gene-addition therapy that not only produces efficient gene transfer but can also transduce basal cells [115, 182].

In some regions of the trachea, cells within the lamina propria appeared to be transduced. While a certain degree of epithelial injury was expected, these findings suggest that in some

areas the physical perturbation extended too far into the airway surface, likely due to forces produced by the wire basket or repetitive contact. Goblet cell hyperplasia was also noted in some animals, a protective response that can occur as a result of mucosal irritation [183]. Notably, the animals that displayed goblet cell hyperplasia had many LacZ-positive goblet cells within these regions, suggesting that intact basal cells were transduced and subsequently differentiated into goblet cells upon epithelial regeneration. Our previous short-term assessments have shown that VSV-G pseudotyped LV vectors rarely transduce goblet cells [115, 163], so their presence further indicates likely expansion and differentiation of transduced basal cells.

One major benefit of physical perturbation noted in this study is the ability to achieve high transduction levels from a relatively small number of LV particles per animal (1×10^8 TU). By using epithelial disruption methods to improve gene transfer efficacy, the LV titres required to achieve therapeutic gene correction are reduced, thus improving the feasibility of an *in vivo* gene therapy approach. Moreover, such reductions in vector titre would provide the benefits of lower LV production time and cost, as well as lessened immune responses. The advantages of physical perturbation could also be applied to improving the efficacy of other lung-based genetic therapies including delivery of gene editing machinery, nanoparticle-based gene transfer systems, and stem cell transplantation strategies. For instance, airway engraftment of transplanted cells could be facilitated via use of physical perturbation rather than chemical agents, which have previously been employed preclinically to achieve epithelial disruption [57], but have disadvantages such as toxicity and poor delivery control.

Physical perturbation appears to be a promising method for enhancing airway gene transfer in rats, however, there are valid concerns that translation of this procedure to patients will be complicated by the infected and inflammatory environment of CF airways and subsequent risk of systemic infection. On the contrary, flexible bronchoscopy procedures including bronchoalveolar lavage, suction, and biopsy are routinely performed clinically and are reported to cause mild damage and bleeding within the airways. Despite this, the procedures are well tolerated, with a low proportion of patients (6-8%) documenting bacteraemia post-procedure [184]. These experiences with routine clinical bronchoscopy indicate that a physical perturbation airway gene transfer procedure may be feasible in humans.

While the physical perturbation study performed here shows proof-of-principle data from the trachea, the target for CF airway gene-addition therapy will be the lower large-medium sized airways, as this is where disease pathophysiology occurs. Further work is needed to assess the feasibility of this approach in lower airways of rats, with our previously established rat bronchoscopy methods offering a potential option for enabling access to these small airways for perturbation. This minimally-invasive miniature bronchoscope techniques provide access to at least the fourth generation of branching in an adult rat, therefore perturbation-devices could be guided via the bronchoscope working channel to the lower conducting airways [109]. Future studies in larger animals such as pigs or sheep will also be valuable for identifying the features of a perturbation device that would be considered safe and effective for human clinical use. A device that can be used in combination with a standard clinical bronchoscope could provide an avenue for rapid translation of this approach to humans.

Another limitation of this work was the lack of immunophenotyping of transduced cells. Due to method optimisation issues (high background staining caused by non-specific primary antibody binding), immunohistochemical verification of cell types could not be performed here. Histological assessment of the airway tissue immediately post-damage and the time-course of epithelial regeneration following this method of physical perturbation will also provide value in understanding of the mechanisms that result in improved gene transfer. Future studies should aim to establish more refined techniques to improve control and reduce the intensity of the perturbation, so that off-target cell transduction and disruption to the subepithelial tissue is minimised.

In summary, although LPC conditioning initially increased gene expression levels in rat lungs, it did not appear to improve reporter gene expression longevity. Even without conditioning, a proportion of rats had measurable *luciferase* flux present in the lungs 12 months following dosing, indicating that a single LV delivery can provide persistent gene expression, albeit at low levels. Physical perturbation to the trachea immediately prior to gene transfer resulted in a 1000-fold increase in LacZ staining when compared to animals that did not receive any airway perturbation, demonstrating that this approach can provide significant benefit for improving gene transfer efficacy and warrants further development and investigation.

