Gene Therapy for

α_1-antitrypsin deficiency

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Abstract

α_1-antitrypsin deficiency (AATD) is an autosomal recessive disorder characterised by low levels of the circulating anti-protease α_1-antitrypsin (hAAT). Low levels of pulmonary hAAT pre-disposes individuals to early-onset emphysema, and the efficacy of intravenous augmentation therapy with purified protein is the subject of debate. Gene therapy could be an alternative treatment strategy. In this thesis, "gold standard" viral and non-viral agents for airway gene transfer were assessed for their ability to produce α_1-antitrypsin.

Key findings in this thesis are:

1. A single dose of gene therapy with the non-viral agent GL67A was not sufficient to produce hAAT in murine lung tissue homogenate or epithelial lining fluid (ELF). A repeat dosing regimen resulted in significant ($p<0.01$) but sub-therapeutic production of hAAT in ELF (*treated:* median 331 (range 0.0-1107) ng/ml hAAT, compared to the therapeutic target of 70 μg/ml in ELF, n=6 per group) (*Chapter 3*).
2. A lentiviral vector, pseudotyped with Sendai virus surface proteins (rSIV.F/HN-hCEF-sohAAT) resulted in therapeutic levels of hAAT in murine ELF (*treated:* median 70.0 (range 53.8-175.7 μg/ml, *untreated:* 0.19 (0.12-0.34) μg/ml, $p<0.05$, n=6 per group) (*Chapter 5*).
3. A single dose of rSIV.F/HN resulted in sustained transgene expression for at least 20 months in murine lung and ELF ($p<0.05$) (*Chapter 5*).
4. In an *ex vivo* assay, broncho-alveolar lavage fluid (BALF) from rSIV.F/HN-treated mice inhibited neutrophil elastase significantly more than BALF from untreated mice ($p<0.05$) (*Chapter 7*).
5. rSIV.F/HN-hCEF-sohAAT successfully transduced human lung tissue *ex vivo*; precision-cut human lung slices (*treated:* median 320 (range 207-1307) ng/ml, *untreated:*

9.3 (7.5-10) ng/ml, $p<0.01$, n=6 per group) and primary human nasal epithelial cells (*treated:* 389 (339-502) ng/ml, *untreated:* 140 (103-176) ng/ml, $p<0.01$, n=6 per group) (*Chapter 6*).

In summary, data generated in this thesis supports further progression of rSIV.F/HN-hCEF-sohAAT towards a clinical trial in hAAT deficient patients.

Declaration of Originality

I hereby declare that the work presented in this thesis was performed by me, or else has been appropriately referenced, in the Department of Gene Therapy, National Heart and Lung Institute, Imperial College London.

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Abbreviations

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$
****	$p<0.0001$
AATD	α_1-antitrypsin deficiency
AAV	Adeno-associated virus
AEC	Airway epithelial cell
ALI	Air liquid interface culture
Amp	Ampicillin
BAL	Broncho-alveolar lavage
BALF	Broncho-alveolar lavage fluid
BALT	Bronchus-associated lymphoid tissue
β-gal	β-galactosidase
BGH polyA	Bovine growth hormone poly-adenylation sequence
BLI	Bioluminescent imaging
CAR	CAR receptor
CBA	Chicken β-actin
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CMV	Cytomegalovirus promoter
CMV IE	Cytomegalovirus Immediate/early enhancer
COPD	Chronic obstructive pulmonary disease
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9
CT	Computed tomography
D-PBS	Dulbecco's phosphate-buffered saline
DMEM	Dulbecco's minimal Eagle's Medium
DMEM-20	Dulbecco's minimal Eagle's Medium with 20% fetal calf serum
DMPE-PEG5000	Dimyristoylphosphatidylethanolamine coupled to 5000 kDa molecular weight polyethylene glycol
DOPE	Dioleoylphosphatidylethanolamine
DTT	Dithiothreitol
EBC	Exhaled breath condensate
EIAV	Equine infectious anaemia virus
ELF	Epithelial lining fluid
ELISA	Enzyme-linked immunosorbent assay
F	Fusion protein from Sendai virus
FCS	Foetal calf serum
FEV_1	Forced expiratory volume in one second

FIV	Feline immunodeficiency virus
FLux	Firefly luciferase
FRC	Functional residual capacity
GFP	Green flourescent protein
GFPLux	Green flourescent protein and Firefly luciferase (chimeric protein)
GLux	Gaussia luciferase
GMP	Good manufacturing practice
GTA	Gene transfer agent
hAAT	Human a_1-antitrypsin
hCEF	Human CMV enhancer and human elongation factor 1a promoter (hybrid promoter)
HEK293	Human embryonic kidney 293 cells
HIV	Human immunodeficiency virus
HN	Hemagglutinin neuraminidase protein from Sendai virus
hPIV	Human parainfluenza virus
IL-8	Interleukin 8
IM	Intramuscular
IN	Intranasal
iPSC	Induced pluripotent stem cell
IV	Intravenous
IVIS	In vivo imaging system
kDa	Kilodalton
LCI	Lung clearance index
LF2000	Lipofectamine 2000
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
LTR	Long terminal repeat
Lux	Luciferase
Lux	Firefly luciferase
mCftr	Murine cystic fibrosis transmembrane conductance regulator
miRNA	Micro RNA
mOD	Mean optical density
MOI	Multiplicity of infection
ND	Not determined
NE	Neutrophil elastase
ns	Not significant
OD	Optical density
p-NA	p-nitroaniline
PBS	Phosphate-buffered saline
pCIK	CMV immediate/early promoter/enhancer plus hybrid intron juxtaposed between promoter and transgene
pCMV	CMV immediate/early promoter/enhancer

Q-PCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
rAAV	Recombinant adeno-associated virus
RLB	Reporter lysis buffer
RLU	Relative light units
rSIV	Recombinant Simian immunodeficiency virus
SERPINA1	Serine protease inhibitor, group A, member 1
SeV	Sendai virus
SIN	Self inactivating
SIV	Simian immunodeficiency virus
SIVagm	Simian immunodeficiency virus from African green monkey
so	Sequence optimised
sol	Soluble
TLC	Total lung capacity
TLR-9	Toll-like receptor 9
TTU	Taqman transducing units
TU	Transducing units
UKCFGTC	UK Cystic Fibrosis Gene Therapy Consortium
VP	Viral particles
VSV	Vesicular stomatitis virus
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element

1 Introduction

1.1 α_1-antitrypsin deficiency

1.1.1 α_1-antitrypsin: discovery, structure and function

α_1-antitrypsin is an anti-protease first identified in 1955 [1]. It is a 52 kDa protein, which is encoded on the long arm of chromosome 14 (14q31-32.1) [2]. α_1-antitrypsin (hAAT) is the second-most abundant serum protein in humans [3], and 80% of the circulating protein is synthesised in the liver [1]; however, it can also be made locally, and production of hAAT by neutrophils [4], macrophages [5], bronchial epithelial cells [6], type II airway epithelial cells [7] and pancreatic islet cells [8] has been documented. The half-life of hAAT in serum is approximately 4-5 days [1] [9].

The major role of hAAT is the inhibition of the serine proteinase neutrophil elastase (NE). Neutrophil elastase is one of several proteinases produced by neutrophils to allow them to migrate through the extra-cellular matrix in response to chemotactic signals, such as leukotriene B_4 (LTB_4) and interleukin 8 (IL-8), produced in response to inflammation or infection [10] [11] [12]. The main physiological function of hAAT is to 'shield' the lung from excess damage caused by neutrophilic proteinases, restricting the activity of proteinases to the desired site of action; hAAT provides around 90% of the anti-proteolytic shield in the lower respiratory tract [13]. However, chronic inflammation of the lung in disease states such as emphysema and cystic fibrosis overwhelms this anti-proteolytic shield, leading to a "protease:antiprotease" imbalance that eventually leads to the proteolytic destruction of lung tissue [14]. Furthermore, a lack of circulating hAAT due to a genetic defect leads to unopposed action of neutrophil elastase in the airways, predisposing patients to emphysema

at a younger age and following less cigarette smoke exposure than in typical chronic obstructive pulmonary disease (COPD) [10] [15] (discussed below).

The α_1-antitrypsin molecule features a reactive centre loop, which recognises proteases such as neutrophil elastase [16]. The hAAT molecule is naturally in a high-energy, unstable state; when NE docks to the reactive centre loop, it triggers a conformational change in hAAT which distorts and inactivates the NE molecule, eventually leading to the proteolytic destruction of the complex [17] [18]. This high affinity reaction is destructive to both molecules, but as there is normally an excess of hAAT in the airway, free NE is usually adequately contained.

α_1-antitrypsin is also known as α_1-proteinase inhibitor (which more strictly describes its major physiological function, discussed above), and SERPINA1 (serine protease inhibitor, group A, member 1); however, as the term α_1-antitrypsin is most commonly used I will use the protein's original name in this thesis. A number of different abbreviations are used for the protein, including AAT, hAAT, A1AT, α_1-AT, etc. The most common abbreviation for α_1-antitrypsin in the literature is hAAT ('human' α_1-antitrypsin), which is the abbreviation I will use throughout this thesis; occasionally, when referring to α_1-antitrypsin from a non-human source, the 'h' is omitted.

1.1.2 Genetics of α_1-antitrypsin deficiency

α_1-antitrypsin deficiency (AATD) is an autosomal recessive disorder, first described by Laurell and Eriksson in 1963 [19]. To date, around 100 genetic variants of hAAT have been identified [20]. The different hAAT genotypes are described by the 'protease inhibitor' (Pi) type [21]; the most common genetic variants are as follows:

- M – a normal hAAT allele; the Pi*MM genotype accounts for about 95% of the Caucasian population worldwide.
- Z – an allele which arose in the Viking population [20]. Homozygous inheritance of the Z allele (Pi*ZZ) is the most common cause of AATD, accounting for around 95% of hAAT deficiency-related pathology [22] [23]. The genotype results in the synthesis of a mutant protein which polymerises in hepatocytes [24], with two major consequences: (a) a loss-of-function effect due to the lack of neutrophil elastase inhibitory capacity in the lung, which leads to emphysema, and (b) a gain-of-function effect due to the toxic influence of hAAT polymers in hepatocytes, leading to liver cirrhosis [25]. Polymers of Z-hAAT are also released into the circulation, and are present in all Pi*ZZ individuals [26]. Polymers of Z-hAAT have been identified in the lung of Pi*ZZ individuals [27], and their presence in a Pi*ZZ individual post-liver transplant implies that they may arise from local hAAT production in the lung [28]. Pro-inflammatory Z-hAAT polymers in the lung are chemotactic to neutrophils, which may further contribute to AATD-related lung disease [29], although the extent to which they do so remains undetermined [25].
- S – an allele which arose on the Iberian peninsula [1]. Pi*MS and Pi*SS genotypes are not associated with disease; whereas Pi*SZ and Pi*MZ are associated with a moderately increased risk of cirrhosis and emphysema.
- Null alleles – several mutations have been described which result in complete absence of hAAT production (Null mutations), leading to particularly severe emphysema in almost all patients [1] [20], but patients do not develop liver cirrhosis, due to the lack of hepatic polymerisation.

There are also interesting 'dysfunctional' mutations described, for example 'hAAT Pittsburgh' where an amino acid substitution converts hAAT from an inhibitor of elastase to an inhibitor of thrombin, resulting in a severe bleeding disorder [30].

1.1.3 Clinical features of α_1-antitrypsin deficiency

AATD can be considered as a monogenic disorder, which pre-disposes patients to different pathologies when other environmental or genetic factors are present [1].

- *Lung disease:* AATD is associated with early onset (35-45 years) emphysema, which tends to be panacinar (i.e. involving the alveoli), in contrast to chronic obstructive pulmonary disease (COPD) in which lung destruction follows a panlobular (i.e. involving the small airways) pattern [22]. The penetrance of Pi*ZZ emphysema (in other words, the number of individuals with Pi*ZZ who go on to develop emphysema) is 60% [1]. IV augmentation therapy is a specific treatment for AATD-related emphysema, although much debate surrounds its clinical efficacy (discussed below).
- *Liver disease:* Z-hAAT has been shown to polymerise and is sequestered within the endoplasmic reticulum (ER) of hepatocytes [24]. 2.5% of children and 30% of adults with Pi*ZZ have AATD-associated liver disease, typically cirrhosis. 2-3% of individuals with PiZZ will develop hepatocarcinoma. There is no specific treatment for AATD-related liver disease, which accounts for around 3% of liver transplants performed in the UK [31].
- *Systemic vasculitis*: AATD-associated vasculitis is seen in approximately 2% of AATD patients due to low levels of circulating protein [32].
- *Panniculitis*: in approximately 0.1% of individuals with AATD, necrotising panniculitis (inflammatory lesions in subcutaneous fatty tissues) occurs [33].

AATD is an under-diagnosed condition, with some sources claiming that only 2% of individuals with AATD worldwide have been identified [1]. It is believed that many patients with a diagnosis of COPD or cirrhosis may be deficient in α_1-antitrypsin.

1.1.4 Levels of circulating hAAT

The severity of α_1-antitrypsin deficiency is determined by the level of hAAT in serum. Severe AATD is defined by hAAT serum levels below 11 μM (0.5 g/l) [34]. This 'threshold' level was originally determined based on the observation that patients with intermediate deficiency (Pi*SZ), in whom serum hAAT is above 11 μM, rarely develop emphysema [34]. This has been investigated further, including in a study by Ferrarotti and colleagues, published in 2012 [21], which accurately measured hAAT levels in over 6000 individuals with Pi*MM and various intermediate deficiency phenotypes (Pi*MS, Pi*SS, Pi*MZ, Pi*SZ); the results of this study are summarised on Figure 1-1.

The critical site of action of hAAT is in the interstitial space of the lung [13]. Studies of albumin diffusion [35] have shown that the endothelial layer presents a minor barrier to protein movement, and levels of hAAT in the interstitium are approximately 80% of serum levels [13]. However, the epithelial layer presents a major barrier to protein movement, and studies have determined that levels of hAAT in the epithelial lining fluid of the lung are 10% those of circulating levels in serum [13]. These observations have been used to build a picture of the levels of hAAT seen in serum, lung interstitium and the epithelial lining fluid (ELF) for individuals with different phenotypes, which I have summarised on Figure 1-2. Importantly, the 'threshold' level of hAAT in serum (11 μM) determined by observations in Pi*SZ individuals equates to a level of 1.1 μM (70 μg/ml) in epithelial lining fluid.

It should be noted that hAAT is an acute phase protein [36] [37], and it has been shown that levels of hAAT are higher during the acute phase response (up to fourfold above normal levels [38]), during inflammatory disease states (e.g. rheumatoid arthritis [39]) and during pregnancy [40] [41].

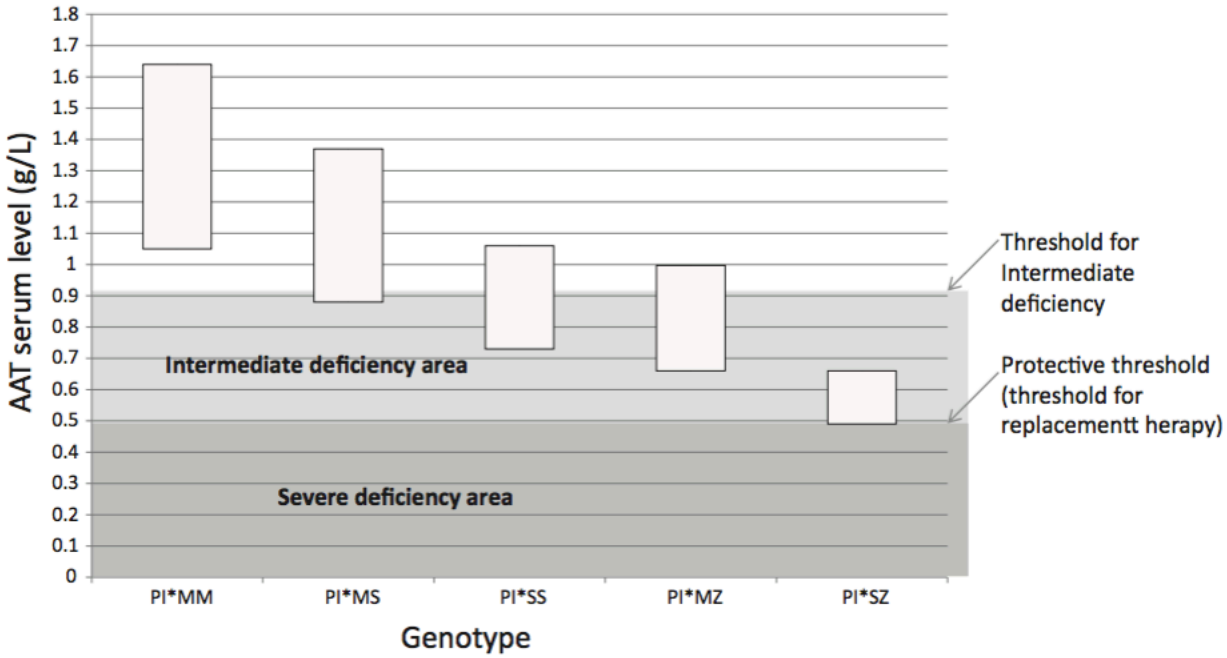


Figure 1-1 Serum levels of human α_1-antitrypsin

In a study performed by Ferrarotti and colleagues [21], the serum levels of human α_1-antitrypsin (AAT) were determined in approximately 6000 individuals. Here, the serum levels are displayed according to hAAT genotype (bars represent 5th and 95th percentiles). Reproduced from [21].

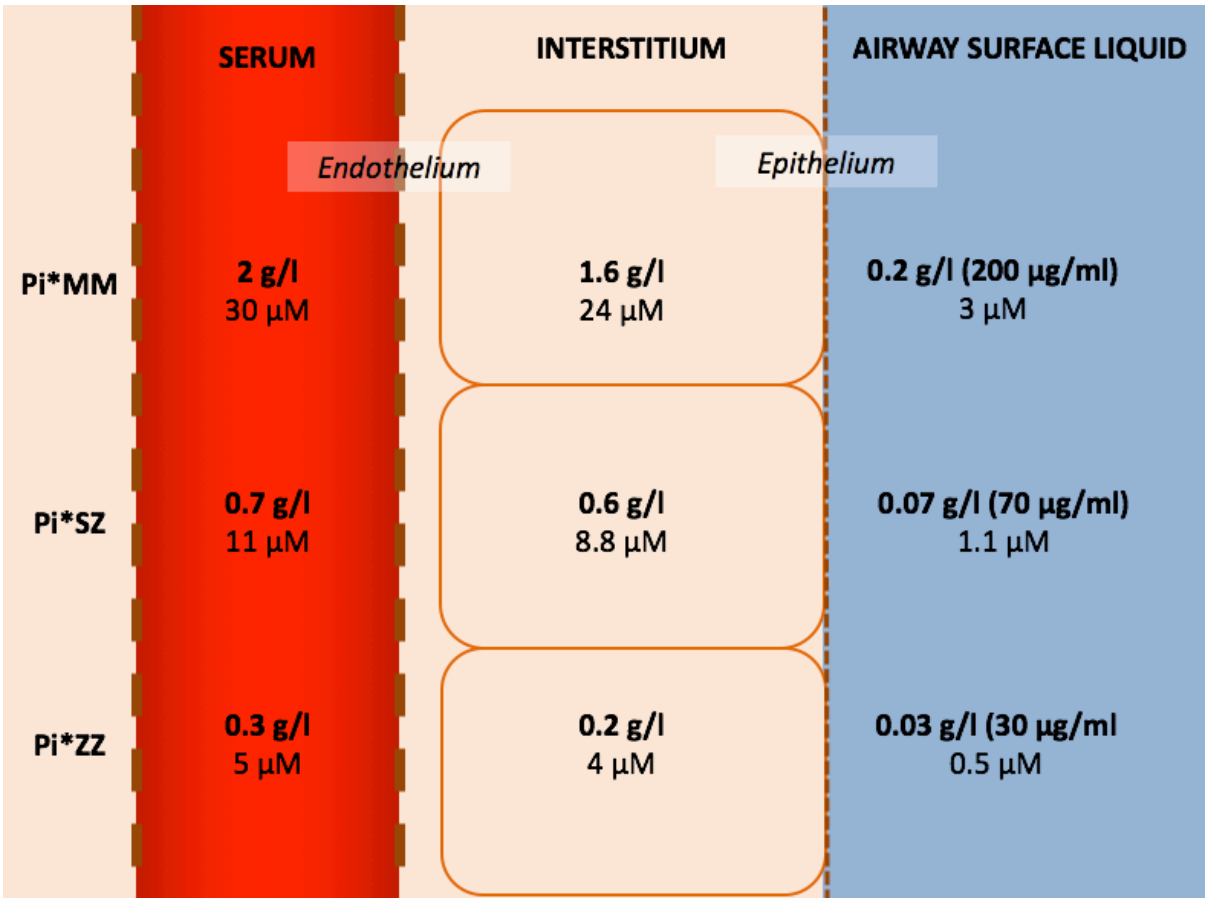


Figure 1-2 Concentrations of human α_1-antitrypsin in serum, lung interstitium and airway surface liquid in different hAAT phenotypes

Levels of human α_1-antitrypsin are shown in serum, lung interstitium and airway surface liquid (epithelial lining fluid) in normal (Pi*MM), intermediate deficiency (Pi*SZ) and severe α_1-antitrypsin deficiency (Pi*ZZ). Measurements of hAAT in intermediate deficiency (Pi*SZ) are used as a threshold level, below which α_1-antitrypsin deficiency is defined as severe; therefore, these threshold values are also used as therapeutic target levels.

Values are shown in both µM and g/l, and are based partly on clinical observations and partly on the derivation of protein movement between tissues described in [35]. This diagram is adapted from [13].

1.1.5 Current treatment options for α_1-antitrypsin deficiency

1.1.5.1 Protein replacement therapy

The only specific treatment for α_1-antitrypsin deficiency currently available in some countries is augmentation therapy, where patients are given purified hAAT with the aim of augmenting serum hAAT levels above the therapeutic threshold of 11 μM. Various preparations of purified hAAT are available, and are usually administered intravenously. This treatment is expensive, and reports on the costs involved vary widely – the most recent figures estimate a cost of between US $60,000 to US $160,000 per patient per year [42] [43]. Augmentation therapy has been available in the USA since 1988 and Germany since 1989 [43], and a number of other countries worldwide, but it is currently not licensed in the UK, with regulatory authorities citing a lack of evidence of the clinical effectiveness [44]. This has been a subject of great debate. To date, only three randomized clinical trials of IV augmentation therapy have been reported (summarised on Table 1-1), none of which have shown a statistically significant benefit of augmentation therapy.