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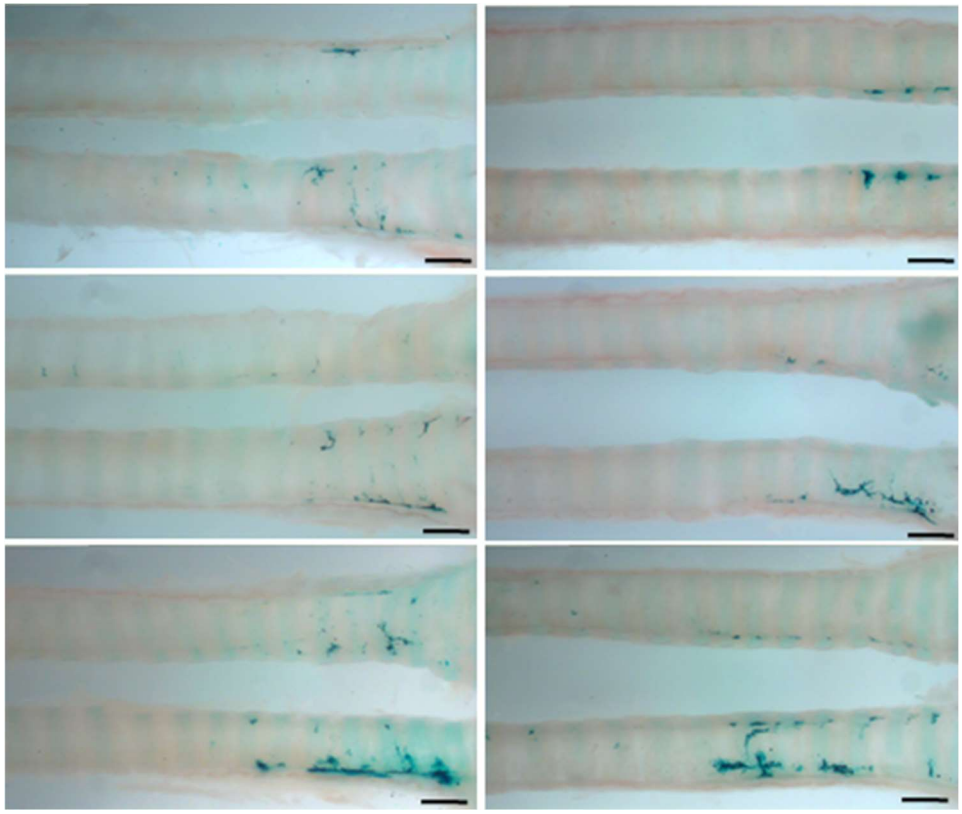

Supplementary Figure 1: *En face* images from animals receiving LPC conditioning or LV vector only indicate strong regions of LacZ staining in the proximal trachea at the site of unintentional ET tube damage.

Chapter 8: Conclusion

As we enter the era of highly effective CFTR modulator therapies for CF patients, it is critical to acknowledge that a significant proportion of individuals are being left without access to these life-preserving treatments. Airway gene-addition therapy is an alternative that has real potential to effectively treat lung disease in any CF patient, irrespective of their mutation type. As gene therapy corrects the underlying basic defect – the faulty CFTR genes – this type of approach could provide a cure for pulmonary disease, particularly if given early in life before irreversible lung damage occurs. Airway disease remains the leading cause of hospitalisation and premature death in CF patients, therefore an effective treatment for the lungs will meaningfully improve patient prognosis. Despite advances in CF airway gene-addition therapy programs worldwide, there are several hurdles to overcome before this type of therapy will become widely available for patients. This thesis addressed some of the key challenges that must be tackled for successful translation of an airway gene-addition therapy for CF.

One of the biggest challenges facing the gene therapy world today is producing enough LV vector for human *in vivo* clinical trials and if successful, product commercialisation. Existing two-dimensional LV production platforms have remained relatively unchanged for decades and are not amenable to large-scale production. Academia and industry alike are investing and innovating rapidly in this space to develop LV production methods that can sustain both clinical trial and commercialisation phases. Many are beginning to employ bioreactors, a commonly used technology in the biopharmaceutical sector.

To overcome the scalability issues associated with existing monolayer dish production, two LV production methods were established and presented in this thesis. The first method employed multilayer cell factories. Cell factory systems are suited to generating gene vector for preclinical investigations in animals, which is particularly relevant as we move to performing airway gene therapy studies in our newly developed CF rat models, as their larger lung size necessitates higher volumes of LV vector than mice. The use of cell factories is a valuable method for producing LV vectors, but it is not without shortcomings. Although labour input is significantly reduced when compared to traditional flask-based methods, a substantial amount of user input is still necessary and there is no integration with automation. While this type of approach is suitable for generating vector for use in small-animal research

studies and some *ex vivo* gene therapy clinical trials [185], it is not capable of sustaining production for human *in vivo* lung gene therapies, which will require much greater quantities of LV.

The second LV production method employed a packed-bed bioreactor system, which achieved supernatant titres of up to 10^6 TU/mL and concentrated yields up to 10^9 TU/mL. Described here was the first report that employed this particular packed-bed bioreactor for producing LV vector. This bioreactor protocol is valuable for use in academic settings and has potential for large-scale production by employing a scale-out approach. With further alterations for achieving GMP, this method could also be suitable for manufacturing a clinical product. Bioreactors provide a range of advantages over traditional plastic-based systems, making them attractive for use in both clinical and commercial manufacturing phases. Bioreactor technologies allow for real-time monitoring and fine control of the culture conditions. Improved efficiency and reduced human input can also be achieved by introducing automation into both the upstream and downstream processes. The system selected here has an extremely large surface area enabling high productivity from a single unit, while minimising the volume of supernatant to be processed.

One disadvantage of a packed-bed bioreactor system is that cells cannot be directly sampled for counting and viability assessments as they as they are attached to substrate. This means that more complex instrumentation is required, such as glucose measurements to approximate cell growth and yields. This type of system was not employed in the described method but would be beneficial to implement in future studies. Moreover, while packed-bed systems allow for increased production capacity with a scale-out approach, this can be more technically challenging and expensive than a scale-up scheme. In this project, a suspension-based production system was explored as an alternative approach for achieving large-scale production by scaling-up. While the suspension bioreactor method did not produce high titres with the in-house suspension-adapted HEK 293T cell line, the LV-MAX kit demonstrated the capability to produce high titres (up to 3×10^6 TU/mL) in a small-scale trial. Suspension-based LV production remains a viable option for clinical and commercial manufacturing that should continue to be pursued.

It is important to note that the LV production methods developed here only employed LV vectors carrying reporter genes. Accordingly, future studies should assess these newly

established production methods with LV vectors carrying the therapeutic *CFTR* gene, as it is possible that *CFTR* gene vectors may not perform in the same manner as those bearing reporter genes. LV-*CFTR* vectors produced using up-scaled methods should subsequently be trialled *in vitro* and *in vivo* to validate titre and functionality.

A major bottleneck that remains with the method presented here and for LV production generally is the downstream processing. This is one aspect that was not addressed in this work. LV vectors are extremely fragile, particularly when compared to other viral vectors such as AAV and AdVs. The delicate nature of LV particles means they are susceptible to damage and inactivation during the purification and concentration stages, therefore achieving a balance of high vector recoveries and purity can be challenging. Ideally, purification processes should be kept to a short duration and the number of steps limited to retain vector functionality. The LV vector manufacturing protocol will not be considered economically viable if losses following downstream processing are too significant.

In this work the downstream processing consisted of clarification, anion-exchange chromatography, and ultracentrifugation. Notably, ultracentrifugation is not a scalable technology and should be eliminated if large-scale manufacturing is intended. Despite employing some level of purification, the LV vector produced in this work is relatively crude and significant process refinement would be needed to produce material that is sterile and of clinical-grade. Not only is this necessary for human use but certain preclinical investigations (e.g. immunological studies) may benefit from using highly purified LV vector as the outcomes would more accurately predict those of clinical trials [186].