Between 1991 and 1997, Dirksen and colleagues performed the first randomised, placebo-controlled trial of augmentation therapy in α_1-antitrypsin deficiency in 56 Danish and Dutch patients with a Pi*ZZ genotype and moderate emphysema (FEV_1 between 30-80% predicted) and who were ex- or never-smokers. The data was reported in 1999 [45]. The patients received either hAAT (250 mg/kg every four weeks) or albumin for at least three years. At the end of the study, there was no significant difference between the treatment and placebo groups for the primary outcome measure, forced expiratory volume in one second (FEV_1) percentage predicted; however, when the authors examined the loss of lung tissue measured by computed tomography (CT), there was a trend towards improvement in the treatment group, although this still did not reach statistical significance (a treatment difference of 1.1 g/L per year, 95% confidence interval -0.08 to 1.78, p=0.07). One criticism of this study,

which the authors acknowledge in their discussion, is that the treatment interval of 4 weeks, chosen for logistical reasons, meant that trough levels of hAAT had often fallen below the therapeutic threshold of 11 μM between treatments. At 28 days post-treatment, the mean levels of hAAT were sub-therapeutic (8.8 μM in the treated group, compared to 6.2 μM in the untreated group), which could explain the lack of protective effect of IV augmentation therapy in this study.

The second randomised trial of IV augmentation therapy for α_1-antitrypsin deficiency, called 'EXACTLE', was performed between 2003 and 2006 and reported in 2009 [46]. A similar group of patients were treated (77 patients with hAAT concentrations below 11 μM in Demark, Sweden and the UK who were ex- or never-smokers) but received IV augmentation therapy at weekly intervals (60 mg/kg) for 2 years, with an optional 6-month extension. Part of the focus of this study was to explore different methods of examining CT lung density data. When the same method as the 1999 study was used, a treatment difference in lung density of 0.70 g/L (95% confidence interval -0.028 to 1.427 g/L, p=0.059) was observed.

The following year, a combined analysis of the two randomised clinical trials was published [47]. This combined analysis was based on data from 119 patients, as 6 patients participated in both studies; therefore, their data from the second study was excluded. In this combined analysis, the authors compared the change in lung density at the start and end of the trial ('end-point analysis') rather than the mean annual change from baseline ('slope analysis'), which was the measure reported in the Danish-Dutch trial. The authors reported a treatment difference of 2.297 g/L (95% confidence interval 0.669 to 3.926, p=0.006) over the course of the trial, supporting the use of IV hAAT to halt the progression of emphysema. Whether or not such a combined analysis of two trials with methodological differences is appropriate is unclear; a meta-analysis of both trials, performed by the Cochrane Collaboration, did not

support the use of IV hAAT augmentation therapy [48] and sparked considerable debate in the field.

In May 2015 a much-anticipated randomised trial called RAPID (Randomised, placebo-controlled trial of Augmentation therapy in alpha-1 Proteinase Deficiency) was reported. This trial included 180 non-smokers from 28 study centres in 13 countries, with severe α_1-antitrypsin deficiency (serum concentration <11 μM) and an FEV_1 of 35-70% predicted. Patients in the treatment group received 60 mg/kg IV hAAT weekly for two years between 2006 and 2010. The previous studies in the field reported CT lung density at total lung capacity (TLC); however, at the request of regulatory bodies, this latest trial reported CT lung density at TLC and functional residual capacity (FRC) combined as its primary outcome. Additional primary outcomes were CT lung density at TLC *or* FRC. Strictly speaking, the trial did not meet its primary outcome, as there was no difference in lung density loss between the two groups at TLC and FRC combined. However, the annual rate of lung density loss at TLC alone (the primary end-point in previous trials) was less in the treatment group than the placebo group (a treatment difference of 0.74 g/L per year (95% confidence interval -0.06 to 1.42, p=0.03)), making interpretation of this trial difficult. In their discussion, the authors first concentrate on the pitfalls of CT lung density at FRC, claiming that TLC recordings have smaller variation and are therefore more reliable.

One possibility raised by the authors of the RAPID study is that a higher dose of IV hAAT may be more appropriate. A safety trial of a doubled dose (120 mg/kg/week of IV hAAT (Prolastin-C, an improved, highly purified IV hAAT formulation)) performed in 30 patients raised no safety concerns [49] and showed that patients treated with 120 mg/kg/week had higher serum concentrations of hAAT at both 7 days and 14 days than patients treated with the lower dose of 60 mg/kg/week; at 14 days, the mean level of serum hAAT in patients treated with 60 mg/kg purified hAAT was below the 11 μM therapeutic target level.

A randomised, double-blind, placebo-controlled trial is currently being performed to evaluate the efficacy of two separate doses of Prolastin-C. In this trial, SPARTA (Study of ProlAstin-C Randomised Therapy with Alpha-1 augmentation), 339 patients will be randomised 1:1:1 to receive 60 mg/kg/week hAAT, 120 mg/kg/week hAAT, or placebo, and patients will be treated for 3 years, with the study due to be completed in 2021 [50].

There are several reasons why it has been difficult to prove effectiveness of IV augmentation therapy for AATD. AATD is a rare disease (defined as a prevalence of less than 1:2000), and recruitment of enough patients for properly powered clinical trials is very difficult. In addition, the best primary end-point for clinical trials remains the subject of much debate, with clinical outcomes such as CT lung densitometry being preferred over patient-centered outcomes such as mortality (this was the conclusion of the Cochrane review published in 2010 [48]). The lack of clinical effectiveness of IV augmentation therapy may also imply that AATD-related lung disease is not only caused by the loss-of-function mutation from lack of circulating hAAT, but also is due (at least in part) to the gain-in-function arising from the presence of circulating hAAT polymers in the lung [25] [51].

Importantly, the lack of evidence for IV augmentation therapy justifies research into other approaches for treating AATD, including gene therapy.

Table 1-1 Completed and ongoing randomised trials of IV α_1-antitrypsin augmentation therapy

Author	Study Name	Status	Location	Patient numbers	Lung function (FEV_1 % predicted)	Brand name	Dose (mg/kg)	Dose interval	Study duration	Primary endpoint	Other endpoints
Dirksen 1999 [45]	N/A	Reported	Netherlands, Denmark	56	30-80%	Alfalastin (Laboratoire Français du Fractionnement)	250	Monthly	3 years	FEV_1 (home measurements)	CT lung density at TLC
Dirksen 2009 [46]	EXACTLE	Reported	Denmark, UK, Sweden	77	25-80%	Prolastin* (Talecris Biotherapeutics)	60	Weekly	2 years	CT lung density at TLC	Exacerbations, lung function, quality of life
Chapman 2015 [52]	RAPID	Reported	Multi-centre (13 countries)	180	35-70%	Zemaira (CSL Behring)	60	Weekly	2 years	CT lung density at TLC and FRC combined	CT lung density at TLC and FRC separately
Sorrells 2015 [50]	SPARTA	Ongoing (completion 2021)	Multi-centre	339	30-80%	Prolastin-C (Talecris Biotherapeutics)	60, 120	Weekly	3 years	CT lung density at TLC	Exacerbations

Completed and on-going trials of IV augmentation therapy in α_1-antitrypsin deficiency. FEV_1 = forced expiratory volume in 1 second, TLC = total lung capacity, FRC = functional residual capacity.

* In Dirksen 2009, it unclear which hAAT preparation (Prolastin or Grifols) was used.

1.1.5.2 Aerosolised hAAT for augmentation therapy

An alternative strategy for the treatment of emphysema caused by AATD would be to give the purified hAAT by inhalation. Inhaled hAAT is an attractive option not only because it directly treats the organ affected in AATD-related emphysema, but also because (a) it would likely reduce the amount of drug needed, thereby reducing the cost and (b) home inhalation devices are more convenient for patients than IV infusion, which requires a hospital visit [53] [43]. Arguments against inhalation of hAAT as a treatment strategy are that (a) the half-life of inhaled hAAT in lung is approximately 3 days [54] whilst the half-life in serum is 5 days, which would necessitate more frequent treatment (probably twice weekly [53]); and (b) it is uncertain whether inhaled hAAT would penetrate the lung interstitium in sufficient quantities to be protective [55]; whilst several trials have demonstrated the presence of purified hAAT in serum following inhalation (Table 1-2), it is not possible to determine the concentration of hAAT in lung interstitium and, as discussed above, the epithelial layer is relatively impermeable to proteins such as hAAT [13] [35]. Nevertheless, as it has been suggested that only 2-3% of hAAT administered intravenously reaches the lung interstitium [56] [43], investigation of inhaled hAAT therapy remains important.

Pre-clinical studies in sheep [57] [58] and dogs [58] showed that aerosolised, purified hAAT reaches the epithelial lining fluid in the lower airways, supporting progression to clinical trials. The first trials of inhaled hAAT for emphysema were performed in 1989 [59] [60], and to date 7 trials have been reported, summarised on Table 1-2 (it should be noted that one "trial" was a study in healthy individuals). Most of these trials have been performed in small numbers of patients, and none have been randomised, meaning that it is difficult to draw conclusions about the effectiveness of inhaled hAAT in the treatment of AATD-associated emphysema. However, taken together the trials performed have shown that inhaled hAAT is safe, well-tolerated, and leads to deposition of purified in hAAT in the epithelial lining fluid with restoration of anti-neutrophil elastase activity [60] and penetrance of purified hAAT into serum.

A recent trial of inhaled hAAT for AATD was performed by Kamada between 2012 and 2014 [61]. This trial was completed in December 2014, and has yet to be formally published in a peer-reviewed journal. However, data presented by the investigators at the 2015 meeting of the American Thoracic Society in Denver, Colorado [62] showed that after one year, treatment with inhaled hAAT had a statistically significant effect on FEV_1 % predicted. The company has submitted a Marketing Authorisation Application to the European Medicines Agency, the outcome of which is pending at the time of writing [63].

1.1.5.3 Inhaled α_1-antitrypsin for the treatment of cystic fibrosis

α_1-antitrypsin is a multi-functional protein, with a range of anti-proteolytic and anti-inflammatory roles. As a result, it has been proposed as a treatment for a range of pulmonary and systemic diseases [38]. Of particular interest is the potential role of hAAT in the treatment of cystic fibrosis.

A key feature of the pathogenesis of cystic fibrosis is chronic lung infection, particularly by *Pseudomonas aeruginosa.* Bacterial inflammation stimulates neutrophil chemoattraction to the lung; activated neutrophils then release NE [64]. NE has a number of effects in the lung; for example, it impairs bacterial killing by cleaving the complement receptor CR1 on neutrophils, and it cleaves IgG immunoglobulins, impairing the innate immune response. It has also recently been shown to cleave the cystic fibrosis transmembrane conductance regulator (CFTR) molecule [65].

In healthy individuals NE is inactivated by hAAT, as described above; however, in the CF lung there is an abundance of free NE, which cannot be completely neutralised by hAAT [66]. Several groups have therefore proposed that treatment with hAAT may restore the protease:antiprotease balance and ameliorate CF lung disease. There have been several trials of hAAT protein delivery via aerosol in CF patients, with mixed results as summarized on Table 1-3. hAAT produced by two different manufacturing processes has been investigated for the treatment of CF: purifying hAAT from pooled human plasma, and producing recombinant protein from transgenic animals. The recombinant protein has been shown to be under-glycosylated, which potentially alters its

biological activity; this method of producing hAAT is also not currently available. To date, no clinical trial has shown a statistically significant improvement in lung function of CF patients following hAAT administration. However, most of the trials have been of very short duration (most have not lasted more than 4 weeks), and have been in older patients (mean age 25). It is likely that such patients have established lung disease, and it is therefore less likely that clinical improvement will be observed.

The trials have also suffered from small patient numbers, and generally a lack of placebo control groups. The two placebo-controlled trials have also been suboptimal; one has only been reported in abstract form [67], whilst in the other the randomised placebo and treatment groups were poorly matched [68]. Despite decades of work, it is still unclear whether inhaled α_1-antitrypsin may be of clinical benefit in cystic fibrosis; however, it theoretically remains a viable treatment option.

In summary, there are insufficient treatment options for α_1-antitrypsin deficiency, with a lack of clinical evidence for the effectiveness of protein replacement therapy. New treatment options are desperately sought, and gene therapy may be a suitable option.

Table 1-2 Completed studies of inhaled α_1-antitrypsin for the treatment of α_1-antitrypsin deficiency related emphysema

Trial	AAT Product	Dose	n	Key findings
Hubbard 1989 [59]	Recombinant AAT (yeast)	10-200 mg (single dose)	16 Pi*ZZ	hAAT detected in ELF above baseline for at least 24 hours. ELF anti-neutrophil elastase capacity increased in dose-dependant manner. Recombinant AAT detected in serum post-treatment.
Hubbard 1989 [60]	Purified hAAT	100 mg twice daily, 7 days	12 Pi*ZZ	hAAT detected in ELF and serum post-treatment. ELF anti-neutrophil elastase capacity increased.
Vogelmeier 1997 [54]	Purified hAAT (Prolastin)	200 mg (single dose)	29 healthy (no AATD)	hAAT detected in ELF for at least 36 hours. ELF anti-neutrophil elastase capacity increased. Half-life of hAAT in lungs 69.2 hours (3 days).
Kropp 2001 [69]	^{123}I labelled purified hAAT (Prolastin)	100 mg (single dose)	18 Pi*ZZ	Peripheral deposition of hAAT. Increased deposition and longer half-life of hAAT in patients with FEV_1 % predicted > 60% normal compared to patients with worse lung function.
Brand 2003 [70]	^{99m}Tc labelled purified hAAT (Prolastin)	150 mg (single dose)	6 Pi*ZZ	Peripheral deposition of hAAT using four different inhalation devices.
Geraghty 2008 [71]	Purified hAAT (Zemaira)	6 – 96 mg once daily, 14 days	11 Pi*ZZ	Reduced Cathespin B and MMP-2 activity in broncho-alveolar lavage (BAL) fluid.
Brand 2009 [72]	^{99m}Tc labelled purified hAAT (Prolastin)	70 mg (single dose)	6 healthy, 7 Pi*ZZ, 7 CF	~70% filling dose deposited in lungs in all groups (higher peripheral than central deposition).

Completed studies of inhaled α_1-antitrypsin in patients with α_1-antitrypsin deficiency (AATD) related emphysema or healthy controls. ELF=epithelial lining fluid, FEV_1=forced expiratory volume in 1 second, MMP-2=matrix metalloproteinase 2, CF=cystic fibrosis.

Table 1-3 Clinical trials of inhaled alpha-1-antitrypsin therapy in cystic fibrosis (continued on last page)

Trial	AAT Product	Dose	N (CF)	Key findings
McElvaney 1991 [73]	Purified hAAT (Prolastin)	1.5 - 3 mg/kg Twice daily for 1 week	12	AAT level in ELF raised 2-3 fold above baseline Free NE activity was suppressed when AAT concentration rose above 8μM. (This study also included IV hAAT administration in 5 CF patients and 12 healthy controls).
Berger 1995 [74]	Purified hAAT (Prolastin)	100, 200, 250 mg Twice daily for 4 weeks	26	Reduction in level of free NE. No significant change in inflammatory markers. One serious adverse event, probably drug-related.
Bilton 1999 [67]	Transgenic recombinant hAAT	62.5/125/250 mg hAAT or placebo 6 months	131	Increased time to first pulmonary exacerbation in treatment group. Fewer exacerbations in top two dose groups than placebo. Improved fatigue score.
Griese 2001 [75] [76]	Purified hAAT (Prolastin)	100 mg twice daily 8 weeks	8	Partial restoration of protease:antiprotease imbalance.
Martin 2006 [68]	Transgenic recombinant hAAT	125/250/500 mg hAAT or placebo 4 weeks, 2-4 weeks off treatment then 2 week re-challenge	39	No significant effect on sputum NE activity, inflammatory cytokines or lung function.
Cantin 2006 [77]	Purified hAAT (Prolastin)	250 mg daily 10 days	17	No effect on sputum neutrophil elastase activity or pulmonary function.
Griese 2007 [64]	Purified hAAT (Prolastin)	25 mg daily 4 weeks	52	Treatment reduced free NE activity and reduced levels of various pro-inflammatory cytokines. No effect on lung function seen.
Hartl 2007 [78]	Purified hAAT (Prolastin)	25 mg daily 4 weeks	10	Inhalation reduced free NE levels and improved bacterial killing/numbers of *Pseudomonas aeruginosa*.

Brand 2009 [72]	^{99m}Tc labelled purified hAAT (Prolastin)	70 mg Single dose	7	~70% filling dose deposited in lungs in all groups (higher peripheral than central deposition). No effect on FEV_1 % predicted

To the best of my knowledge, this table summarises the data on all clinical trials of inhaled AAT in cystic fibrosis (CF) patients. N numbers show number of cystic fibrosis (CF) patients in each study. Two AAT products have been used: Prolastin (hAAT purified from pooled human plasma) and transgenic recombinant AAT (purified from sheep milk; no longer available).
BD=twice daily, ELF=epithelial lining fluid, NE=neutrophil elastase, tg-AAT=transgenic recombinant human AAT.

1.2 Gene Therapy

Gene therapy is defined as "the introduction… of genetic material within a cell or organism with the intention of curing or treating a disease" [79]. After several decades of intensive research, gene therapy-based drugs are beginning to show measurable clinical benefits. In 2012 Glybera, an adeno-associated virus used to treat adults with lipoprotein lipase deficiency, was approved by the European Medicines Agency, becoming the first gene therapy to receive marking authorisation in the Western world [80]. As a relatively common genetic condition with insufficient treatment options, AATD therefore represents an appropriate target for gene therapy.

1.2.1 Gene therapy for α_1-antitrypsin deficiency

1.2.1.1 Clinical trials of gene therapy for α_1-antitrypsin deficiency

There have been previous attempts to deliver hAAT via gene therapy to treat alpha-1-antitrypsin deficiency, summarised in Table 1-4. The earliest trial, performed by Brigham and colleagues and reported in 2000, used a plasmid-cationic liposome complex to deliver hAAT to the nasal epithelium of 5 patients in an unblinded, non-randomised study [81]. One nostril was transfected, with the other left untransfected as a control; this led to hAAT protein expression in the transfected nostril, peaking at day 5 post-treatment. The study also showed that levels of IL-8, a pro-inflammatory cytokine, were reduced to normal levels post-treatment. Gene therapy raised levels of hAAT in nasal lavage fluid to one third of the 'normal' range, defined as patients receiving weekly IV hAAT augmentation therapy. The authors acknowledged some methodological flaws in this trial, particularly the use of the untransfected nostril as the control, which complicated the data analysis.

Subsequent trials of gene therapy for AATD have used recombinant adeno-associated viral (rAAV) vectors to deliver hAAT cDNA. The first viral trial was performed by Brantly and

colleagues, reported in 2006 [82]. In this unblinded, non-randomised study, 12 patients with AATD were treated with an rAAV2 vector carrying hAAT cDNA under the control of the chicken β-actin promoter (rAAV2-CBA-AAT), given by intramuscular injection. The authors selected intramuscular administration as the method of delivery based on the observation that rAAV2 had previously been used to deliver protein to the serum after muscle transduction. As commonly occurs following treatment with rAAV vectors [83], anti-capsid antibodies to the vector were produced in response to treatment, but otherwise the therapy was well-tolerated. Unfortunately, low-level expression of wild-type hAAT was only observed in one patient post-therapy; background levels of wild-type hAAT from IV augmentation therapy (which was discontinued 28 days before treatment) masked any vector-related hAAT expression in the other 11 patients.

The same group performed a further trial, reported in 2009 [84]. In this trial 9 patients were treated with a similar vector that was packaged in an rAAV serotype 1 capsid (rAAV1), chosen because of increased efficiency of murine muscle transfection with this vector compared to other rAAV serotypes [85] [86]. In the two higher dose cohorts, expression of wild-type hAAT was observed above background levels in all subjects, despite the development of an effector T-cell response to the vector [3]. In the higher dose cohorts, expression of hAAT was around 0.1% of normal levels.

In the most recent study [87] the same rAAV1 vector was used but it was produced by a herpes simplex virus complementation method, generating a higher yield and therefore enabling a substantial increase in dose [88]. 9 patients were treated with different doses of vector (with a switch to weight-based dosing); patients in the top dose group received 100 intra-muscular injections (1.35 ml per injection). In these patients, mean hAAT expression peaked at day 30, and persisted for at least 90 days; however, the peak concentration of hAAT in serum was 36 μg/ml, around 3% of the therapeutic target [3]. In at least one patient expression of hAAT has persisted

for five years [89]. Because further increases in dose would not be possible with this technique, the authors of this study are now investigating an isolated limb infusion method, allowing a substantially greater volume of vector to be delivered to the muscle [90].

Taken together, the results of previous trials of gene therapy for α_1-antitrypsin provide proof-of-concept for a gene therapy approach for the production of hAAT. However, the fact that a huge dose of rAAV vector is only sufficient to produce protein expression at around 3% of the required level demonstrates that further improvements are needed.

1.2.1.2 Pre-clinical studies of delivery of human α_1-antitrypsin to the lung

In my opinion, there are a number of limitations of the previous trials of gene therapy for α_1-antitrypsin deficiency:

1. *The vector used:* the trials reported above used rAAV vectors. Many clinical studies in other diseases have shown that rAAV vectors cannot be repeatedly administered, which limits their use for the treatment of lifelong chronic diseases such as AATD;
2. *The route of administration:* in the trials reported above, an intramuscular route of delivery was used. However, as discussed above, most of the pathology caused by AATD is in the lung; therefore, I feel that treating the lung directly may be more efficient and could reduce the dose of vector needed.

To my knowledge, three groups have performed pre-clinical studies of hAAT gene transfer to the lung. A recent study used an AAVrh.10 vector, which was administered by the intrapleural route to mice and non-human primates, resulting in production of vector-specific mRNA for at least one year [22]. Even though the authors did not report post-treatment production of hAAT protein, a Phase I/II trial using this vector has been approved [91] and is due to begin recruiting in July 2016 (NCT02168686).

An alternative AAV serotype, AAV6, was investigated in a study by Halbert and colleagues [92]. In this study, the authors reported secretion of hAAT into the serum and epithelial lining fluid of mice and dogs following administration of the vector to the lung; however, the immune response to AAV capsids limited the use of this vector, and animals were required to be immunosuppressed in order to observe sustained transgene expression.

One group used a lentiviral vector for hAAT gene therapy to the lung [93] [94]. Wilson and colleagues applied a lentiviral vector to the trachea of mice, showing that gene therapy resulted in secretion of hAAT into the epithelial lining fluid; gene therapy was also shown to ameliorate a mouse model of emphysema [94]. The authors showed that the vector selectively transduces macrophages; however, it required co-transfection with Lipofectamine 2000 which may limit the use of this strategy in humans.

Due to the limitations of these studies, there is scope for investigating alternative gene therapy strategies for AATD. The UK Cystic Fibrosis Gene Therapy Consortium, with whom I performed the research in this thesis, have developed viral and non-viral gene transfer agents for airway gene delivery, which could be used for the treatment of AATD. I will therefore consider each in turn below.

Table 1-4 Completed trials of gene therapy for α_1-antitrypsin deficiency

Trial	Gene transfer agent	Delivery method/Dose	n	Key findings
Brigham 2000 [81]	Non viral (DOTMA:DOPE-pCMV-AAT)	Nasal instillation (unilateral)	5 Pi*ZZ	Significantly higher hAAT concentration in nasal lavage from transfected nostril compared to untransfected nostril. Peaked at 5 days, returning to normal within 14 days. hAAT in nasal lavage fluid reached approximately 30% of level in patients receiving IV augmentation therapy. Reduction in nasal lavage IL-8 to normal levels.
Brantly 2006 [82]	rAAV2-CBA-AAT	Intramuscular injection 2.1e12 – 6.9e13 vg	12 Pi*ZZ or Pi*Z with another disease-causing mutation	Vector-specific DNA in blood of 7 out of 9 subjects in higher dose cohorts. No expression of hAAT in 11 out of 12 patients, with background hAAT from IV augmentation therapy observed. Most patients developed antibody response to rAAV2 capsid.
Brantly 2009 [84]	rAAV1-CBA-AAT	Intramuscular injection 6.9e12 – 6.0e13 vg	9 Pi*ZZ	Vector-specific hAAT expressed above background in higher dose cohorts. Sustained at levels of 0.1% normal for one year, despite development of neutralising antibody response.
Flotte 2011 (interim analysis [87], Mueller 2013 [95]	rAAV1-CBA-AAT (HSV-complementation method)	Intramuscular injection 6.0e11 – 6.0e12 vg/kg	9 AATD	Dose-dependant expression of hAAT. Mean hAAT expression peaked at day 30, persisting for around one year. All subjects developed antibody response to AAV. Peak hAAT concentration in serum of 36 μg/ml in highest dose group, below therapeutic target of 572 μg/ml.

A summary of previously reported trials of gene therapy for α_1-antitrypsin deficiency (AATD). IL-8 = interleukin 8, VG = vector genomes, HSV = herpes simplex virus.

1.3 Viral and non-viral vectors for airway gene transfer

One of the most common genetic conditions in the world is cystic fibrosis (CF), caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) [96] [97] [98] which has a number of pulmonary and extra-pulmonary effects.

Since CFTR was cloned in 1989, treating the underlying genetic defect in CF has been at the forefront of gene therapy research. Despite initial optimism throughout the scientific community, gene therapy for CF has proven considerably more challenging than once anticipated. The success in recent years of a small molecule drug targeting the CFTR molecule (Ivacaftor, trade name Kalydeco) has provided proof-of-concept that treating the underlying molecular defect in CF can lead to clinical improvements [99] [100] and has renewed interest in CF gene therapy, resulting in a successful Phase IIb trial of gene therapy for cystic fibrosis (discussed below).

The UK Cystic Fibrosis Gene Therapy Consortium ("the Consortium") is an alliance of three research groups from London, Oxford and Edinburgh, formed with the intention of pooling funding and expertise to improve patient outcomes [101]. Over the past 15 years the Consortium has investigated non-viral and viral vectors for the transfer of genes to the airway, resulting in the selection of two "gold standard" gene transfer agents:

- The non-viral, cationic lipid formulation GL67A;
- A lentiviral vector, based on simian immunodeficiency virus (SIV), pseudotyped with proteins from Sendai virus (named rSIV.F/HN).

The pre-clinical and clinical development of these vectors is discussed below.

1.3.1 Non-viral gene therapy for the treatment of cystic fibrosis

Non-viral gene transfer agents (GTAs) consist of two parts, (a) the therapeutic cDNA component and (b) the carrier molecule that envelopes and binds the DNA. These two components have undergone extensive development throughout the history of non-viral CF gene therapy, with the aim of creating a potent GTA that is not only capable of delivering the CFTR gene to airway epithelial cells, but which can also enter cells without activating an immune response, thus allowing for the repeated administration which is necessary for the treatment of a chronic disease.

In the first trial of non-viral gene therapy for CF, reported in 1995 [102], gene transfer agents were administered to the nose of CF patients and led to a partial correction of the nasal potential difference. The first trial to deliver a non-viral GTA to the lung was reported in 1999 [103], and again showed correction of the chloride abnormality in CF patients; the results of this trial were replicated by a different group in 2001 [104].

The Consortium's translational research pathway led to a Phase IIb trial of gene therapy, which was reported in 2015 [105]. The following milestones in the translational pathway are particularly significant:

- The selection of the most appropriate non-viral GTA. After extensive research, the Consortium determined that GL67A, first used in the 1990s, remained the most potent non-viral GTA for airway gene transfer some two decades later [106].
- The plasmid vector (pGM169) carrying the CFTR cDNA was developed, leading to a novel plasmid that was completely devoid of immuno-stimulatory CpG-dinucleotides [107]. The Consortium also selected a novel promoter hCEFI, consisting of the elongation factor 1α promoter coupled to the cytomegalovirus (CMV) enhancer.
- Regulatory-compliant toxicology studies using pGM169 were performed in mice [108] and sheep [109], with no chronic inflammatory responses or extra-pulmonary responses to the vector observed.

The resulting Phase IIb multi-dose trial was the largest trial of gene therapy performed in man to date. The trial was double-blinded and placebo controlled, and approximately 140 patients were recruited to the study from 18 sites in the UK. Patients were randomly assigned 1:1 to receive 5 ml of nebulised pGM169/GL67A or 5 ml 0.9% saline every 28 days (±5 days) for 12 months through a Trudell AeroEclipse II nebuliser. The primary endpoint was the change in FEV_1 % predicted.

Data from 116 patients per-protocol (who received at least 9 doses of treatment) was analysed [105]. Importantly, there were no differences in treatment-attributable adverse reactions between the placebo and active group, demonstrating that gene therapy was well-tolerated at the 5 ml dose.

There was a significant, albeit modest, treatment effect in the pGM169/GL67A group compared to the placebo group. In the treatment group, the change in FEV_1 % predicted throughout the 12-month study period was close to 0%, compared to a mean deterioration of lung function of about 4% relative change in the placebo group. The overall treatment effect was a 3.7% difference in FEV_1 % predicted in the treatment group compared to placebo (95% confidence interval 0.1-7.3, p=0.046). Similarly, every single secondary outcome investigated showed improved values of lung function in the treatment group compared to the placebo group, although none of these outcomes reached statistical significance.

Conclusions taken from the multidose trial were positive. The active and placebo group were closely matched and no safety concerns were seen in response to the treatment. Importantly, there was a significant stabilisation in lung function in treated patients and secondary outcomes were also supportive of a protective effect on lung function. This trial, therefore, demonstrated for the first time that repeated administration of non-viral gene therapy over a 12-month period can lead to stabilisation in the lung function of CF patients.

The clinical improvement in patients treated with gene therapy in the Phase IIb trial was significant, but modest; yet it remains the first proof-of-concept that repeated administration of a non-viral GTA carrying CFTR can safely change clinical parameters, which is encouraging for future research. The results of this trial prove that non-viral gene transfer to the lung is feasible despite the numerous intra- and extra-cellular barriers to airway gene transfer; and the results therefore provide a rationale for the assessment of GL67A for other lung diseases, such as α_1-antitrypsin deficiency.

1.3.2 Viral gene therapy for the treatment of cystic fibrosis

Viruses are an alternative method for delivering the CF gene into epithelial cells. Zabner *et al* were the first group to administer an adenovirus to the nose [110], whilst Crystal *et al* were the first group to administer gene therapy to the lungs of four CF patients using a similar vector [111]. 8 further trials using an adenovirus were performed between 1993 and 2001, however taken together the results show that there was inefficient and transient gene transfer with adenoviral gene therapy to the lung, possibly due to the localisation of the necessary receptor for adenoviral entry (Coxsackie and adenovirus receptor, CAR) on the basolateral membrane of airway epithelial cells. Furthermore, the induction of an immune response to the virus reduced efficacy on repeated administration. Recently developed helper-dependent adenoviruses, which are devoid of viral coding sequences, may hold promise for lung gene therapy, but are currently too early in development to warrant clinical assessment.

In the 2000s, adeno-associated virus (AAV) began to supersede adenovirus as the viral vector of choice in many trials. In the largest trial of AAV in CF, 100+ subjects were given repeated doses of AAV carrying CFTR [112]. However, the trial did not meet its primary endpoint and patients failed to show an improvement in lung function. One reason for this might be that AAV has a packaging capacity limited to approximately 5 kB, making it difficult to

package the 4.7 kB CFTR gene along with a promoter and other regulatory elements. In addition, an antiviral immune response is seen on repeat administration, which may limit the use of rAAV for CF gene therapy to the lung, and pre-existing immunity to rAAV vectors could also limit their use as neutralising antibodies block vector transduction [83]. It remains to be seen if rAAV vectors have a role in pulmonary gene therapy, although intriguingly it has been demonstrated that AAV is able to transduce progenitor cells in the lung [113], which feasibly could overcome the problems with repeat adminstration of rAAV vectors.

Alternative viral vectors are RNA viruses such as human parainfluenza virus (hPIV) [114] and Sendai virus (SeV) [115]. Sendai virus has previously been investigated by the Consortium as it is a murine paramyxovirus with high airway tropism, due to the presence of two envelope proteins (fusion (F) and hemagglutinin neuraminidase (HN)) on the viral surface. Previous Consortium work has demonstrated that treatment of the murine airway with Sendai viral vectors leads to a high, albeit transient (~1 week) level of gene expression; unfortunately, as with other viral vectors, an immune response to the virus significantly reduces its efficacy on repeat administration [116].

1.3.3 Lentiviral vectors

Lentiviral vectors can transduce dividing and non-dividing cells, and have therefore been a source of great interest for gene therapists. They have proven clinical efficacy following *ex vivo* transduction and transplantation of haematopoietic stem cells in patients with metachromatic leukodystrophy [117] and Wiskott-Aldrich syndrome [118], and have been shown to be safe following direct injection into the brain of patients with Parkinson's Disease [119]. Several groups have investigated lentiviral vectors for the treatment of CF, including research using human immunodeficiency virus (HIV) [120], simian immunodeficiency virus (SIV) [121], feline immunodeficiency virus (FIV) [122] and equine infectious anaemia virus (EIAV) [123] for airway gene transfer. To the best of my knowledge there has been far less research using

lentiviral vectors for the treatment of α_1-antitrypsin deficiency, with just one group reporting preclinical studies using a lentiviral vector [94].

Lentiviral vectors share some properties with γ-retroviral vectors, which are amongst the oldest vectors used in clinical trials of gene therapy. Over the past decide lentiviral vectors have gradually superseded γ-retroviral vectors because of their favourable safely profile; in trials using γ-retroviral vectors for the treatment of X-linked severe combined immunodeficiency (SCID-X1), 5 out of 19 patients developed acute lymphoblastic leukaemia, with two patients dying [124]. This was subsequently shown to be due to the upregulation of proto-oncogenes as a result of the presence of transcriptionally active, strong enhancer sequences in the 5' long terminal repeats (LTRs) of γ-retroviral vectors [125]. γ-retroviral vectors have also been shown to integrate in a non-random way, with clusters of integration events observed close to transcription start sites [126] [127], which is believed to enhance clonal proliferation [128]. It is believed that lentiviral vectors have a better safety profile as insertion is random and not biased towards transcription start sites; in the three trials that have been reported to date there has been no obvious clustering of vector insertions [117] [118] [128]. However, the small number of patients and the short follow-up time in these trials limits the validity of these conclusions, and it will be important to closely monitor the safety profile of lentiviral vectors in future pre-clinical and clinical studies. To further improve the safety profile of these vectors, they have been rendered self-inactivating (SIN) by a deletion in the 3' LTR [129], internal promoter sequences have been used which have decreased the risk of insertional mutagenesis [130].

Lentiviral vectors have no natural lung tropism, so require pseudotyping with various envelope proteins to facilitate airway gene transfer. The vesicular stomatitis virus G (VSV-G) protein is commonly used for pseudotyping lentiviral vectors, however for efficient transduction of the airway epithelium it is necessary to co-administer tight junction openers

such as lysophosphatidylcholine (LPC), which allows access to the basolateral membrane [131]; this may have considerable safely implications in man, particularly in patients with chronic lung infection as seen in CF. Several groups have investigated the use of lentiviral vectors pseudotyped with other proteins, including the gp64 protein from baculovirus [132]; however, this vector also requires co-administration of a second agent, methylcellulose, to enable *in vivo* gene transfer, which could affect its safety profile.

The UK CF Gene Therapy Consortium has developed a novel SIV vector which has been pseudotyped with the F and HN surface proteins from Sendai virus [121]. Pseudotyping lentiviral vectors with these Sendai virus proteins has shown promise in pre-clinical studies [133]; a single dose of pseudotyped virus, named rSIV.F/HN, administered to the airway leads to expression of an intracellular reporter gene for the life-time of a mouse (~2 years), and surprisingly it has also been shown that repeated monthly administration is feasible without loss of gene expression, with no evidence of toxicity in murine studies. The vector has also been shown to transduce human tissues *ex vivo*. This vector has been placed on a clinical translational pathway, with a first-in-man trial of lentiviral gene therapy for CF planned for 2017.

In summary, the UK Cystic Fibrosis Gene Therapy Consortium has developed two technologies for gene transfer to the lung:

- *non-viral* gene therapy with the cationic lipid GL67A, which has proven efficacy in a Phase IIb clinical trial [105];
- *viral* gene therapy with a pseudotyped rSIV.F/HN lentiviral vector, which has favourable characteristics compared to other viral vectors used in pre-clinical studies [134].

The potential applications of these platform technologies are not limited to the treatment of cystic fibrosis, but could be used to for the delivery of other genes to the lung either (a) to treat

lung disease, or (b) with the aim of using the lung as a factory organ to produce proteins to treat systemic disease. An example of a genetic disease that primarily affects the lung, and which could be amenable to a gene therapy approach, is α_1-antitrypsin deficiency; this thesis will concentrate on gene therapy for α_1-antitrypsin deficiency, with the aim of using the Consortium's platform technologies to produce therapeutic levels of α_1-antitrypsin in the lung.

1.4 Other treatment strategies for α_1-antitrypsin deficiency

A number of other therapies for α_1-antitrypsin deficiency are in clinical and pre-clinical development and should be briefly considered. A strategy to knock down the expression of Z-hAAT in hepatocytes could be beneficial in AATD-associated liver disease, and experiments in mice using small interfering (siRNA) constructs targeted at hAAT mRNA have recently been published [135] [136]. The authors showed that treatment with siRNA (a) reduces circulating hAAT, (b) reduces soluble and aggregated hAAT in the liver, and (c) prevented or reversed liver disease in a mouse model of Z-hAAT liver disease. Further experiments in non-human primates have been conducted and have shown that treatment with siRNA reduces circulating hAAT by 80%; this data has been reported at scientific meetings, but has not been published in peer-reviewed journals at the time of writing [137]. Alnylam Pharmaceuticals is now performing a Phase I/II clinical trial, which commenced in July 2015 [138]. Their product, ALN-AAT, uses a small interfering RNA (siRNA) construct targeted at hAAT mRNA; it is conjugated to an N-acetylgalactosamine ligand, to increase efficiency of delivery to hepatocytes. The trial is due to be completed in 2017.

It has also been proposed that the mutated hAAT gene could be corrected *ex vivo*. Yusa and colleagues [139] successfully achieved bi-allelic correction of the Z-hAAT point mutation in human induced pluripotent stem cells (iPSCs) derived from AATD patients, using a zinc

finger nuclease/piggyBac combination approach. iPSCs were then differentiated into hepatocyte-like cells, and secretion of normal hAAT demonstrated *in vitro* and *in vivo*. It is possible that newer technologies such as CRISPR/Cas9 could refine this approach further [140] [141].

It has also been proposed that an rAAV vector which includes (a) inhibitory miRNA sequences targeting Z-hAAT and (b) wild-type hAAT cDNA could achieve simultaneous knockdown of the mutated Z protein and increased expression of the normal M protein [142]. This dual-therapy approach has been shown to reduce levels of Z-hAAT in the serum of mice expressing the PiZ allele, without affecting the overall liver miRNA profile, so may hold promise for future therapies.

Other approaches to treat AATD liver disease include using small molecule drugs or antibodies either to block intracellular polymerisation of Z-hAAT, or to clear pre-existing polymers [25]. Pharmaceutical approaches have also been proposed for the treatment of AATD lung disease: a molecule called AZD9668 has been identified as a selective oral inhibitor of NE, and would theoretically reduce the elastase burden in the lung of patients with emphysema caused by AATD. It has been investigated in patients with bronchiectasis [143], COPD [144] [145] and cystic fibrosis [146].

In summary, whilst many treatment strategies have been proposed for AATD, there remain insufficient options for patients with AATD. Taken together, the controversy and high cost associated with intravenous or inhaled augmentation therapy and the lack of sufficient hAAT production following gene therapy with rAAV vectors justify further investigations into other options for the treatment of α_1-antitrypsin deficiency.

1.5 Aims & Objectives

The aim of this research project is to investigate gene therapy for α_1-antitrypsin deficiency using the "gold standard" viral (rSIV.F/HN) and non-viral (GL67A) vectors for airway gene therapy, developed by the UK Cystic Fibrosis Gene Therapy Consortium, which have previously been shown to be the most efficient vectors for delivery of genes to the lung.

To complete this aim, I plan to use these vectors *in vitro*, *in vivo* and *ex vivo*, specifically:

- To demonstrate the functionality of viral and non-viral vectors in *in vitro* experiments;
- To administer the vectors to the lungs of mice, and demonstrate the production of α_1-antitrypsin in lung tissue homogenate, epithelial lining fluid, and serum;
- To investigate the ability of these vectors to transduce primary human tissue *ex vivo* using air liquid interface cultures, lung slices and nasal brushings;
- If the above experiments are successful, to investigate the vectors in large animal models e.g. sheep.

I also aim to demonstrate that the recombinant hAAT protein produced by gene therapy is functional and inhibits neutrophil elastase, placing gene therapy for α_1-antitrypsin deficiency using rSIV.F/HN and/or GL67A on a translational pathway to the clinic.

Finally, as sputum samples from stable cystic fibrosis patients are available to me, I plan to analyse them for α_1-antitrypsin, which could offer new insights into the disease.

2 General Materials & Methods

In this chapter I provide a broad description of the experimental techniques used frequently throughout the thesis, to improve the clarity of each section and to avoid repetition. Each chapter has its own specific Material & Methods section, where more detail is provided, for example on the viral titre used in each experiment.

2.1 Plasmid production

2.1.1 Production of non-viral human α_1-antitrypsin vector plasmids

Plasmids coding for human α_1-antitrypsin (hAAT) were produced at the Consortium's Core Facility in Oxford by Jean-François Gelinas, as part of his PhD project. In-depth details of plasmid production will be presented in his thesis (currently unpublished); here, I provide brief details.

hAAT cDNA was a gift from C. Halbert (Seattle, USA) [92]. The transgene was sub-cloned into the Consortium's plasmid backbones, described in [107], using standard molecular biology techniques. A codon-optimised version of hAAT (sohAAT) was designed in-house, purchased from GeneArt (LifeTechnologies), and this codon-optimised transgene was also sub-cloned into plasmid backbones. A full description of codon-optimised plasmids is provided below (Section 3.3.2). In total, four plasmids encoding hAAT were generated under the control of different promoters, summarised on Table 2-1.

GT115 cells (Invivogen) were then transformed and cultured with the appropriate antibiotic; these cells are a strain of *E. coli* which are optimised for the production of artificial CpG-free plasmids (Jean-François Gelinas, personal communication). Colonies were isolated on selective agar plates, and sub-cultured for pDNA extraction using Wizard Plus SV MiniPrep

Columns (Promega). Restriction enzyme analysis was performed, and one correct clone from each sub-culture was further amplified. Plasmids were purified using Q2500 EndoFree Mega Columns from QIAGEN (Qiagen, Crawley, UK) and suspended in endotoxin-free water. Plasmids were verified by sequencing (Source Bioscience, Nottingham UK). The pDNA concentration was assessed using a NanoDrop spectrophotometer (Thermo Scientific), and purity confirmed when the OD260/280 ratio was close to 1.8.

Control plasmids coding for Firefly luciferase and *Gaussia* luciferase were used throughout the thesis as appropriate. Control plasmids used are summarised on Table 2-2.

2.1.2 Production of genome plasmids for lentiviral vectors

Genome plasmids for the production of lentiviral vectors were produced by Jean-François Gelinas using standard molecular biology techniques, and described in detail in his thesis (currently unpublished). Briefly, hAAT vector plasmids were digested with restriction enzymes and the transgene ligated into lentiviral backbone plasmids. One Shot Stbl3 cells (Invitrogen) were transformed and plasmids amplified as described in Section 2.1.1; Stbl3 cells are a strain of *E. coli* optimised for the production of large plasmids (Jean-François Gelinas, personal communication).

Two plasmids were made:

- SIV1 hCEF-soGLux
- SIV1 hCEF-sohAAT

Production of lentiviral vectors using these plasmids is described in Section 2.3.

Table 2-1 Human α_1-antitrypsin vector plasmids

Full plasmid name	Promoter	Transgene	CpG-free?	Gene Medicine production code
hCEFI-hAAT	hCEFI	hAAT	No	pGM383
hCEFI-sohAAT	hCEFI	sohAAT	Yes	pGM385
pCIK-hAAT	CMV	hAAT	No	pGM387
pCIK-sohAAT	CMV	sohAAT	Transgene only	pGM397

Plasmids produced by the UK CF Gene Therapy Consortium coding for human α_1-antitrypsin. "Gene Medicine production codes" are shorthand codes used by the Consortium. In this thesis, I will refer to all plasmids by their full plasmid name, but include the production codes here to aid cross-referencing with other sources.