Another issue facing the rapidly-growing gene and cell therapy sectors is the unsustainable demand for clinical-grade viral vector products. Analysts indicate that the demand for viral vector will soon exceed the global manufacturing capacity [187] and those currently in clinical trial phases are already experiencing lengthy waiting periods to receive products from contractors. Substantial investment in the expansion of gene vector manufacturing infrastructure will be essential in the coming years to minimise this potential bottleneck.

As we advance airway gene therapy preclinical studies, not only do we need sufficient gene vector quantities, but we also require suitable test models that will provide meaningful results for human translation. Several CF animal models have been developed for research purposes, and these vary significantly in the disease phenotypes they exhibit, particularly in regard to

lung disease. Given this, the process of selecting the right model for a particular research investigation can be complex. It is therefore critical to understand each model, as well as their advantages and limitations before undertaking a research study.

The lack of available CF animal models in Australia has hindered trials of airway gene-addition therapies. Australia has strict quarantine and biosecurity legislation that prevents live import of many animal species, with the exception of laboratory mice and rats. Some CF animal models – particularly commercially developed genetically modified models – may also have licensing agreements that prevent their use or restrict the types of research that can be performed. To overcome limited availability of CF animal models in Australia, two new CF rat models were generated using CRISPR/Cas9 gene editing. One model is a *CFTR* KO rat and the second harbours the common Phe508del mutation, the first mutation-specific CF rat model to be developed worldwide.

CF rat models provide a number of advantages for airway gene therapy studies. Logistically, rats are easy to breed to large numbers allowing for statistically well-powered investigations to be performed. Moreover, the disease severity observed in these CF rat models is relatively mild compared to other CF animal models, enabling longitudinal studies and repeated measures to be performed. Rat airways have a similar cellular architecture to humans including the presence of submucosal glands in the trachea, which is important for assessing transduction of specific cell types, levels of gene expression, and overall efficacy of the gene transfer. Compared to the mouse, the larger physical size of the rat enables improved access to the airways for techniques such as bronchoscopy.

Overall, Phe508del rats demonstrated milder disease than KO rats. In terms of recapitulating specific CF traits, both KO and Phe508del rat models exhibited impaired survival, reduced weight-gain, electrophysiological defects in the nasal epithelium, and gastrointestinal obstruction and histopathology. KO rats additionally demonstrated disease of the exocrine pancreas and increased submucosal gland area in the trachea. Spontaneous lung disease has not been observed in either CF rat model to date. Accordingly, a more complete understanding of the lung characteristics of these CF rat models will be imperative in informing the conception of future airway gene therapy investigations.

Key studies are currently ongoing to further characterise these CF rat models. Investigations include determining if pulmonary disease develops with age or whether intra-lung challenge

with CF-relevant pathogens will be required to induce CF lung pathology. Work is also underway to determine whether CF-like ion transport abnormalities are present in the tracheal airways of these rat models by generating air liquid interface (ALI) cultures and performing Ussing chamber measurements. Using rat-generated ALIs, the efficacy of LV-*CFTR* mediated gene transfer can be assessed in the lower airways of the CF rat models to compliment recent studies that have shown successful correction of the bioelectric defect in the KO rat nasal epithelium via use of potential difference measurements (unpublished). In the future it will be important to determine how the Phe508del rat responds to relevant CFTR modulator therapies, as this will increase the utility of the model for both mechanistic investigations and the assessment of novel therapeutic strategies where modulators are considered the current gold standard.

With access to the right animal model, we can explore and assess strategies for developing an efficacious airway gene-addition therapy. Effective lung-based gene therapies are challenging to develop due to the physical, biological, and immunological obstacles that the gene vector must overcome to reach the target epithelial cells. The airway epithelium itself has barrier properties that are designed to resist infiltration by pathogens and foreign bodies, including gene vectors. A range of techniques have previously been explored to improve gene transduction efficiency in the airways, and one such strategy involves disrupting the integrity of the airway epithelium prior to gene vector delivery. Preclinically, chemical conditioning and physical perturbation approaches have demonstrated improved transduction levels when used in conjunction with viral vectors.