Transgenes preceded by the code 'so' are codon-optimised (details provided in Section 3.3.2).

hAAT: human α_1-antitrypsin
CMV: cytomegalovirus immediate/early promoter
hCEFI: human CMV enhancer and human elongation factor 1α promoter (hybrid promoter)

Table 2-2 Control plasmids used in this thesis

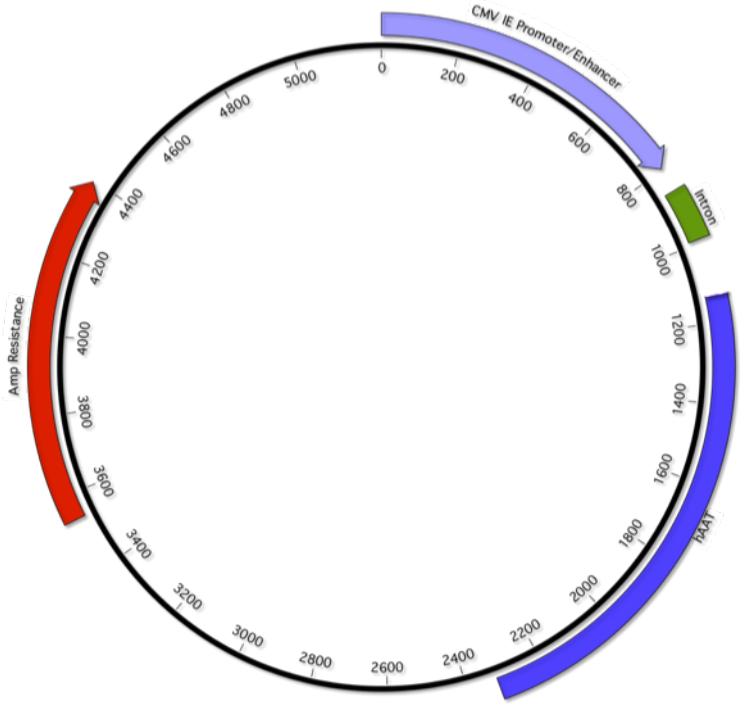
Full plasmid name	Promoter	Transgene	CpG-free?
pCIK-GLux	CMV	GLux	No
hCEFI-soGLux	hCEFI	soGLux	Yes
pCIK-FLux	CMV	GLux	No

Plasmids produced by the UK CF Gene Therapy Consortium used as controls throughout this thesis. "Gene Medicine production codes" are shorthand codes used by the Consortium. In this thesis, I will refer to all plasmids by their full plasmid name, but include the production codes here to aid cross-referencing with other sources.

Transgenes preceded by the code 'so' are codon-optimised (details provided in Section 3.3.2).

CMV: cytomegalovirus immediate/early promoter
hCEFI: human CMV enhancer and human elongation factor 1α promoter (hybrid promoter)
GLux: Gaussia luciferase
FLux: Firefly luciferase

A. pCIK-hAAT



B. pCIK-sohAAT

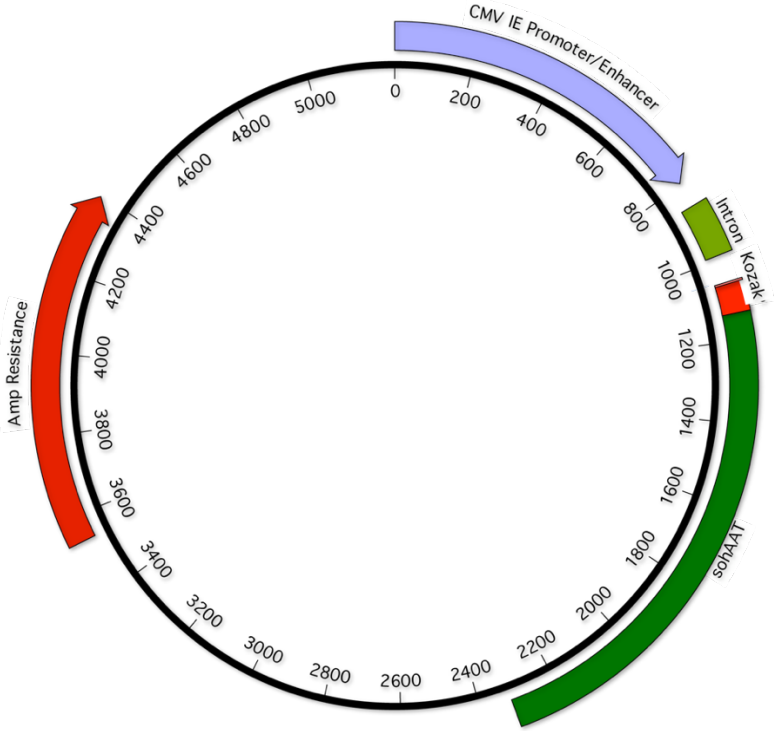
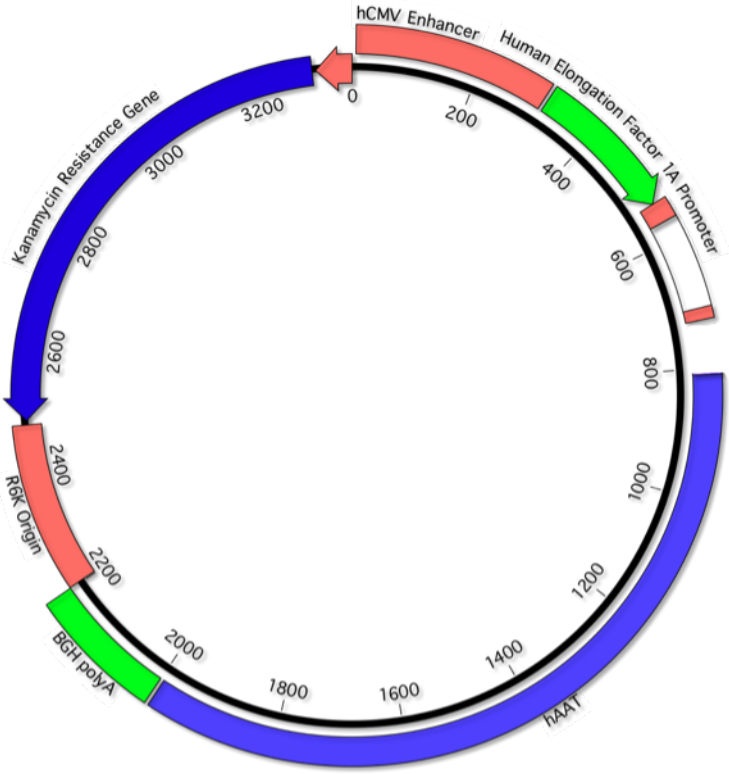


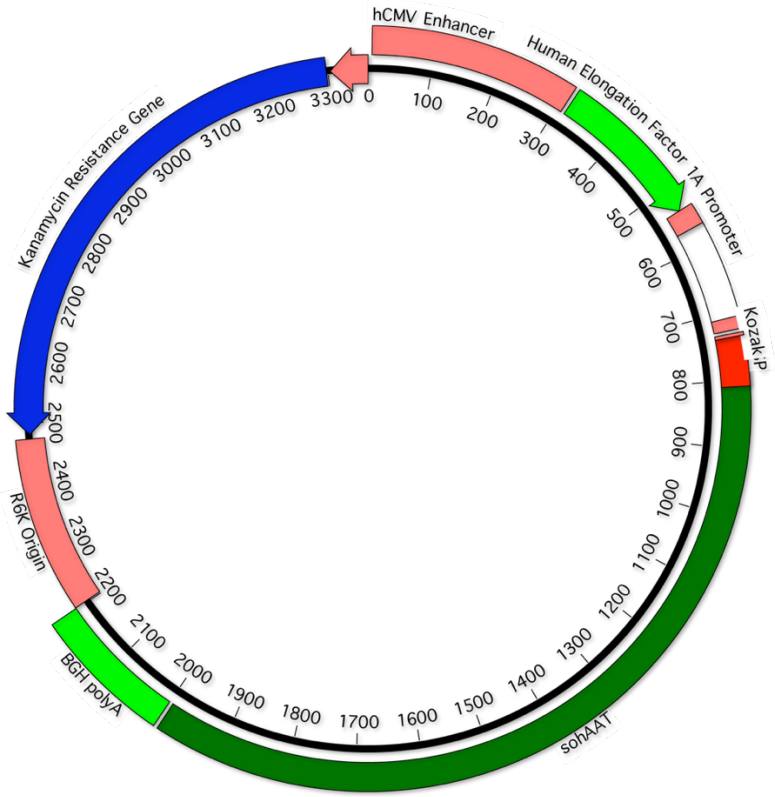
Figure 2-1 Human α_1-antitrypsin vector plasmids (continued on next page)

Four human α_1-antitrypsin (hAAT) vector plasmids were produced as described in Table 2-1. CMV IE: cytomegalovirus immediate/early promoter; hAAT: human α_1-antitrypsin; Amp: ampicillin.

C. hCEFI-hAAT



D. hCEFI-sohAAT



hCMV: human cytomegalovirus enhancer; sohAAT: sequence-optimsed human α_1-antitrypsin; BGH polyA: bovine growth hormone poly-adenylation sequence; R6K: plasmid R6K origin of replication.

2.2 Preparation of lipid:plasmid complexes for transfection studies

GL67A: the cationic lipid GL67A has been extensively used by the UK Cystic Fibrosis Gene Therapy Consortium in pre-clinical and clinical studies. It consists of three components: GL67 (Genzyme Haverhill, UK), dioleoylphosphatidylethanolamine (DOPE) and dimyristoylphosphatidylethanolamine coupled to 5000 kDa molecular weight polyethylene glycol (DMPE-PEG5000) (both Avanti, Alabaster, Alabama, USA). GL67A was generated by formulation at molar ratios of 1:2:0.05 (GL67:DOPE:DMPE-PEG5000) and freeze-drying (performed by OctoPlus, Leiden, The Netherlands).

On the day of the transfection experiments, one vial containing 63 µM GL67A was removed from the freezer and thawed. To prepare GL67A at 12mM, 5.25 ml water for injection (Braun, Germany) was injected through the vial septum using an 18-gauge needle. The vial was then vortexed on a multi-tube vortexer (VWR, Leicestershire, UK) at room temperature for 40 minutes, or longer if necessary to completely dissolve the lipid pellet.

To generate lipid:plasmid complexes at 1:4 molar ratios: For *in vivo* transfection experiments and some *in vitro* experiments, GL67A was diluted 10-fold with water for injection to a final concentration of 1.2 mM. pDNA was then diluted to 1.6 mg/ml (equivalent to 4.8 mM) with water for injection and added to GL67A in a 1:1 ratio by volume. The complexes were allowed to form by incubation at 30°C for 15 minutes, and the tubes then gently shaken to homogenise the solution. The final formulation contained approximately 0.8 mg pDNA per ml; animals were treated with 100 µl lipid:plasmid complexes, equivalent to 80 µg pDNA in total (described below). Complexes were used within two hours of preparation.

To generate lipid:plasmid complexes at 6:8 molar ratios: For some *in vitro* experiments, pDNA was diluted to 5.6 mg/ml (equivalent to 16 mM) with water for injection, and added to GL67A in a 1:1 ratio by volume. Complexes were allowed to form as before. The final formulation contained approximately 2.65 mg pDNA per ml. The reasons for using two different molar ratios are discussed in Section 3.3.1.

***Lipofectamine*:** Lipofectamine 2000 (Invitrogen, Paisley, UK) was complexed at a ratio of 2.5 µg Lipofectamine 2000 to 1 µg pDNA (w:w) ratio, according to the manufacturer's recommendation and based on previous experience in the laboratory (Mario Chan, personal communication).

2.3 Production of lentiviral vectors

Replication defective self-inactivating lentiviral vectors were based on the non-pathogenic simian immunodeficiency virus (SIV) from African green monkey (SIVagm) [147] and pseudotyped with Sendai virus F/HN proteins. Vectors were produced either in London by Mario Chan, or in Oxford by the Consortium's Core Facility.

2.3.1 Production of London-made virus in adherent cell cultures

London-made virus was generated by HEK293T/17 cell transient co-transfection as previously described [148] based on a method from DNAVEC (Japan) [149]. Briefly, adherent HEK293T/17 cells were maintained in complete media (Dulbecco's minimal Eagle's medium (DMEM, Invitrogen, Carlsbad, CA), 10% fetal calf serum, supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml). The viral vector was produced by transient transfection of five plasmids complexed to Lipofectamine reagents (Invitrogen) according to the manufacturer's recommendations:

- Packaging vectors: two plasmids coding for viral genes *rev* and *gag-pol* under the control of the cytomegalovirus (CMV) immediate/early promoter, and the chicken β-actin (CBA) promoter, respectively;
- pCAGGS-Fct4, encoding the truncated F pseudotype protein, under the control of the CAGGS promoter (a hybrid promoter containing elements of chicken β-actin promoter, cytomegalovirus immediate-early enhancer, and chicken β-actin intron 1/exon 1 [150]);
- pCAGGS-SIVct+HN, encoding the HN pseudotype protein fused with cytoplasmic tail of SIV transmembrane protein;
- Genome plasmids: one plasmid carrying the transgene of interest (e.g. hAAT cDNA, *Gaussia* luciferase cDNA).

The plasmid constructs are described in more detail in the Consortium's viral production paper (awaiting publication).

12 hours after transfection, the culture medium was replaced with serum-free DMEM containing 5 mM/l sodium butyrate. The culture supernatant was harvested at 48 hours, filtered through a 0.45 μm filter membrane, and trypsin added (50 μl per 10 ml product) to activate the F protein.

When possible, the supernatant was concentrated by high-speed concentration (20,000 g_{av} for 4 hours at 4°C). Vector pellets were suspended in PBS to 100- to 200-fold concentration and stored at -80°C.

2.3.2 Concentration of supernatant in Millipore spin columns

In some experiments, viral vectors were further concentrated to improve the titre. When this was performed, vectors are referred to as 'concentrated'. Concentration of viral vectors was

performed in Amicon Ultra-15 Centrifugal filter units (Millipore, Watford, UK) according to the manufacturer's recommendations. Vector supernatant (up to 10ml) was added to the tubes, which were spun at 4000 g_{av} for 30 minutes at room temperature. Approximately 10% of the original volume is retained, which is the 'concentrated' virus used in the experiment.

Data presented in Section 4.3.2 suggests that this process concentrates rSIV.F/HN lentiviral vectors approximately 10-fold.

2.3.3 Production of Oxford-made virus in serum-free cell suspension cultures

In order to scale-up the production of lentiviral vectors in a GMP-compliant manner, viral vectors were produced in cells grown in animal-free suspension culture using a WAVE Bioreactor (GE Healthcare). The protocol is described in the viral production paper (awaiting publication). Virus produced in Oxford was shipped to the London laboratory on dry ice using a same-day courier service.

2.3.4 Vector titration

Where possible, the particle and functional titre for each vector batch was determined by the Consortium's Core Facility in Oxford. Occasionally it was not possible to determine the functional titre of a particular batch of virus, as it was below the lower limit of detection of the assay. Where this is applicable, it is clearly stated in the text and figure legends. In some experiments, financial and personnel constraints at the Core Facility prevented determination of particle and functional titre; where this is the case it is clearly stated in the text along with a discussion of whether or not it affects interpretation of the data in question.

Particle titre, when performed, was determined using quantitative PCR (Q-PCR). Viral RNA was extracted using QIAamp RNA mini-kit (QIAGEN, Strasse, Germany) and was reverse transcribed (Superscript II, Invitrogen). PCR was performed with primers directed at

a 131 nucleotide section of the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) sequence, as previously described [148], against a standard curve of RNA mimics containing the same sequence. Particle titre is given as viral particles (VP) per ml.

Functional titre was performed to determine the number of viral particles capable of transducing cells, based on a method by Segura *et al* [151]. Functional titre in TaqMan transducing units (TTU) per ml was determined by transducing suspension-growing human embryonic kidney (HEK) 293F cells with serial dilutions of vector stock. 72 hours later DNA was extracted using QIAamp blood DNA kit (QIAGEN). Viral genomes were quantified by DNA TaqMan (with primers detected towards the WPRE sequence, as above) against a standard curve of plasmid DNA containing the same sequence. This was then normalised to total DNA concentration to calculate WPRE copies (i.e. Transducing Units) per well of cells. This was plotted against total µl virus applied per well of cells, and the slope of best-fit line was used to determine the TaqMan Transducing Units (TTU) per ml virus.

Scientific notation: When reporting viral titres and other large numbers in this thesis, I have used a commonly accepted shorthand where "e" represents "times ten to the power of". For example, 1e7 is equivalent to $1x10^7$.

2.4 *In vitro* gene transfer studies

In vitro gene transfer studies were performed in human embryonic kidney (HEK) 293 cells or A549 lung adenocarcinoma cells as indicated in the text. The cell density used in each experiment is detailed in the relevant chapter. Cells were cultured in the indicated volume of complete media (Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (all regents from ThermoFisher, Waltham, USA)). Cells were incubated in a 37°C incubator at 5% CO_2.

Non-viral or viral vectors were applied to the cells as described in the relevant chapter. At indicated timepoints, supernatant was removed and stored at -80°C pending further analysis.

At the indicated time-point post transduction, cells were lysed with the addition of 300 µl reporter lysis buffer (Promega) and freeze-thawed three times. The samples were centrifuged at 13,000 g_{av} and the supernatant stored at -80°C pending further analysis.

2.4.1 Quantification of total protein

Total protein in cell lysate and lung tissue homogenate was measured using a Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK) based on the Bradford method of protein quantification [152]. Samples and standards were analysed in duplicate, and the assay performed according to the manufacturer's instructions.

2.5 Animal studies

All animal procedures were performed in accordance with the conditions and limitations of the UK Home Office Project and Personal license regulations under the Animal Scientific Procedure Act (1986).

All mice used in this study were purchased from Charles Rivers Laboratories, UK, or Harlan, UK, and were maintained at the animal facility at Imperial College London under the ethical guidelines established by the College. Unless otherwise stated in the relevant chapter, mice used were C57Bl/6 wild type females, 6-8 weeks old at the time of treatment.

2.5.1 Anaesthetic procedures

In most experiments, light sedation with isoflurane was deemed sufficient. Mice were placed in an anaesthetic chamber with 1 litre/minute oxygen and 2 to 3% isoflurane (Baxter,

Staines, UK). Mice were usually unconscious within 60 seconds of being placed in the chamber. Treatment was then administered, and the animal observed until fully recovered.

For longer procedures (such as nasal perfusion techniques in Chapter 8), animals were anaesthetised with 76 mg/kg Ketaset (Fort Doge Animal Health, Southampton, UK) and 1 mg/kg Domitor (Orion Pharma, Hampshire, UK), injected intraperitoneally in 100 µl total volume water for injection (Braun, Germany). After the procedure, anaesthetic was reversed with 1 mg/kg Antisedan (Orion Pharma, Hampshire, UK) injected intraperitoneally in 20 µl water for injection, and the animal observed until fully recovered.

When necessary, mice were sacrificed with an overdose of Euthatal (Merial, Essex, UK) (200 µl total volume of 200 mg/ml phenobarbital, intraperitoneal injection). When the pedal withdrawal reflex was absent, the femoral artery was dissected and snipped to exsanguinate the animal and thereby confirm death.

2.5.2 Routes of vector delivery

Various methods were used to deliver genes to mice in this thesis.

Nasal sniffing: this is the easiest and quickest method for delivering gene transfer agents to the murine lung used in this thesis. Mice were anaesthetised with isoflurane as described above, removed from the anaesthetic chamber and placed supine on the palm of the hand, with light thumb pressure used to close the animal's mouth. 100 µl of solution (i.e. gene therapy product or negative control) was administered to the nostril in small aliquots over approximately 60 seconds, using a standard laboratory pipette. After the procedure, the animal was placed in a recovery chamber and observed until fully conscious.

Slow nasal sniffing: mice were anaesthetised in an isoflurane chamber. Every two minutes, the animal was removed and placed supine on the palm of the hand as described above. 10 µl

of negative control or gene transfer agent was administered to the nose using a laboratory pipette, and the animal returned to the anaesthetic chamber. The process was repeated at two minute intervals until the total dose of 100 µl had been delivered.

Nasal perfusion: mice were anaesthetised with Ketaset and Domitor (as described in Section 2.5.1), placed supine on a heat pad, and secured in place using surgical tape. A 0.5 mm diameter catheter was placed 2.5 mm within the left nostril. Mice were then positioned in a slightly inverted position (approximately 30º) in an attempt to avoid perfusion of the lung. 100 µl total volume of negative control or gene transfer agent was perfused at a rate of 6.67 µl per minute (15 minutes total) using a syringe pump (Cole-Parmer, Vernon Hills, IL) as previously described [134]. After the procedure the anaesthetic was reserved, using Antisedan as described in Section 2.5.1.

2.5.3 Tissue harvesting

After death had been confirmed, the thorax was dissected, and blood was collected by puncturing the heart with a needle. Using this method, approximately 1 ml of blood could be collected per animal into 1.8 ml Eppendorf tubes, which were transported back to the laboratory at room/outdoor temperature. Immediately on return to the laboratory (between 30 and 120 minutes after sample collection), blood was spun at 3000 g_{av} to obtain serum, which was frozen at -80°C pending further analysis.

Broncho-alveolar lavage (BAL) was then performed. The neck was dissected and the trachea exposed; a cannula was inserted into the trachea and held in place with a piece of knotted suture thread. Subsequently, 500 µl PBS was instilled and aspirated three times. Broncho-alveolar lavage fluid (BALF) samples were immediately snap frozen in liquid nitrogen, and subsequently transported back to the laboratory and stored in the -80°C freezer.

Occasionally, it was not possible to obtain a serum and/or BALF sample from a particular animal.

After BAL was performed, the thoracic cavity was further dissected to allow removal of the lungs, which were also snap frozen in liquid nitrogen and subsequently stored at -80°C. Before analysis, the lung tissue samples were homogenized in 300 µl reporter lysis buffer (RLB, Promega, Southampton, UK) using Lysing Matrix D tubes and a Fast Prep machine (ThermoFisher Scientific, Waltham, USA) at 4.0 m/s for 40 seconds.

In some experiments, the nasal turbinate was dissected. After dissection, tissue was immediately snap frozen and stored at -80°C. The tissue was then homogenised by adding 100 µl reporter lysis buffer, cutting the tissue into small sections, and free-thawing three times. The lysate was spun at 10,000 g_{av} for 10 minutes and the supernatant analysed.

2.6 Quantification of proteins following gene transfer

Gaussia luciferase: *Gaussia* luciferase (GLux) was quantified using a GLux assay kit (New England Biolabs, UK), performed according to the manufacturer's recommendations. 15 µl of each sample was analysed in duplicate on a white 96-well plate. An automated plate reader (Appliskan, Fisher Scientific, Leicestershire, UK) was programmed to dispense 50 µl of the *Gaussia* luciferase substrate coelenterazine, diluted in assay buffer as recommended, to each well. After a 1 second shake, the plate reader read the luminosity for 10 seconds, reported as relative light units (RLU) per µl of sample volume. For lung tissue homogenates, GLux expression was corrected for protein levels using a Bio-Rad protein assay as described above.

Firefly luciferase: Firefly luciferase (FLux) was quantified using a luciferase assay kit (Promega, WI, USA), performed according to the manufacturer's instructions. Luciferase assay reagent was diluted in assay buffer, and 100 µl dispensed to each well containing 20 µl sample, analysed in duplicate. Luciferase expression was reported as RLU/mg protein.

Human α_1-antitrypsin (hAAT): in experiments described in Chapters 3 to 8 a commercially available sandwich ELISA kit from Abcam (catalogue number ab108799) was used. Plates were pre-coated with hAAT specific antibodies. hAAT standards were prepared with concentrations of 0.39, 1.56, 6.25, 25 and 100 ng/ml. 50 µl of each standard or sample was analysed in duplicate.

After an initial two-hour incubation at room temperature, the standards and samples were removed and each well washed 5 times with wash buffer (provided with the kit). 50 µl of secondary, biotinylated hAAT antibody was added, incubated for one hour, and washed as before. 50 µl streptavidin-biotin-peroxidase complex was then added, incubated for 30 minutes, and washed as before.

50 µl chromogen substrate was then added to each well, and a blue colour change was observed. After 10 minutes, and without washing the plate, 50 µl of stop solution was added to each well and a blue to yellow colour change observed. The plate was then immediately read in an Appliskan plate reader (Fisher Scientific, Leicestershire, UK) at 450 nm wavelength.

An alternative hAAT ELISA kit was used to examine sputum samples in Chapter 9. The method for this is described in the relevant chapter.

2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 6). The statistical test used in each experiment is described in the relevant Materials & Methods section and/or the figure legends. Briefly, the distribution of data was assessed using the D'Agostino-Pearson omnibus normality test; in most experiments, n numbers were too small for this test and therefore non-parametric tests used in subsequent analysis. Therefore, I have usually expressed data as median (range) rather than mean ± standard error of the mean.

For two-group comparison, a Student's t-test was performed for parametric data and a Mann-Whitney *U* test for non-parametric data. For multi-group analysis, a Kruskal-Wallis test for non-parametric data with Dunn's post-hoc multiple comparisons test was used (there are no multi-group analyses of parametric data in this thesis).

The null hypothesis was rejected at $p<0.05$.

3 Lipid-mediated gene therapy for the production of α_1-antitrypsin

3.1 Introduction

The UK CF Gene Therapy Consortium has developed vectors for gene transfer to the lung, for the treatment of cystic fibrosis. The overall aim of this thesis is to explore further applications of these technologies, for example to produce secreted α_1-antitrypsin (hAAT) for the treatment of α_1-antitrypsin deficiency and possibly other diseases.

GL67A was the non-viral vector of choice in the recent Phase IIb trial of cystic fibrosis performed by the UK Cystic Fibrosis Gene Therapy Consortium [105]; extensive pre-clinical analysis identified this as the most effective non-viral vector for gene transfer to the airway [106], justifying my selection of it in these studies. GL67A has also passed regulatory-compliant toxicology studies [108] [109], meaning there is a clear translational pathway for hAAT gene therapy using this vector. For these reasons, my research started with an assessment of non-viral gene therapy using the cationic lipid GL67A complexed to plasmids coding for human α_1-antitrypsin.

Hypothesis: I hypothesise that GL67A:pDNA complexes carrying human α_1-antitrypsin cDNA will produce secreted human α_1-antitrypsin.

Aim: I aim to demonstrate production of human α_1-antitrypsin *in vitro* and *in vivo* following administration of lipid:plasmid complexes.

Conclusion: Gene therapy with GL67A:pDNA complexes leads to significant levels of human α_1-antitrypsin *in vitro* and *in vivo*; however, levels of hAAT in murine lungs are sub-therapeutic, even following repeated vector administration.

3.2 Materials & Methods

Plasmids coding for human α_1-antitrypsin were produced as described in Section 2.1.1.

3.2.1 In vitro *experiments*

Human embryonic kidney (HEK)293 cells (ATCC) were seeded on 6-well plates (Corning, USA) at a cell density of 250,000 cells per well. Cells were cultured in 2 ml complete media (Dulbecco's Modified Eagle Medium (DMEM), 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (all regents from ThermoFisher, Waltham, USA)). Cells were incubated in a 37°C incubator at 5% CO_2.

Lipid:plasmid complexes were prepared as described in Section 2.2 and diluted in Optimem (ThermoFisher, Waltham, USA). Cells were transfected with 1 µg pDNA per well, complexed to Lipofectamine 2000 (Invitrogen, Paisley, UK) or GL67A as indicated, in 400 µl total volume Optimem (n=3 to 6 per group). Negative control wells received 400 µl Optimem alone. Six hours post-transfection, the lipid:plasmid complexes were removed and replaced with 1 ml Optimem plus 10% foetal calf serum. 24 hours post-transfection, the media was changed and replaced with 2 ml complete media.

48 hours post-transfection, the media was collected and stored at -80°C pending further analysis. Cells were lysed with reporter lysis buffer and analysed for total protein concentration as described in Section 2.4. Transgene expression was assessed in supernatant as described in Section 2.6, and corrected for total protein.

3.2.2 In vivo *experiments*

3.2.2.1 Single dose experiments

BALB/c mice were treated with plasmids carrying hAAT or GLux cDNA, complexed to GL67A in a 1:4 molar ratio. Details of the precise plasmid constructs used in each experiment accompany each figure, and the method for preparing lipid:plasmid complexes is described in General Materials & Methods (section 2.2).

Three different promoters were used in the studies in this chapter:

- pCMV (CMV immediate/early promoter/enhancer)
- pCIK (CMV immediate/early promoter/enhancer plus hybrid intron juxtaposed between promoter and transgene [153])
- hCEFI (a chimeric promoter comprised of the human elongation factor 1α promoter and the CMV immediate/early enhancer)

Each animal was given 100 µl lipid:plasmid complexes (containing approximately 80 µg pDNA) by nasal sniffing. Animals were usually culled 48 hours post-transfection, apart from in the first experiment where some animals were also culled 14 days post-transfection. Protein expression (hAAT or GLux) was determined in lung tissue homogenate and broncho-alveolar lavage fluid as described in Section 2.6.

3.2.2.2 Repeat dose experiments

In the first repeat dosing experiment, BALB/c mice were treated with either (a) 6 doses of GL67A:hCEFI-sohAAT (100 µl/dose) at four-day intervals, (b) 6 doses of pCIK-GLux (100 µl/dose), or (c) 6 doses of PBS (100 µl/dose).

In the second repeat dosing experiment, C57Bl/6 mice were treated with either (a) 6 doses of GL67A:hCEFI-sohAAT (100 µl/dose) at 10-day intervals, (b) 1 dose of GL67A:hCEFI-

sohAAT (100 µl/dose) at the same time as the animals receiving the sixth dose, or (c) 6 doses of PBS (100 µl/dose). Animals were harvested 6 days after the final dose, and hAAT expression determined in lung tissue homogenate and broncho-alveolar lavage fluid as described in General Materials & Methods (section 2.6).

3.2.2.3 Detection of mRNA

In one experiment, vector-specific mRNA was measured. At the time of harvest, the right lung was homogenised as previously described (Section 2.5.3); the left lung was placed in 5 ml RNAlater (Sigma, UK) and stored at 4°C for three days. The tissue was then removed and homogenised in 600 µl RLT-Plus/β-mercaptoethanol solution (10 µl 14.3M β-mercaptoethanol (Sigma, UK) per 1ml RLT-Plus (Qiagen, Crawley, UK)) in Lysing Matrix D tubes using a FastPrep homogeniser (ThermoFisher Scientific, Waltham, USA) at 6.0 m/s for 60 seconds. Homogenate was incubated at room temperature for 15 minutes and then transferred to QIAshredder columns (Qiagen, Crawley, UK) and centrifuged at at full speed for 2 minutes. Columns were removed and the homogenate centrifuged for a further 5 minutes, after which the supernatant was collected. Total RNA was extracted using a QIAgen AllPrep DNA/RNA extraction kit, performed according to the manufacturer's recommendations with on-column DNase treatment (Qiagen) and in-solution DNase (Ambion DNA*free*).

Reverse transcription PCR (qRT-PCR) was performed be Jean-François Gelinas at the Consortium's Core Facility, based on a previously reported method [153]. Absolute levels of mRNA were quantified by two-step real-time quantitative TaqMan RT-PCR. Primers and probes for vector-specific and endogenous mRNA are were as follows:

Vector (primers directed to intron in the 5' untranslated region common to both plasmids used):

- Forward: TGAGGCACTGGGCAGGTGT

- Reverse: GTCGTATTAAGTACTCTAGCCTTAAGA
- Probe: CCACTCCCAGTTCAATTACAGC

Endogenous (primers directed to murine cystic fibrosis transmembrane conductance regulator (mCftr)):

- Forward: AGCCAGCTTTATCTCCAAACTCTTC
- Reverse: GCTGTCTGTACCCTTTCCTCAAA
- Probe: TCAGCTGGAGCACAGC

'No template' and 'no reverse transcriptase' controls were included. An RNA standard curve was prepared, and used to calculate the number of copies of plasmid-derived and endogenous mRNA. The amount of plasmid-derived mRNA is expressed as percentage of vector specific mRNA/endogenous mCftr mRNA. Samples that were positive for mRNA but below the concentration of the lowest standard are reported as 'positive but not quantifiable' (PBNQ).

3.2.2.4 Urea assay

To control for the dilution of airway surface liquid when performing broncho-alveolar lavage, a commercially available urea assay kit was purchased from Abcam (Cambridge, UK) and performed according to the manufacturer's instructions. The principle of the kit is that urea (in the test samples/standards) acts as a substrate for specific enzymes supplied in the kit. The resulting enzymatic product reacts with an OxiRed probe to generate colour at 570 nm.

50 µl of murine serum or broncho-alveolar lavage fluid was analysed on a 96-plate plate and compared to a standard curve ranging from 0 to 5 nM/well urea. A 'reaction mix' was added to each sample or standard well, consisting of OxiRed probe, enzyme mix, developer and converter enzyme diluted in equal volumes in assay buffer according to the manufacturer's recommendations. "Sample control wells" received no converter enzyme; due to the lack of converter enzyme in these wells, the final colour-generation step with OxiRed cannot occur, and they therefore provide a baseline reading of optical density at 570 nm for each sample.

All samples and standards were analysed in duplicate. The plate was incubated for 60 minutes at 37°C, after which the optical density at 570 nm was measured in a plate reader (Appliskan, Fisher Scientific, Leicestershire, UK).

To analyse the data, the mean optical density reading for the blank (0 nM/well urea) was subtracted from all readings. The mean optical density for each urea standard was plotted against the amount of urea in that well. The mean optical density readings for sample control wells were subtracted from sample readings to remove the effect of interfering agents on the optical density readings; corrected sample readings were then read off the standard curve, and the concentration of urea calculated by dividing the amount of urea (nM) by the volume of sample (µl) and corrected for the dilution factor. The urea assay experiments were designed by me and performed with Caroline Moran, an undergraduate student who was on a summer placement in our laboratory, under my supervision.

3.3 Results

3.3.1 Lipid:plasmid complexes carrying hAAT cDNA produce secreted human α_1-antitrypsin in vitro

To assess the functionality of human α_1-antitrypsin (hAAT) plasmids before performing *in vivo* experiments, an *in vitro* cell transfection experiment was performed.

In the first experiment, two plasmids carrying the hAAT transgene were assessed: one under the control of the pCIK promoter (consisting of the cytomegalovirus (CMV) promoter and an intron to drive higher levels of gene expression [154] [155]), and the other under the control of the hCEFI promoter. hCEFI is a chimeric promoter developed by the UK CF Gene Therapy Consortium, consisting of the human CMV immediate/early enhancer and the elongation factor 1α promoter [107]. This promoter/enhancer been shown to lead to sustained, long-lasting levels of gene expression *in vitro* and *in vivo*, and in addition is free of CpG dinucleotides, which are pro-inflammatory [107]. Some cells were given an irrelevant plasmid (pCIK-GLux) coding for Gaussia luciferase to assess whether transfection with lipid:plasmid complexes stimulates production of endogenous hAAT in HEK293 cells.

It has previously been demonstrated that gene transfer with lipid:plasmid complexes is log orders more efficient than naked plasmids alone [156]. In this experiment, I assessed two different lipid vectors, Lipofectamine 2000 and GL67A. Lipofectamine 2000 is a commonly used lipid vector which has been optimised for *in vitro* gene transfer. Therefore, using it in this experiment gave me the best possible chance of seeing protein expression. However, the UK CF Gene Therapy Consortium has previously determined that the lipid formulation GL67A [157] is the most efficient lipid for pulmonary gene transfer and was therefore also assessed in

the first experiment. For GL67A, lipid:plasmid complexes were prepared at two different molar ratios:

- *1:4 lipid:plasmid molar ratio:* A 1:4 molar ratio has been shown to be better tolerated when delivered by nasal perfusion to mice [133]
- *6:8 lipid:plasmid molar ratio:* this molar ratio is more efficient in aerosolisation experiments, which will ultimately be used in clinical trials; therefore, it is useful to assess the efficiency of gene transfer at a 6:8 molar ratio here.

In these *in vitro* experiments, human embryonic kidney (HEK)293 cells were transfected as they are a commonly used, easy-to-transfect cell line. Cells were transfected on day 1 and the production of human α_1-antitrypsin assessed in cell culture supernatant 48 hours later using a commercially available ELISA, selected on the basis of its sensitivity in a wide range of tissue samples (Abcam, personal communication).

The results of the *in vitro* transfection experiment are shown on Figure 3-1. In all cells receiving lipid:plasmid complexes carrying hAAT cDNA, there were higher levels of hAAT in supernatant than in untransfected controls; this reached statistical significance following multi-group analysis in all but one group of cells (receiving GL67A:hCEFI-hAAT at a 1:4 molar ratio). The highest level of expression was seen in cells given LF2000:hCEFI-hAAT:

- *LF2000:hCEFI-hAAT:* median 540 (range 224 – 940) ng hAAT per mg protein;
- *Untreated:* median 0.78 (range 0.78 – 5.5 ng hAAT per mg protein; $p<0.0001$)

In cells treated with GL67A:hCEFI-hAAT at a 1:4 molar ratio, there was a trend towards expression of hAAT (median 208 ng hAAT per mg protein) which did not reach statistical significance. It is likely that the lack of statistical significance is due to the adjustment of the p value to account for multiple group comparisons, and I do not feel that this result should prevent future experiments using this vector.

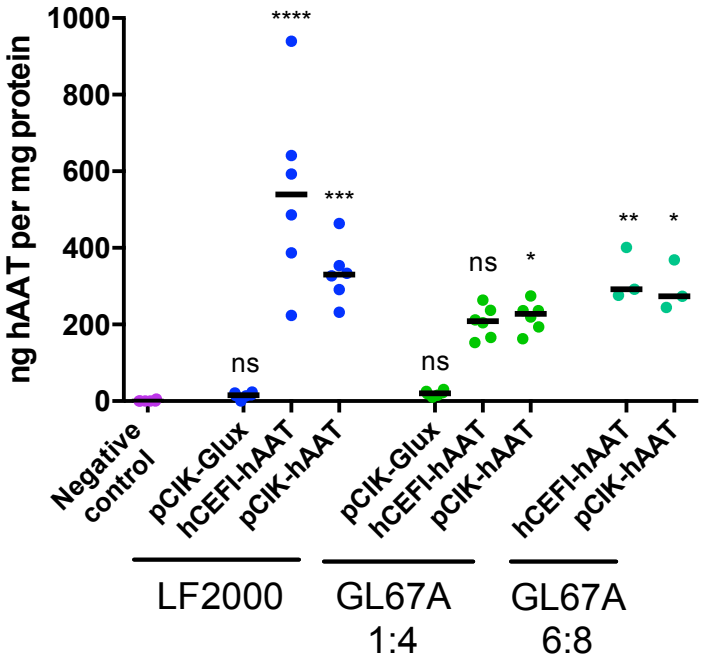


Figure 3-1 Production of human α_1-antitrypsin following *in vitro* transfection of HEK293 cells

HEK293 cells were transfected with lipid:plasmid complexes diluted in Optimem. Lipid vectors used were Lipofectamine 2000 (LF2000) or GL67A. For GL67A, 1:4 and 6:8 lipid:plasmid molar ratios were used as indicated. Negative control cells received Optimem alone. 48 hours post-transfection, the concentration of human α_1-antitrypsin (hAAT) in supernatant was assessed by ELISA and normalised to the total protein per well from cell lysates.

Each data point represents one well of cells on a six-well plate. Horizontal bars represent group medians.

Statistics shown are compared to negative control. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant (Kruskal-Wallis test with Dunn's post-hoc analysis).

In cells receiving an irrelevant plasmid (pCIK-GLux), there was no statistically significant expression of hAAT compared to untransfected controls. However, there was a trend towards increased hAAT production:

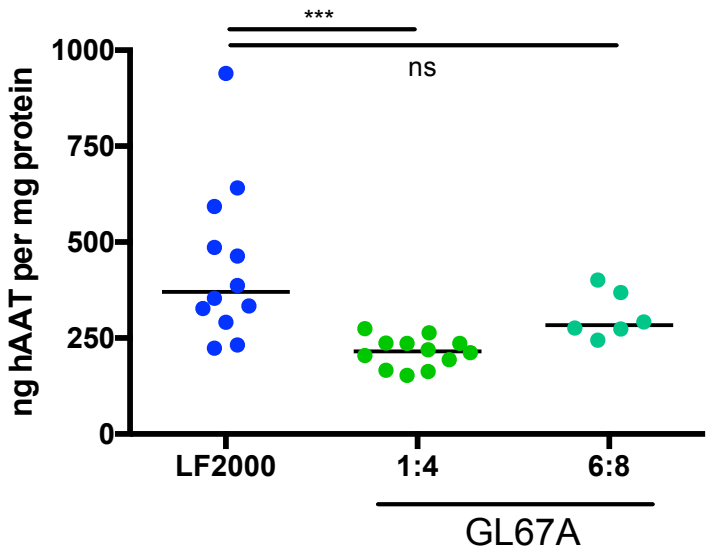
- *LF2000:pCIK-GLux:* median 15.5 (range 0.78-22.3) ng hAAT per mg protein;
- *GL67A:pCIK-GLux:* median 21.0 (range 10.6-30.1) ng hAAT per mg protein;
- *Untreated:* median 0.78 (range 0.78 – 5.5 ng hAAT per mg protein.

Based on this data, it is not possible to conclude whether or not transfection of cells with lipid stimulates production of human α_1-antitrypsin, so this question was investigated further in subsequent experiments, described throughout this thesis.

I then performed further analysis to determine if the promoter or the vector had any effect on gene expression in this pilot experiment. Cells treated with hAAT plasmids complexed to Lipofectamine 2000 showed significantly higher ($p<0.001$) hAAT expression than cells treated with plasmids complexed to GL67A in a 1:4 molar ratio, although there was no significant difference between Lipofectamine 2000 and GL67A in a 6:8 molar ratio (Figure 3-2A).

There was no statistically significant difference between cells given hAAT plasmids driven by the hCEFI promoter compared to the pCIK promoter (Figure 3-2B).

A.



B.

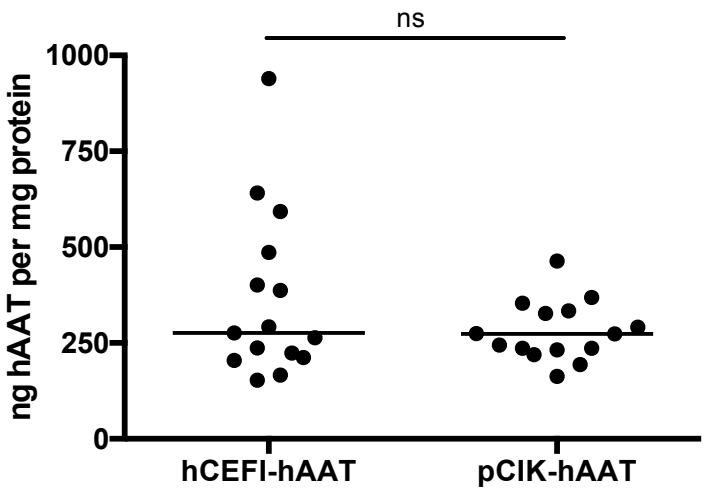


Figure 3-2 Production of human α_1-antitrypsin following *in vitro* transfection of HEK293 cells

Here, data from Figure 3-1 is grouped to assess the effect of the vector or promoter on gene expression.

(A) For cells given plasmids carrying human α_1-antitrypsin (hAAT) cDNA, results are grouped according to the vector to which the plasmids were complexed: Lipofectamine 2000 (LF2000) or GL67A (in 1:4 or 6:8 lipid:plasmid molar ratios as shown). Expression of human α_1-antitrypsin (hAAT) was determined by ELISA. ***=$p<0.001$, ns=not significant (Kruskal-Wallis test with Dunn's post-hoc analysis).

(B) For cells given plasmids carrying hAAT cDNA, results are grouped according to the promoter used (hCEFI or pCIK, described in the text). ns=not significant (Mann-Whitney *U* test).

Each data point represents one well of cells on a six-well plate. Horizontal bars represent group medians.

The results of this experiment confirm that the hAAT vector plasmids produced by the UK CF Gene Therapy Consortium are functional, and produce a secreted human α_1-antitrypsin that is detected by ELISA when given to HEK293 cells *in vitro*. This supports the progression of studies with these plasmids into *in vivo* models. There was more human α_1-antitrypsin production when hAAT plasmids were complexed to LF2000, compared to plasmids complexed to GL67A. However, there was still significant hAAT expression in most cells given GL67A:pDNA complexes, and as it has previously been determined that this vector is the most suitable non-viral vector for gene transfer to the lung, GL67A alone was carried forward to future experiments.

3.3.2 Codon-optimised plasmids produce secreted human α_1-antitrypsin in vitro

In the second *in vitro* experiment, I assessed two further hAAT plasmids carrying a codon optimised, CpG-depleted hAAT transgene to ensure these plasmids were also functional. Previous work from the Consortium has shown that CpG dinucleotides are pro-inflammatory *in vivo*; a plasmid containing even a single CpG dinucleotide resulted in a significantly higher inflammatory response than a plasmid with zero CpG dinucleotides [107]. In addition, it is known that optimising the sequence of each DNA triplet code can lead to higher levels of protein expression in mammalian cells [158]. Therefore, the Consortium produced a CpG-depleted, codon optimised hAAT cDNA, termed sohAAT, which was sub-cloned into the two plasmids used in previous experiments. Of the resulting plasmids, hCEFI-sohAAT is entirely CpG-depleted; in pCIK-sohAAT, the transgene contains no CpG residues but CpG islands are present in the plasmid backbone. Importantly, recent Consortium research has shown that CpG-depletion of the transgene, but not necessarily the backbone, is critical for sustained and high level gene expression [159].

Before performing *in vivo* experiments, I wished to assess the functionality of these plasmids *in vitro*. Whilst I would not expect to see greater levels of gene expression from these plasmids compared to non-CpG-depleted plasmids *in vitro*, where there is no immune response (i.e. no advantage of CpG-depletion), it is still important to assess whether or not CpG depletion and codon optimisation have affected hAAT production before progressing to large scale *in vivo* experiments. Because this pilot experiment was intended to lead to *in vivo* experiments, I used the vector of choice for *in vivo* lung transfer, GL67A, in a 1:4 lipid:plasmid molar ratio.