As we transition to using CF rat models for airway gene therapy development, it was important to first validate the efficacy of our standard two-step airway-dosing regimen in rats. While previous studies in mice have shown enhanced LV-mediated gene transfer from LPC airway conditioning [108, 171], it was unknown whether this effect would be replicated in rats. When assessed 1-week post gene vector delivery, LPC conditioning was found to significantly improve lung gene expression levels in rats. However, the longevity of transgene expression was not enhanced with the use of LPC, suggesting that LPC conditioning did not improve transduction of basal cells. Due to method optimisation issues, immunohistochemical verification of transduced cell types (including basal cells) could not be completed in this thesis, with this work still ongoing. Further investigation is also needed to

determine the optimal concentration of LPC for rat airways, as it is possible that greater enhancement would have been obtained using a higher concentration or volume.

This thesis describes the first report of using physical perturbation in conjunction with LV-mediated airway gene transfer. Physical epithelial disruption was found to provide significant and substantial enhancement of LV vector transduction levels, with a 1000-fold increase in LacZ staining observed when compared to LV-only control animals. In this study, chemical conditioning with LPC did not provide a significant increase in LacZ staining in the trachea when compared to the LV only group, which is consistent with the *luciferase* assessment where tracheal gene expression levels did not differ between PBS and LPC-treated animals. High levels of gene expression were achieved using physical perturbation and target epithelial cell types including basal cells were transduced. The promising data from this study indicates that use of physical perturbation to transiently disturb the epithelium could provide significant benefit for enhancing the delivery of genetic therapies to the airways. Notably, disruption of the surface epithelial cells allows the delivery vehicle access to deep-lying basal cells, which if transduced, can provide a basis for long-term therapeutic gene expression. Improved longevity of gene expression can subsequently reduce the frequency of repeated gene vector administrations, which is favourable from financial, practical, and immunological perspectives.

While this was a valuable proof-of-principle study, there are limitations to this work that should be noted. Some animals that received physical perturbation exhibited LacZ-positive cells in the subepithelial compartment, indicating that damage occurred below the basement membrane and was therefore too deep in those areas. Further refinement of the technique is required to improve control of the device and minimise damage to off-target tissue. Simultaneous physical perturbation and LV vector delivery would also be desirable in the future to minimise procedural complexity and target vector delivery more directly to the perturbed regions (rather than whole lung) for further enhancement of transduction. In this study in rats, the trachea was used as a surrogate for the lower airways due to ease of access. For CF, the large-medium sized airways will be the target for *CFTR* gene-addition therapy, therefore future studies will assess the feasibility of this approach in the lower airways of rats as well as larger animal models with human-sized airways such as pigs or sheep. Immunohistochemical verification of LacZ-expressing cell types resulting from physical perturbation will also be necessary. Future work will focus on understanding the mechanisms

that lead to improved cell-transduction when physical perturbation is employed prior to LV gene vector delivery.

A physical perturbation strategy appears promising in this animal study, but substantial work would be needed to feasibly translate this technique to human airways. A custom-made bronchoscope device that allows for simultaneous physical perturbation and LV vector delivery would be ideal to minimise procedural steps. While translation of this procedure to humans is seemingly complex, it is important to note that flexible bronchoscopy procedures are routinely performed in CF patients, and despite the infective and inflammatory nature of the CF airways, they are well tolerated [184]. While intentional physical damage to the airways is considered more complicated and higher-risk, the routineness of these procedures demonstrate that such a physical perturbation approach may be feasible in humans.

Significant progress in the development of airway gene-addition therapy programs worldwide means that achieving successful translation to the clinic is now closer than ever before. This accomplishment is not without major challenges: we need to meet the growing demand for large-scale vector manufacturing, obtain meaningful and translatable results in preclinical studies by using suitable research models, and achieve efficient delivery of our gene-cargo to the airway epithelium to restore CFTR function to therapeutically-relevant levels. We are now in the midst of a new era of medicine, with significant interest, innovation, and investment in developing treatments that correct the genetic defect, rather than providing only symptomatic relief. This paradigm shift along with a substantial global research effort brings with it a renewed sense of optimism that an effective genetic-based therapy for CF airway disease is now close to becoming a reality.

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