The results of the experiment are shown in Figure 3-3. In cells treated with hCEFI-hAAT and hCEFI-sohAAT, there was significantly ($p<0.05$) more hAAT expression compared to negative controls and cells transfected with an irrelevant plasmid (pCIK-GLux). In cells treated with pCIK-hAAT and pCIK-sohAAT there was a trend towards expression of hAAT, although this did not reach statistical significance. There was no significant difference in protein expression between cells treated with plasmids carrying CpG-depleted and non-CpG-depleted transgenes (Figure 3-3B).

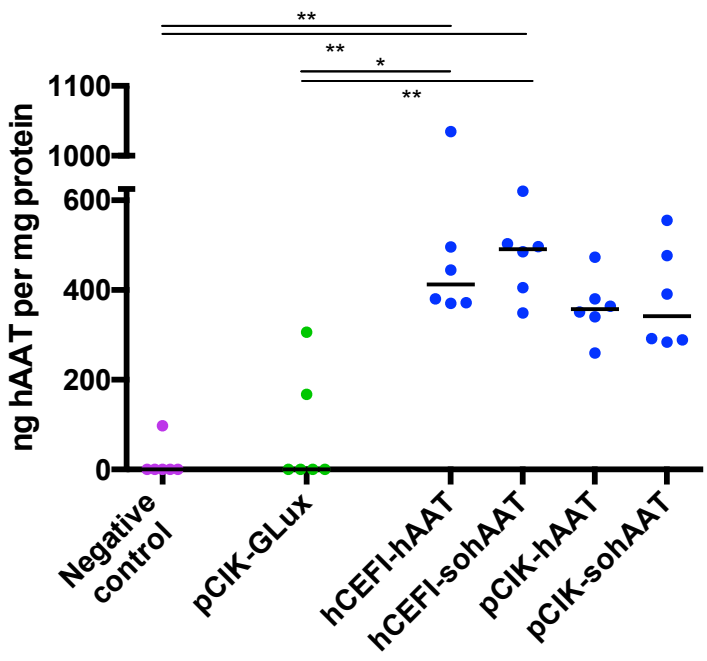
Consistent with the previous results, there was no significant expression of human α_1-antitrypsin in cells transfected with an irrelevant plasmid (pCIK-GLux):

- *GL67A:pCIK-GLux:* median 0.78 (range 0.78-306) ng hAAT per mg protein
- *Untreated controls:* median 0.78 (range 0.78-97) ng hAAT per mg protein

This suggests that, in HEK293 cells, there is no up-regulation of human α_1-antitrypsin in response to challenge with the lipid vector GL67A or plasmid DNA.

The results of this experiment show that all four vector plasmids coding for human α_1-antitrypsin produce the protein *in vitro* when complexed to a non-viral vector, supporting the progression to *in vivo* studies.

A.



B.

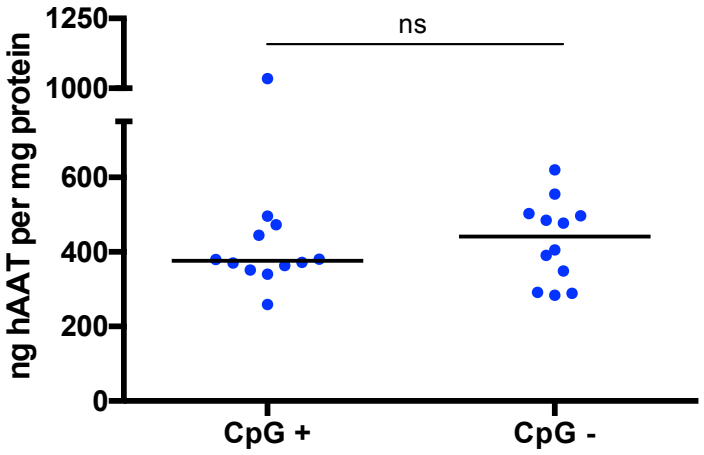


Figure 3-3 Production of human α_1-antitrypsin following treatment with CpG-depleted plasmids

A. HEK293 cells were transfected with plasmids carrying human α_1-antitrypsin (hAAT) cDNA, complexed to GL67A in a 1:4 molar ratio. 48 hours post-transfection, human α_1-antitrypsin (hAAT) in cell culture supernatant was determined by ELISA and normalised to the total protein content in each well.
Each data point represents one well of cells; horizontal bars represent group medians. *=$p<0.05$, **=$p<0.01$ between the groups shown (Kruskal-Wallis test with Dunn's post-hoc analysis). All other multi-group comparisons were not significant.

B. Here, data from panel A is combined according to the CpG status of the transgene. There was no significant difference between plasmids containing CpG-depleted and non-CpG-depleted transgenes.
Each data point represents one well of cells; horizontal bars represent group medians. ns=not significant.

3.3.3 A urea assay to estimate the dilution of murine epithelial lining fluid when performing broncho-alveolar lavage in mice

To aid interpretation of the data from *in vivo* gene transfer experiments, it is first necessary to describe experiments that were performed to calculate the degree of dilution of epithelial lining fluid when performing broncho-alveolar lavage. My studies concentrate on the production of secreted protein following gene transfer to the airway epithelium, so measuring the amount of secreted protein in epithelial lining fluid (ELF) is crucial. However, to obtain ELF samples, it is necessary to perform broncho-alveolar lavage (BAL); a process which significantly dilutes ELF. When broncho-alveolar lavage fluid (BALF) samples are analysed, e.g. for *Gaussia* luciferase or human α_1-antitryspin expression, the readings obtained will therefore be much lower than the true concentration in ELF.

Whilst it may sometimes be acceptable or even desirable to report the concentration of a protein in BALF, ELF is generally speaking more biologically relevant as it reflects the true therapeutically relevant, local concentration. It is also more common in the literature to report protein levels in ELF rather than BALF, as doing so corrects for laboratory-to-laboratory variation in the lavage technique (e.g. if a different volume of PBS is used to perform the lavage wash). To aid comparisons of my data with different studies, I therefore wanted to determine to what degree ELF samples were diluted when performing BAL in mice in my hands.

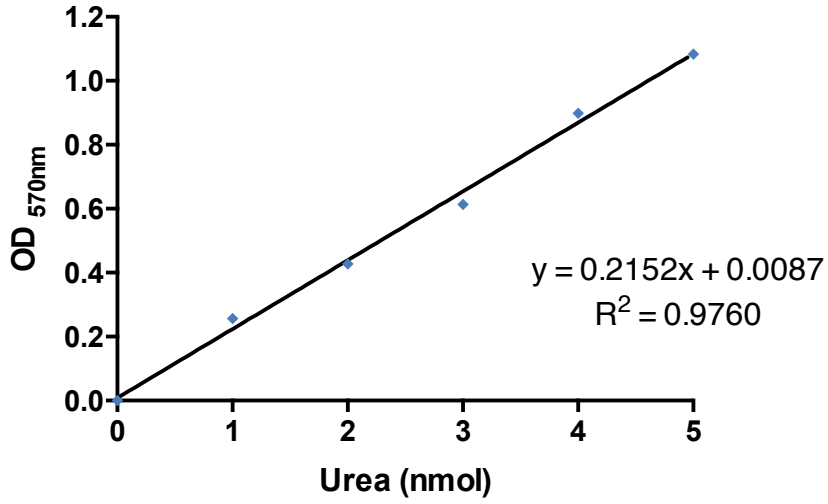
The most commonly accepted method for determining the dilution factor of lavage is to perform a urea assay [160]. Urea is a small molecule which readily diffuses throughout the body's tissues, and is therefore assumed to be at the same concentration in serum, lung tissue and epithelial lining fluid [161]. By measuring the concentration of urea in serum and BAL

samples from one animal, it is possible to calculate the degree to which the ELF has been diluted; the protein concentration measured in BALF can then be multiplied by this dilution factor to determine the protein concentration in ELF.

A number of commercially available urea assay kits were identified; a kit from Abcam was chosen as the most suitable, as several publications had used the kit to measure urea in biological samples [162] [163]. To validate the assay, we first measured a standard curve and compared it to the manufacturer's published data. In our hands, the mean optical densities and equation of the line of best fit were similar to the manufacturer's published data (Figure 3-4).

Murine serum and broncho-alveolar lavage fluid (BALF) samples were then serially diluted and analysed on the assay kit. At a dilution of 1:125, the reported urea concentration in serum fell within the range of the assay kit; BALF samples required 1:5 dilution to fall within the range of the assay kit (Figure 3-5). Therefore, serum samples were diluted 1:125 and BALF samples diluted 1:5 in future urea assays.

A



B

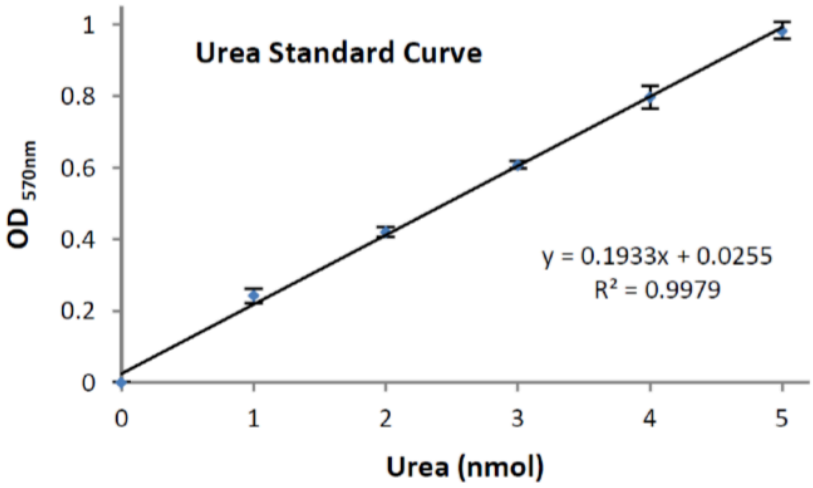
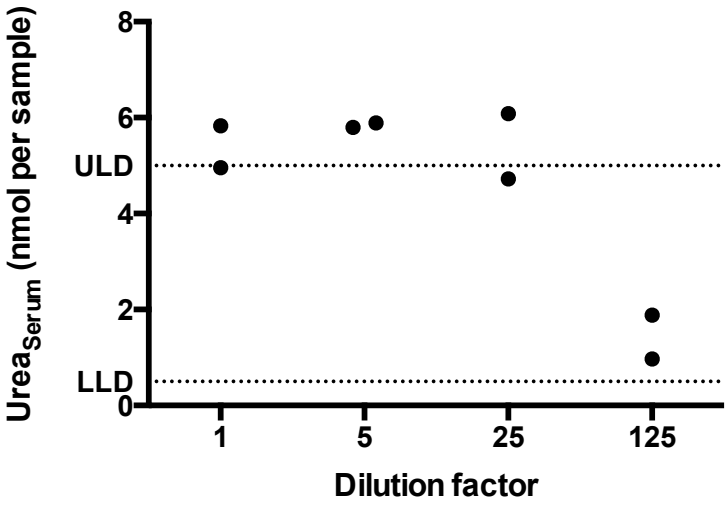


Figure 3-4 Validation of the Abcam urea assay kit

(A) A urea assay kit from Abcam (catalogue number ab83362) was performed according to the manufacturer's instructions. Urea standards (1, 2, 3, 4, 5 nM/well) and a blank were analysed in duplicate. The mean optical density of the blank was subtracted from all readings, and mean optical densities (OD) of each standard plotted against the amount of urea in nM. Each data point represents the mean optical density of n=2 standards. The equation of the line of best fit and the goodness of fit (R^2) are shown.

(B) Urea assay standard curve reproduced from the manufacturer's data sheet. We had similar optical density readings for each standard, and a similar equation for the line of best fit.

A



B

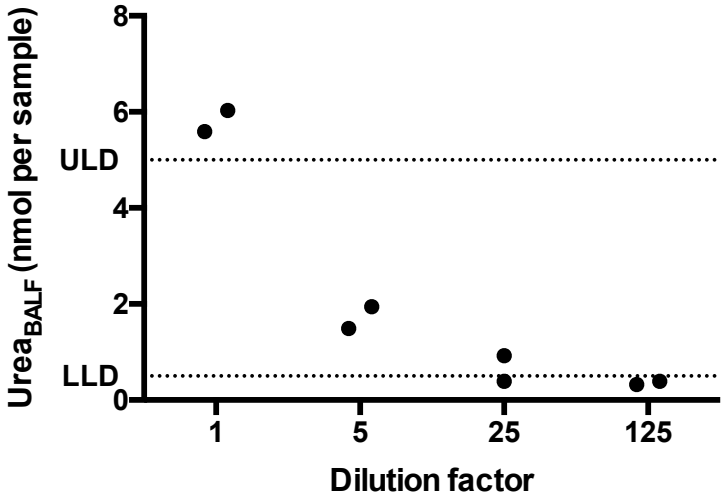


Figure 3-5 Serial dilution of murine serum and broncho-alveolar lavage fluid samples

Serum **(A)** and broncho-alveolar lavage fluid (BALF) **(B)** samples from treated mice were diluted 1:1, 1:5, 1:25 and 1:125 in assay buffer. The amount of urea in each sample is reported as nM per sample. The lower limit of detection (LLD) of the assay is 0.5 nM; the upper limit of detection (ULD) is 5 nM.

Serum samples required 1:125 dilution to fall within the range of sensitivity of the assay kit; BALF samples required 1:5 dilution to fall within the range of sensitivity.

Each data point represents one sample from one animal, analysed in duplicate. Dotted lines represent the upper and lower limit of detection of the assay kit.

3.3.4 Performing broncho-alveolar lavage in mice dilutes epithelial lining fluid approximately 42-fold

Having validated the assay, we then analysed urea concentrations in 14 serum and BALF samples. The concentration of urea in nM/µl is obtained by dividing the amount of urea (nM/well) detected in the assay by the volume of sample per well (µl/well). The concentration was then converted to nM/l, the more commonly used unit of urea concentration in biological samples in the literature.

The mean concentration of urea in serum was 17.4 mM/l, and the mean concentration of urea in broncho-alveolar lavage fluid was 0.458 mM/l. The dilution factor of the lavage procedure was then calculated for each animal. The mean dilution factor (calculated by dividing the serum urea concentration by the BALF serum concentration) was 41.7, and the median dilution factor was 32; other values including the standard error of the mean and standard deviation are given on Table 3-1. This data suggests that in my hands, murine epithelial lining fluid is on average diluted approximately 42-fold when performing broncho-alveolar lavage. This value will be used in this thesis as a conversion factor to obtain protein concentration in epithelial lining fluid following analysis of broncho-alveolar lavage fluid.

Table 3-1 Mean concentrations of urea in murine serum and broncho-alveolar lavage fluid

	Serum (mM/l)	BALF (mM/l)	Dilution factor
N =	14	14	14
Mean	**17.4**	**0.46**	**41.7**
Standard error of the mean	0.97	0.034	18.9
Standard deviation	3.63	0.13	5.16
Median	17.0	0.45	32.0
Range (upper)	10.5	0.28	24.0
Range (lower)	25.0	0.66	88.0

A urea assay was performed on 14 murine serum and broncho-alveolar lavage fluid (BALF) samples, analysed in duplicate. Here, the mean values of urea concentration are reported along with the standard error of the mean, the standard deviation, and the range (upper and lower).

The dilution factor of broncho-alveolar lavage is calculated by dividing the urea concentration in serum by the urea concentration in BALF. This was performed for each matched pair (i.e. serum and BALF from the same animal), and the mean dilution factor of 14 samples is reported.

3.3.5 Transfection with lipid:plasmid complexes leads to significant reporter gene expression in the murine lung

In the first murine gene expression experiment, mice were treated with either (1) a plasmid coding for the secreted reporter gene *Gaussia* luciferase, (2) a plasmid coding for the therapeutic protein human α_1-antitrypsin, or (3) PBS as a negative control.

Gaussia luciferase (GLux) has previously been shown to be a stable, sensitive reporter gene [164]. The plasmid chosen was pCMV-GLux, as it has previously been shown to lead to significant levels of gene transfer in mice [164]. The plasmid chosen for hAAT mice was pCIK-hAAT, which is very similar to pCMV-GLux (pCIK contains a hybrid intron between the CMV promoter and transgene [153]).

BALB/c mice were given 100 µl lipid:plasmid complexes at a 1:4 molar ratio, containing approximately 80 µg pDNA per dose, to the airway by nasal sniffing. The other experimental variables (the molar ratio and volume) were chosen based on extensive previous work performed by the Consortium [165]. Nasal sniffing was chosen as the delivery method because (1) it uses significantly less expensive pDNA and lipid than aerosolisation experiments and (2) the most practical method for aerosolisation in mice is using a whole body nebuliser, which leads to inefficient gene transfer; aerosolisation remains an option to further validate pre-clinical experiments before translation into the clinic in future.

Treated animals were harvested 48 hours and 14 days post-transduction, and levels of *Gaussia* luciferase and human α_1-antitrypsin determined in lung tissue homogenate and broncho-alveolar lavage fluid. At 48 hours post-treatment, expression of *Gaussia* luciferase was significantly higher in treated animals than controls (Figure 3-6). However, by day 14 reporter gene expression was no longer significant and was close to control levels, which is consistent to previous observations of gene expression from the CMV promoter [107]:

- **Lung** (n=4 per group)

  Treated (day 2): 25,371 (12,893-80,537) RLU/mg protein, $p<0.01$ compared to untreated

  Treated (day 14): 207 (92.6-409) RLU/mg protein, not significant compared to untreated

  Untreated: 12.4 (7.8-35.4) RLU/mg protein

- **BALF** (n=4 per group):

  Treated (day 2): 4815 (2499-24,223) RLU/µl, $p<0.05$ compared to untreated

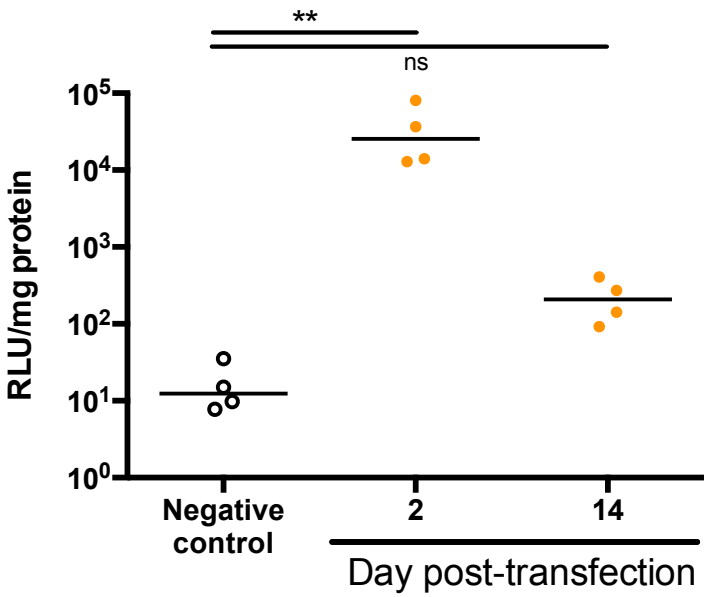
  Treated (day 14): 152 (148-171) RLU/µl, not significant compared to untreated

  Untreated: 147 (100-168) RLU/µl untreated

  Data expressed as median (range)

This data is similar to data previously published by our group, showing *Gaussia* luciferase production in murine lungs following non-viral gene therapy [164]. This is useful validation of my gene delivery technique, justifying progression to experiments delivering human α_1-antitrypsin cDNA.

A



B

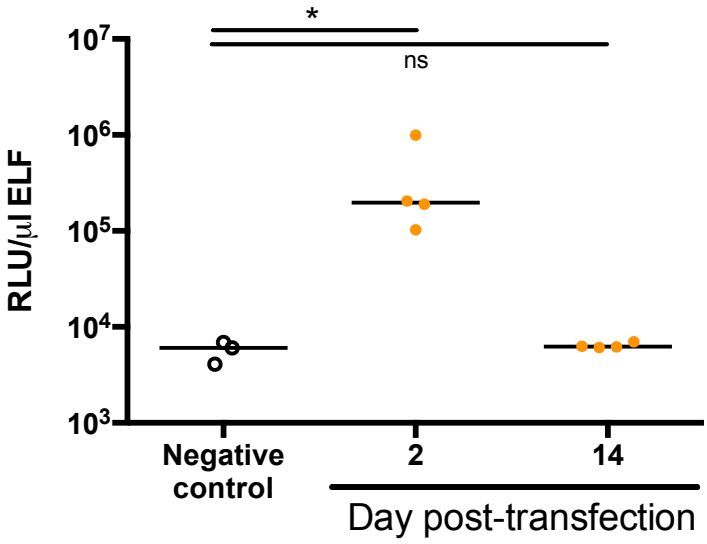


Figure 3-6 Expression of *Gaussia* luciferase in mice transfected with a single dose of GL67A:pCMV-GLux

Mice were treated with 100 µl GL67A:pCMV-GLux (1:4 molar ratio). At the indicated time points post-transfection, *Gaussia* luciferase (GLux) expression was compared in **(A)** lung tissue homogenate and **(B)** epithelial lining fluid (ELF). Negative control animals were given 100 µl PBS and harvested on day 2.

Each data point represents one animal. Horizontal bars represent group medians.

RLU = relative light units. *=$p<0.05$, **=$p<0.01$, ns=not significant (Kruskal-Wallis test with Dunn's post-hoc analysis).

3.3.6 A single dose of GL67A:pCIK-hAAT leads to minimal hAAT expression in mice

I then analysed expression of human α_1-antitrypsin in lung tissue homogenate and epithelial lining fluid. At 48 hours post-transfection, expression of human α_1-antitrypsin in lung tissue homogenate was significantly higher than controls (Figure 3-7A):

- *pCIK-hAAT at day 2:* 0.23 (range 0.18 – 0.31) ng hAAT per mg protein
- *Untreated:* 0.061 (range 0.05 – 0.08) ng hAAT per mg protein, $p<0.05$

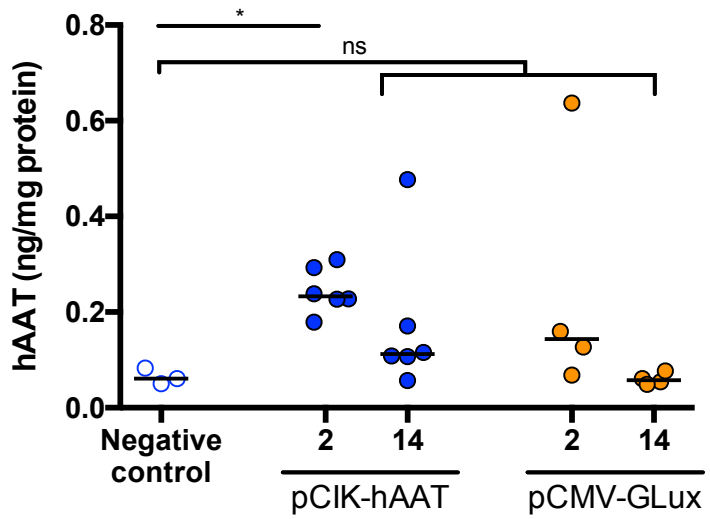
By day 14, levels of α_1-antitrypsin were not significantly higher than untreated animals. In animals treated with pCMV-GLux, human α_1-antitrypsin expression was not significantly higher than in untreated controls.

In epithelial lining fluid (ELF), there was a trend towards increased expression of human α_1-antitrypsin in animals treated with pCIK-hAAT compared to controls; however, this did not reach statistical significance (Figure 3-7B):

- *pCIK-hAAT at day 2:* median 210 (range 0 – 273) ng/ml hAAT
- *Untreated:* median 75 (range 66 – 143) ng/ml hAAT, not significant

I also wanted to determine if non-viral gene therapy with GL67A in mice leads to the up-regulation of any proteins that are detected by the human α_1-antitrypsin ELISA kit. Although there was no statistically significant production of human α_1-antitrypsin in animals treated with pCMV-GLux compared to untreated controls, I couldn't conclusively rule this out due to the small group numbers. However, I also felt that it was difficult to justify the continued use of untreated animals in experiments due to ethical considerations and in order to be compliant with the 3R's [166]. Therefore, untreated animals were omitted from some of the future experiments, but are included in the most important experiments.

A



B

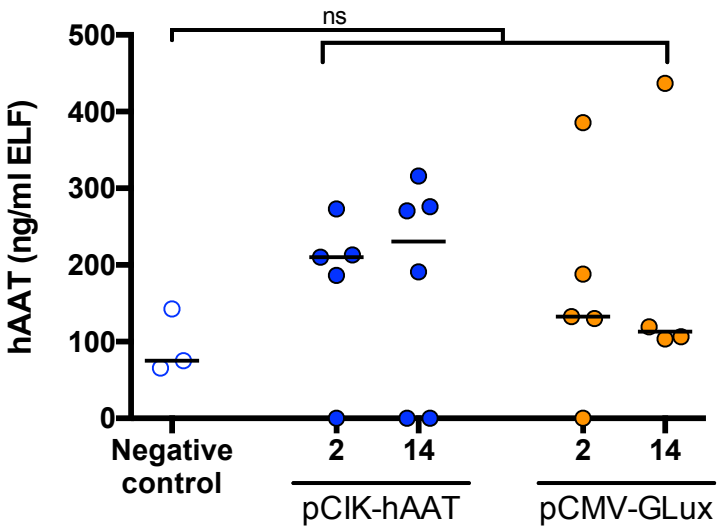


Figure 3-7 A single dose of GL67A:pCIK-hAAT leads to low-level human α_1-antitrypsin expression in murine lung

Mice were treated with either (1) 100 µl GL67A:pCIK-hAAT, (2) 100 µl GL67A:pCMV-GLux or (3) 100 µl PBS (negative control). At the indicated time points post-transfection, human α_1-antitrypsin (hAAT) expression was compared in **(A)** lung tissue homogenate and **(B)** epithelial lining fluid (ELF).

Each data point represents one animal. Horizontal bars represent group medians.

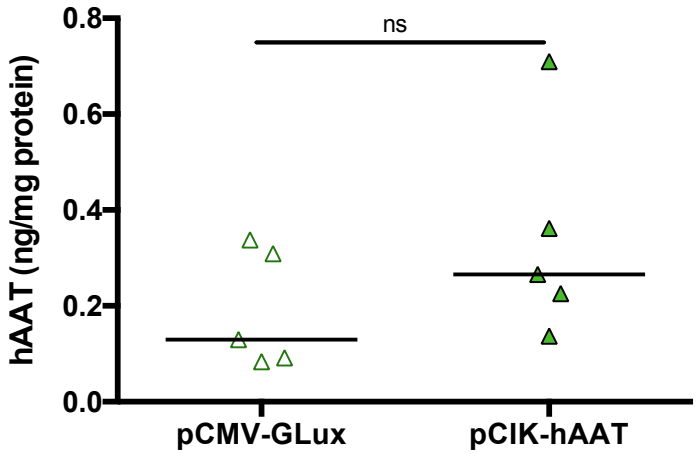
*=$p<0.01$ (pCIK-hAAT group harvested at day 2 compared to negative control), ns=not significant (indicated groups compared to negative control; Kruskal-Wallis test with Dunn's post-hoc analysis).

Because the number of animals used in the previous single dose experiment was relatively small, it was decided to extend the experiment with a further 5 mice in each treated group (pCIK-hAAT and pCMV-GLux). As protein expression was essentially undetectable 14 days post-transfection, it was decided to harvest all animals 48 hours post-transfection; all other experimental variables were kept the same, although no untreated animals were included for the reasons stated above.

hAAT expression in these 10 mice is shown in Figure 3-8. In both lung tissue homogenate and epithelial lining fluid, there was no significant hAAT expression in animals treated with pCIK-hAAT compared to animals treated with pCMV-GLux. There was also no statistically significant difference when data from both single dose experiments was combined (Figure 3-9).

These data show that expression of human α_1-antitrypsin in mice following a single dose of GL67A:pCIK-hAAT is sub-therapeutic (discussed in section 3.4), and it is likely that improvements in plasmid design or the dosing schedule will be necessary to drive higher levels of gene expression.

A



B

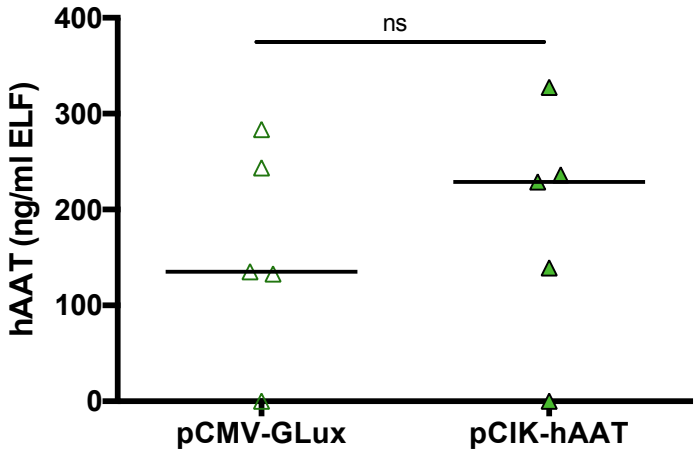


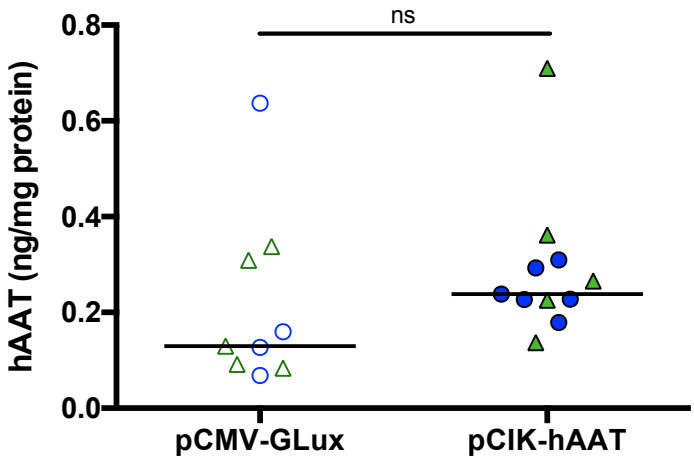
Figure 3-8 A single dose of GL67A:pCIK-hAAT is not sufficient for significant levels of human α_1-antitrypsin in murine lung

To confirm the results of the previous experiment, further mice were treated with (1) 100 µl GL67A:pCIK-hAAT or (2) 100 µl GL67A:pCMV-GLux. 48 hours post-transfection, human α_1-antitrypsin (hAAT) expression was compared in **(A)** lung tissue homogenate and **(B)** epithelial lining fluid (ELF).

Each data point represents one animal. Horizontal bars represent group medians.

ns=not significant (Mann Whitney *U* test).

A



B

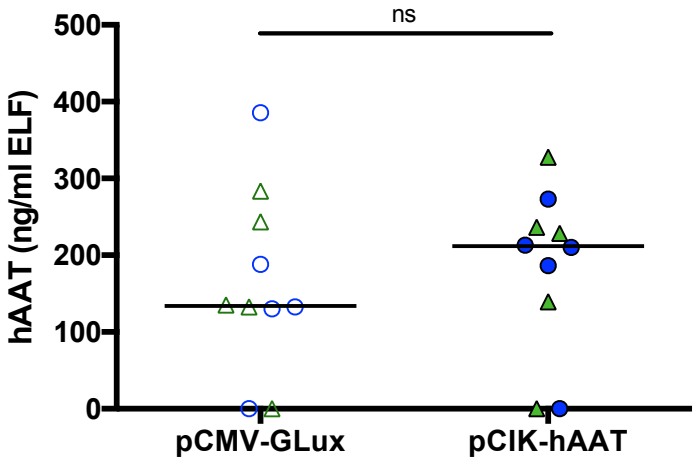


Figure 3-9 Combined analysis of two single dose experiments with pCIK-hAAT confirm that there is no significant expression of human α_1-antitrypsin

Data from the two single-dose experiments with pCIK-hAAT, reported in Figure 3-7 and Figure 3-8, were combined. There was no statistically significant expression of human α_1-antitrypsin in **(A)** lung tissue homogenate or **(B)** epithelial lining fluid (ELF).

Each data point represents one animal. Horizontal bars represent group medians. Triangles represent animals in the first single-dose experiment (Figure 3-7), circles represent animals in the second single-dose experiment (Figure 3-8).

ns=not significant (Mann Whitney *U* test).

3.3.7 A single dose of CpG-depleted plasmid does not lead to significant levels of human α_1-antitrypsin in murine lung

It is possible that improvements in plasmid design could lead to improved levels of α_1-antitrypsin. The UK CF Gene Therapy Consortium has developed CpG-depleted plasmids; previous research has shown that completely removing CpG dinucleotides from plasmids reduced the inflammatory response after treatment and led to sustained transgene expression in lung [107]. The Consortium has also developed codon-optimised plasmids, in which the DNA sequence is modified to improve protein expression in mammalian cells. Codon optimised plasmids are distinguished by the code 'so' added before the transgene to differentiate them from non-codon optimised plasmids (e.g. the codon optimised plasmid used in this experiment is described as pCIK-sohAAT). I hypothesised that CpG-depleted, codon optimised plasmids could lead to improved levels of α_1-antitrypsin following gene therapy.

The data presented so far in this chapter has shown that, after treatment with GL67A:pCMV-GLux, mice produce high levels of the secreted reporter gene *Gaussia* luciferase (approximately 3 log orders than negative controls in lung tissue homogenate, Figure 3-6). However, mice treated at the same time with a similar plasmid coding for hAAT (GL67A:pCIK-hAAT) showed minimal gene expression. To rule out a problem with the hAAT plasmid, I decided in the next experiment that I would also analyse mRNA levels post gene therapy.

Mice (n=6 per group) were treated with 100 µl of pCIK-sohAAT or pCIK-FLux by nasal sniffing. A plasmid coding for FLux (Firefly luciferase) was chosen instead of *Gaussia* luciferase because a pCIK-GLux plasmid was not available at the time of the experiment, and it was

necessary that both groups were treated with plasmids carrying identical promoters if mRNA levels post-treatment were to be analysed.

Expression of Firefly luciferase in lung tissue homogenate was approximately four log orders higher in animals treated with pCIK-FLux compared to pCIK-sohAAT ($p<0.01$, Figure 3-10). Please note that as Firefly luciferase is not secreted, I did not analyse FLux expression in epithelial lining fluid. For human α_1-antitrypsin, expression in lung tissue homogenate was again not significantly higher than controls (Figure 3-11), suggesting that a repeat dosing strategy will be necessary to drive higher levels of gene expression.

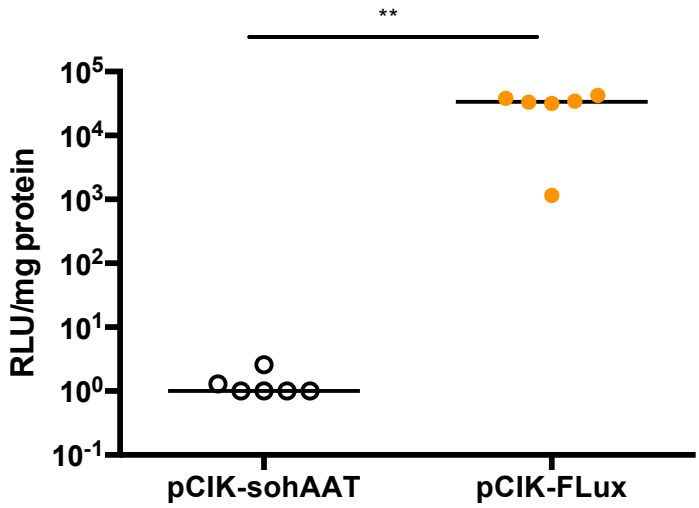


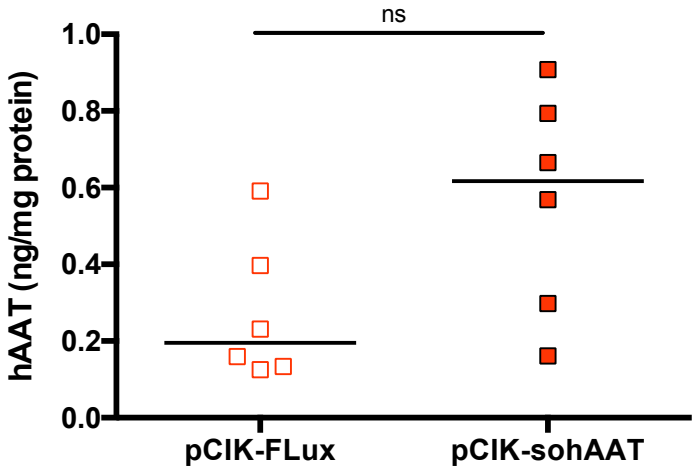
Figure 3-10 A single dose of GL67A:pCIK-FLux leads to significant expression of Firefly luciferase in mouse lung

Mice were treated with (1) 100 µl GL67A:pCIK-FLux or (2) 100 µl GL67A:pCIK-sohAAT. 48 hours post-transfection, Firefly luciferase (FLux) expression was measured in lung tissue homogenate.

Each data point represents one animal. Horizontal bars represent group medians.

RLU=relative light units. **=$p<0.01$ (Mann Whitney *U* test).

A



B

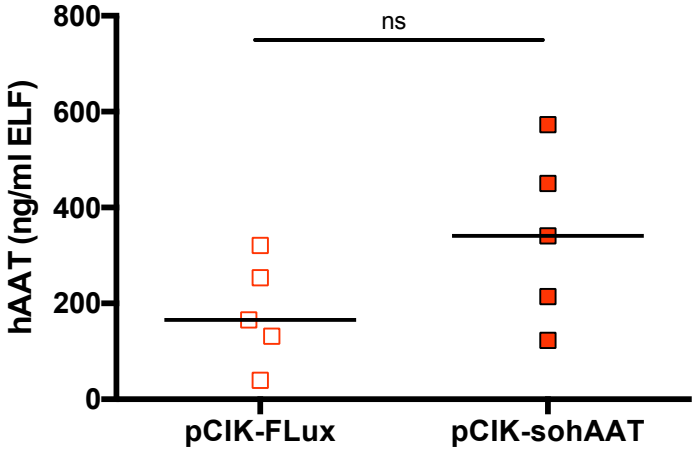


Figure 3-11 A single dose of GL67A:pCIK-sohAAT leads to low-level, non-significant human α_1-antitrypsin expression in murine lung

Mice were treated with (1) 100 µl GL67A:pCIK-FLux or (2) 100 µl GL67A:pCIK-sohAAT. 48 hours post-transfection, human α_1-antitrypsin (hAAT) expression was compared in **(A)** lung tissue homogenate and **(B)** epithelial lining fluid (ELF).

Each data point represents one animal. Horizontal bars represent group medians.

ns=not significant (Mann Whitney *U* test).

3.3.8 Equivalent mRNA levels following treatment with pCIK-FLux and pCIK-sohAAT

The levels of vector-specific mRNA from animals treated with pCIK-FLux and pCIK-sohAAT was determined using quantitative reverse transcription PCR (RT-PCR) with primers directed towards an intron in the 5' untranslated region common to both plasmids. RT-PCR was performed by Jean-François Gelinas at the Consortium's Core Facility in Oxford. The amount of vector-specific mRNA was normalised to endogenous murine cystic fibrosis transmembrane conductance regulator (mCftr) mRNA, which has been used in studies of CF gene therapy in mice and is therefore well established as a control (Jean-François Gelinas, personal communication).

There was no significant difference between the percentage vector/endogenous mRNA in animals treated with pCIK-FLux and pCIK-sohAAT (Figure 3-13). In one animal from each group, vector-specific mRNA was detectable above background levels, but below the lowest RNA concentration on the standard curve.

This confirms that, as expected, transcription from the two plasmids was equivalent; therefore, the reasons that human α_1-antitrypsin is detected log orders of magnitude lower than reporter proteins in the *in vivo* experiments presented previously is due to either (a) reduced translation of hAAT compared to reporter proteins, (b) a shorter half-life or failure of secretion of hAAT compared to reporter proteins, or (c) relatively insensitive assay methods for measuring hAAT compared to reporter proteins. This suggests that treatment with a single dose of lipid:plasmid complexes is not sufficient to lead to therapeutically relevant levels of human α_1-antitrypsin in lung; therefore, the next step was to attempt a repeat dosing experiment in an effort to drive higher protein expression.

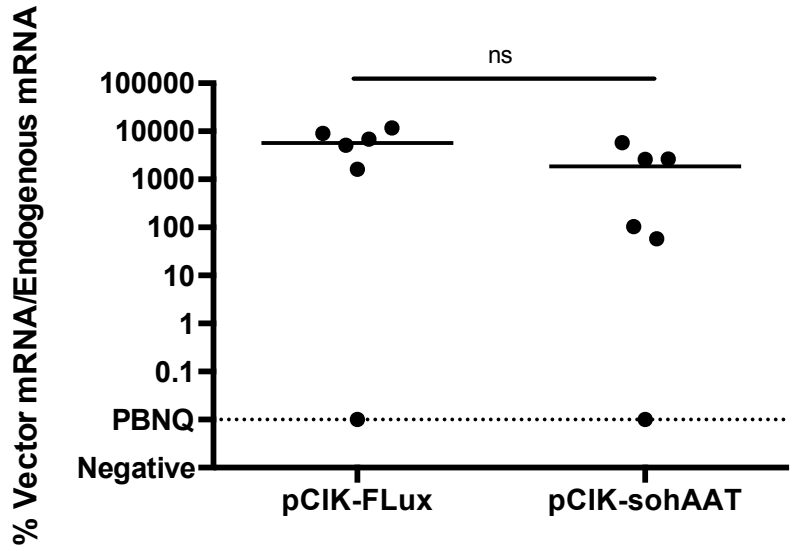


Figure 3-12 Equivalent transgene expression in mice treated with pCIK-FLux and pCIK-sohAAT

Mice were treated with (1) 100 µl GL67A:pCIK-FLux or (2) 100 µl GL67A:pCIK-sohAAT and harvested 48 hours post-transfection. qRT-PCR was performed on total RNA extracted from lung tissue homogenate, with primers directed towards an intron in the 5' untranslated region common to both plasmids. Data are expressed as percentage vector mRNA compared to endogenous mRNA (murine cystic fibrosis transmembrane conductance regulator (mCftr)), such that at 100% levels of vector and endogenous mRNA are equal.

Each data point represents one animal. Horizontal bars represent group medians. PBNQ = positive but not quantifiable (vector-specific mRNA detectable but below the lowest concentration on the RNA standard curve).

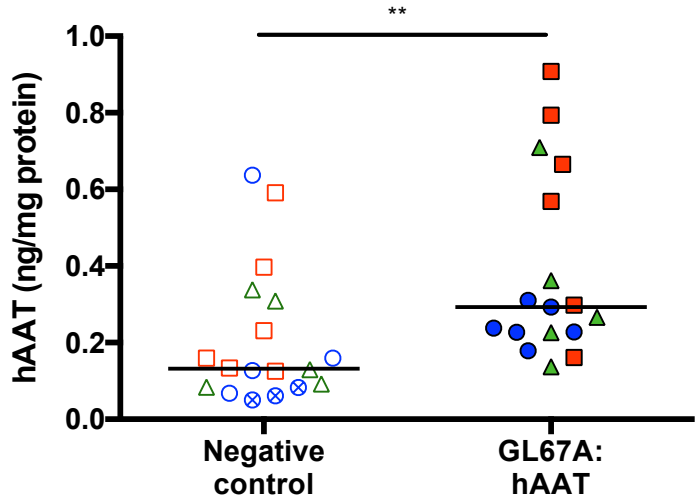
ns=not significant (Mann Whitney *U* test).

However, before carrying out a repeat dosing experiment, I performed a combined analysis of the three single dose experiments with plasmids coding for hAAT to see if higher group numbers could lead to statistical significance in these experiments. There are significant limitations in doing this: firstly, two different plasmids (pCIK-hAAT and pCIK-sohAAT) were used; secondly, different negative control plasmids (pCMV-GLux and pCIK-FLux) were used. Finally, combining experiments performed at different times is not ideal and the results must be interpreted with a degree of caution.

hAAT expression in all mice treated with plasmids coding for hAAT (n=17) was compared to mice treated with irrelevant control plasmids or untreated mice (n=18). In lung tissue homogenate, a statistically significant difference was observed between the two groups (*treated:* 0.29 (0.14-0.91) ng hAAT per mg protein; *untreated:* 0.13 (0.05-0.64) ng hAAT per mg protein; $p<0.01$). There was no statistically significant difference between the groups in epithelial lining fluid.

This combined data analysis suggests that a single dose of lipid:plasmid complexes coding for human α_1-antitrypsin may lead to protein expression. However, the signal is very modest and large group numbers will be needed in future experiments to improve statistical analysis. In the next experiment, I will treat animals with multiple doses of lipid:plasmid complexes in an attempt to drive higher levels of gene expression.

A



B

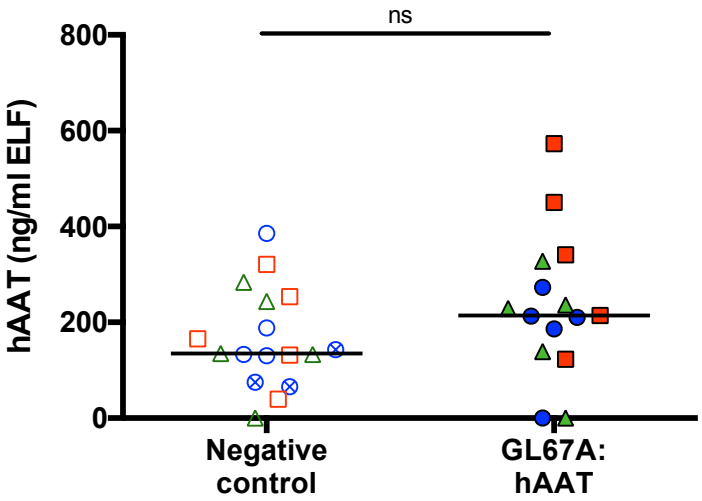


Figure 3-13 A combined analysis of single dose non-viral gene therapy experiments for the production of human α_1-antitrypsin

In three separate experiments, animals were treated with 100 µl lipid:plasmid complexes coding for hAAT (pCIK-hAAT, represented by blue circles and green triangles; pCIK-sohAAT, represented by red squares). Negative control mice were treated with 100 µl irrelevant control plasmids (pCMV-GLux, represented by open blue circles and open green triangles; pCIK-FLux, represented by open red squares) or given 100 µl PBS (crossed blue circles). Human α_1-antitrypsin expression was determined in (a) lung tissue homogenate and (b) epithelial lining fluid (ELF).

Each data point represents a single animal from one of three experiments. Horizontal bars represent group medians.

**=$p<0.01$, ns=not significant (Mann Whitney *U* test).

3.3.9 Mice cannot tolerate bolus administration of GL67A:pDNA at four-day dose intervals

Because a single dose of GL67A:pCIK-hAAT or GL67A:pCIKsohAAT did not lead to significant human α_1-antitrypsin protein expression in murine lung, a repeat dosing experiment was performed. The plasmid selected for the repeat dosing experiment was hCEFI-sohAAT; the hCEFI promoter is a CpG-free chimeric promoter composed of the human elongation factor 1α promoter and the CMV immediate/early enhancer, and was shown to be effective in the recent Phase IIb trial of gene therapy for cystic fibrosis [105]. It has been shown to have a different expression profile than the CMV/CIK promoters used in earlier experiments; whilst CMV leads to a high level of short-term gene expression, published Consortium data has shown that hCEFI leads to long-term gene expression [107]. Therefore, it is reasonable to assume that with repeat doses there may be an accumulation of protein, leading to higher overall levels. The hCEFI-sohAAT plasmid to be used in the repeat dosing experiment was identical to the plasmid used in the cystic fibrosis gene therapy trial, with the exception of the transgene. As a "sixth generation" plasmid produced by the Consortium, it represents the most advanced non-viral gene therapy technology currently available to our group.

Three groups were included in the experiment. Animals received either (1) GL67A:hCEFI-sohAAT (n=8), (2) the irrelevant plasmid GL67A:pCIK-GLux (n=6) or (3) PBS (n=6), by nasal sniffing in 100 µl total volume. A four- to five-day dose interval was chosen for pragmatic reasons, to allow the experiment to be completed within a two month timeframe. It was planned that the mice would receive six doses; at the time of the sixth dose, it was

intended to add an extra single dose group. This group was not included in the final experiment for reasons discussed below.

The mice received their first dose on day 0, and the second dose on day 4. Despite recovering from the anaesthetic, some mice in the two GL67A groups were found dead before the third dose (planned for day 7) could be given. The remaining mice in the two treatment groups showed signs of illness (hunched backs and piloerection). All the animals in the PBS group were alive and appeared well.

Following consultation with the Named Animal Welfare and Care Officers at the animal house, the experiment was terminated and the unwell mice humanely killed. The numbers of mice in each group who had died and appeared unwell after the second dose of GL67A is shown in Table 3-2. It was not possible to perform post-mortem analysis because by the time the animals were discovered, rigor mortis had been set and tissue changes and necrosis would have prevented histological assessment. The reasons for the unexpected deaths observed in this experiment will be discussed later in this chapter.

Table 3-2 Mouse survival in the first repeat dosing experiment

Group	Delivery method	Total volume	*N*	*N dead following second dose*
GL67A: hCEFI-sohAAT	Nasal sniffing	100 µl	8	6 (75%)
GL67A: pCIK-GLux	Nasal sniffing	100 µl	6	3 (50%)
Negative control (PBS)	Nasal sniffing	100 µl	6	0 (0%)

Mice received the indicated vectors or PBS by nasal sniffing in 100 µl total volume, on day 0 and day 4. Before the third dose, a number of mice in the two GL67A groups were found dead.

3.3.10 Repeat dosing with GL67A:hCEFI-sohAAT leads to significant but sub-therapeutic levels of hAAT in murine lung

Before carrying out another repeat dose experiment, the appropriate dose interval was discussed at length. It was decided that a 10-day dose interval should allow the animals enough time to recover from the effects of treatment with bolus GL67A. For humane reasons, a small cohort of three mice was treated with GL67A:pDNA; only once they had survived three doses were further animals treated.

Three groups were included in the experiment. In the first group, mice received six doses of GL67A:hCEFI-sohAAT (100 µl/dose), at 10-day intervals. In the second group, mice received one dose of GL67A:hCEFI-sohAAT, at the same time that the first group received their sixth dose. In the control group, mice received six doses of 100 µl PBS at 10-day intervals. Unfortunately, not enough irrelevant plasmid (e.g. pCIK-GLux) was available to include this group, so it was omitted from the experiment. All mice were harvested six days after the final dose and α_1-antitrypsin expression analysed in lung tissue homogenate and epithelial lining fluid. The animals were harvested at a later time-point than in previous experiments because the hCEFI promoter has previously been shown to lead to longer-lasting gene expression than the CMV promoter.

It was necessary to change the strain of mice used from BALB/c mice to C57Bl/6 mice due to mice stocks available at the time of the experiment and in order to reduce animal usage in line with the 3Rs.

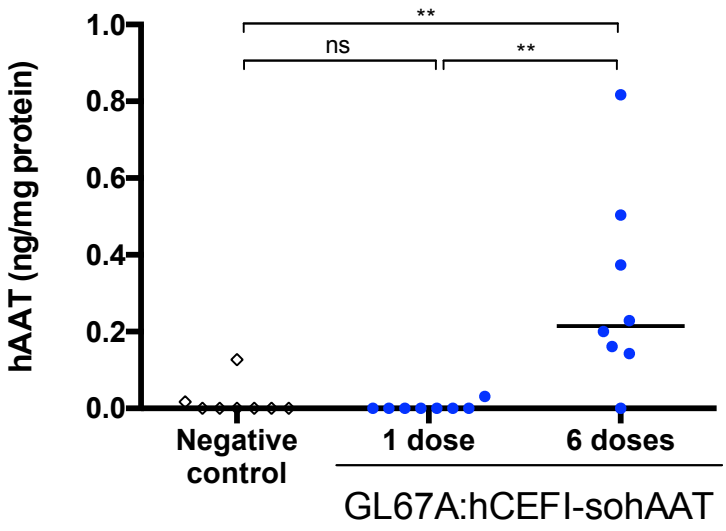
Encouragingly, all mice survived until the end of the experiment. They were carefully observed on a daily basis, and no toxic effects were seen. It would therefore appear that either a longer dose interval (10 or more days) is necessary when treating mice with multiple doses of GL67A:pDNA complexes.

In lung tissue homogenate, there was no detectable hAAT expression in the single dose group. However, in animals treated with 6 doses of GL67A:hCEFI-sohAAT, there was significant expression of hAAT. 6 days after the sixth and final dose, median hAAT expression in treated mice was 0.21 (range 0-0.82) ng hAAT per mg protein, compared to 0.0 (range 0.0-0.13) ng hAAT per mg protein in *untreated* mice ($p<0.01$, Figure 3-14A). Expression of hAAT was significantly higher in animals receiving 6 doses of GL67A:hCEFI-sohAAT, compared to those receiving one dose.

Similar results were observed in epithelial lining fluid, with barely any hAAT expression detectable after one dose, but significant levels of hAAT detectable after six doses ($p<0.01$, Figure 3-14B); however, there was not a statistically significant difference between animals receiving one and six doses. In ELF, the median hAAT concentration was 331 (0.0-1107) ng/ml. The therapeutic target level in ELF, based on patients with intermediate α_1-antrypsin deficiency, is 70 µg/ml or 70,000 ng/ml [13]. The levels of human α_1-antitrypsin seen in epithelial lining fluid in this experiment are therefore approximately 200-fold below the therapeutic target level.

Taken together, the data from the repeat dosing experiment shows that repeatedly administering lipid:plasmid complexes to the murine airways drives higher level of human α_1-antitrypsin expression. However, a different strategy of gene delivery will need to be found to drive hAAT expression to therapeutic levels.

A



B

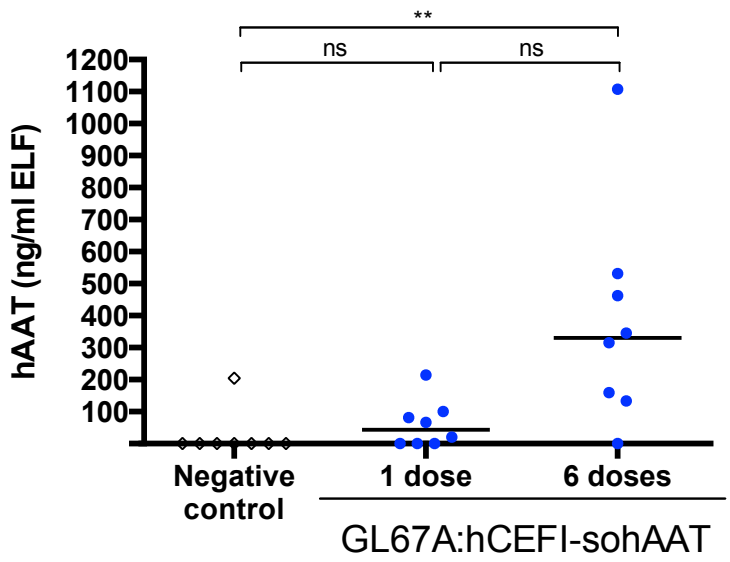


Figure 3-14 Repeat dosing with GL67A:hCEFI-sohAAT leads to significant levels of human α_1-antitrypsin expression in murine lung

Mice were treated with either one dose or six doses of GL67A:hCEFI-sohAAT, or treated with PBS (negative control). Animals were harvested six days after the final dose and human α_1-antitrypsin (hAAT) expression determined in (A) lung tissue homogenate and (B) epithelial lining fluid (ELF).

Each data point represents a single animal. Horizontal bars represent group medians.

**=$p<0.01$, ns=not significant (Kruskal-Wallis test with Dunn's post-hoc analysis).

3.3.11 Expression of human α_1-antitrypsin in murine serum following non-viral gene transfer

In the repeat dose experiment, I also analysed hAAT expression in serum. Although there was no detectable hAAT in the serum of most animals, in three mice serum expression of human α_1-antitrypsin was detectable (Figure 3-15A). The three mice in which hAAT was detectable in serum were the same mice that showed the highest level of hAAT expression in lung and epithelial lining fluid (Figure 3-15B and C), implying that as gene expression in the lung increases, spillover of protein into the serum occurs. Comparing relative levels of hAAT in ELF and serum suggests that hAAT levels in serum following airway gene transfer are approximately 10% of the levels seen in ELF. However, this observation is based on analysis of just three mice so should be interpreted with caution.

This pilot data shows that gene transfer to the lung can produce a protein that is secreted into the serum, which could be useful for the treatment of systemic diseases.

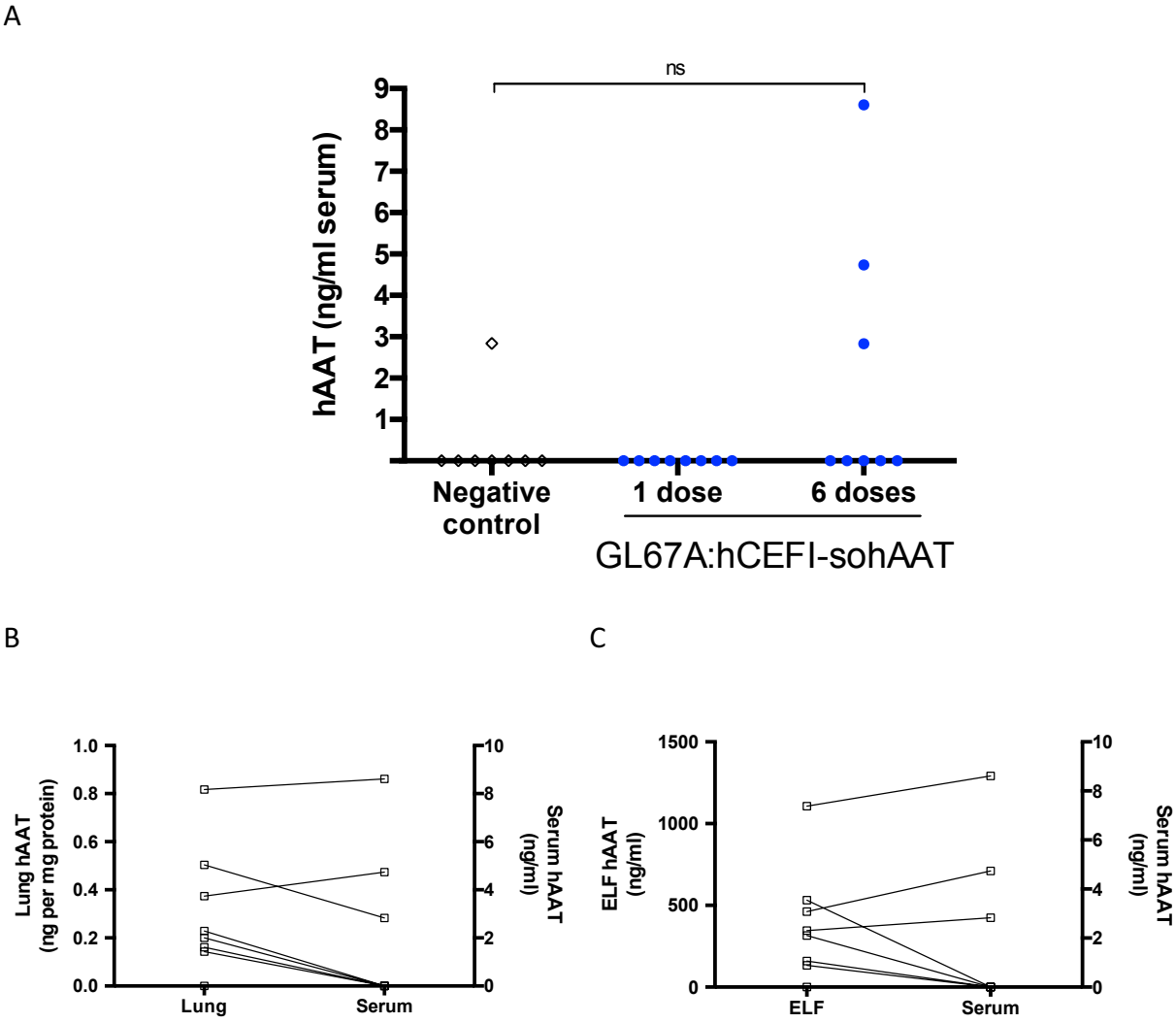


Figure 3-15 Detection of human α_1-antitrypsin in murine serum following gene transfer to the lung

(**a**) Mice were treated with either one dose or six doses of GL67A:hCEFI-sohAAT, or treated with PBS. Animals were harvested six days after the final dose and human α_1-antitrypsin (hAAT) expression determined in serum. Each data point represents a single animal. ns=not significant.

(**b**) hAAT expression was compared between lung and serum for animals receiving six doses of GL67A:hCEFI-sohAAT. The levels of hAAT in lung and serum for each animal are joined. Each data point represents a single animal.

(**c**) hAAT expression was compared between epithelial lining fluid (ELF) and serum for animals receiving six doses of GL67A:hCEFI-sohAAT. The levels of hAAT in ELF and serum for each animal are joined. Each data point represents a single animal.

3.4 Discussion

In this chapter, I have used gene expression cassettes developed by the UK Cystic Fibrosis Gene Therapy Consortium, complexed with the lipid formulation GL67A, to produce human α_1-antitrypsin *in vitro* and in the murine lung. I have shown (1) that all four hAAT plasmids, produced by a colleague in the Oxford laboratory, lead to the production of secreted α_1-antitrypsin *in vitro*; (2) a single dose of non-viral gene therapy leads to barely detectable levels of hAAT expression; (3) increasing the number of doses given from one to six doses leads to significant levels of gene expression in lung tissue homogenate and epithelial lining fluid; and (4) the level of hAAT obtained is approximately 200-fold below the therapeutic target level in epithelial lining fluid. This suggests that alternative treatment strategies or different gene transfer agents will need to be investigated in the future.

Two different lipid vectors were assessed *in vitro*: the commonly used Lipofectamine 2000, and the vector of choice in the recent Phase IIb trial of gene therapy for cystic fibrosis, GL67A [105]. I chose to study both vectors in initial experiments in order to validate my gene transfer technique (there are fewer steps in the preparation of Lipofectamine for cell transfection compared to GL67A). Although Lipofectamine 2000 led to higher levels of protein expression, it was not log orders higher than GL67A. Previous data from the Consortium has shown that Lipofectamine performs less well than GL67A in mice, and it has also not been assessed in man, making GL67A a better candidate for further study throughout this thesis.

The different hAAT plasmids produced are driven by different promoters: pCIK and hCEFI. hCEFI is a chimeric promoter produced by the UK CF Gene Therapy Consortium. It contains the CMV immediate/early enhancer linked to the elongation factor 1α promoter, and is entirely devoid of pro-inflammatory CpG dinucleotides [167]. My experiments

presented here have shown that, 48 hours post transfection, there is no significant difference between protein expression from these promoters. However, these pilot experiments were not designed to measure the duration of gene expression; it has previously been shown that hCEFI leads to sustained levels of gene expression in mice [167], and future experiments presented in this thesis will examine the duration of gene expression from the hCEFI promoter.

Two of the plasmids constructed contained a CpG-depleted, codon-optimised transgene sohAAT. It is important to remember that the plasmid pCIK-sohAAT still contains CpG dinucleotides in plasmid backbone, although data recently published suggests that it is the CpG status of the transgene that has the greatest effect on gene expression [159]; hCEFI-sohAAT is entirely devoid of CpG residues. CpG depletion is of note, because the presence of even one CpG dinucleotide is pro-inflammatory, activating the innate immune system via toll-like receptor 9 (TLR-9) [159] [168] [169], limiting *in vivo* gene expression. Furthermore, these plasmids are codon optimised; each DNA triplet coding for one amino acid is optimised to lead to higher levels of protein expression in mammalian cells.

In this Chapter I reported the results of a urea assay on serum and BALF samples of mice treated either with GL67A (in this chapter) or with the lentiviral vector rSIV.F/HN-hCEF-sohAAT (reported in Chapter 5), to aid interpretation of the *in vivo* gene transfer data presented in this thesis. As this thesis concentrates on the production of secreted proteins, it is important to analyse epithelial lining fluid (ELF) samples for protein expression; however, the process of collecting these samples (broncho-alveolar lavage) significantly dilutes ELF, meaning that the true level of protein expression in ELF is under-reported. The commonly accepted method of calculating the effect of lavage on ELF dilution is performing a urea assay [160] and a commercially available assay kit, supported by published data, was selected. I showed that the mean serum urea concentration in 14 mice was 17.4 mM/l, similar to

previously published data. Stechman and colleagues reported serum urea levels of 10.9 mM/l in female C57Bl/6 mice, the same as used in my studies [170]; in two other publications, the authors reported serum urea levels of approximately 11-12 mM/l in C57Bl/6 mice [171] [172]. Slight variations are likely due to different methods being used to analyse urea concentration.

Surprisingly, I have been unable to find any data in scientific journals regarding the expected concentration of urea in murine epithelial lining fluid, despite extensive searches on PubMed, Google and Web of Science. I have found one source, an abstract discussed at a scientific meeting in Brazil, where researchers reported urea concentrations in BALB/c mice of 50 mg/dl (8.3 mM/l) in serum and 4 mg/dl (0.66 mM/l) in ELF [173], both values which are not dissimilar to my findings of 17.4 mM/l in serum and 0.46 mM/l in ELF. I have also not been able to find any published reports on the dilution factor in murine broncho-alveolar lavage; although many investigators perform a urea assay to calculate a conversion factor from BALF to ELF, they usually do not state what this conversion factor is.

There are limitations to the urea assay data I have presented here. Firstly, the number of samples analysed is small (14); I would have liked to perform a urea assay on each and every matched serum and BALF sample obtained in this thesis, however this was neither practical (due to time and financial constraints; each assay kit, costing approximately £319, only allows 20 samples to be analysed) nor was it feasible (as very small volumes of these biological samples were obtained, and measuring transgene expression is the priority). Therefore, instead of calculating a conversion factor for each separate lavage procedure, I have used a mean conversion factor, based on the results from 14 animals, throughout this thesis. In addition, I performed the urea assay on samples from C57Bl/6 mice, whereas most of the experiments in this chapter (apart from the second multi-dose experiment) were performed on BALB/c mice. I do not anticipate any differences in dilution factor between the two

strains of mice, but it would have been preferable to compare data from the different strains; importantly, in metabolic studies in different strains of mice, the serum urea concentration was nearly identical between female C57Bl/6 and BALB/c mice [170].

Nevertheless, the samples I did analyse on the urea assay are from treated animals in two experiments which I consider to be of particular importance in this thesis: the mice in the multi-dose non-viral experiment (discussed below), and the mice which expressed the highest levels of α_1-antitrypsin in the lentiviral vector experiments presented in Chapter 5.

I started my *in vivo* research using the secreted reporter protein, *Gaussia* luciferase. This is a small luciferase produced by the shrimp *Gaussia princeps*, which is secreted by cells due to the presence of a signal peptide [174]. Because the aim of my studies is to produce a secreted protein, human α_1-antitrypsin, *Gaussia* luciferase is a more appropriate reporter to use in preliminary experiments than non-secreted reporters such as firefly or Renilla luciferase; it is also more sensitive than the commonly used secreted reporter alkaline phosphatase both *in vivo* and *in vitro* [175]. The Consortium has previously reported production of *Gaussia* luciferase in mice following gene therapy with GL67A: 24 hours after a single dose of GL67A:pCMV-GLux, GLux expression was approximately three log orders higher than controls in lung tissue homogenate and broncho-alveolar lavage fluid [164]. The data from my experiments are similar to this report, validating my *in vivo* gene transfer technique. I have also demonstrated production of a different reporter protein, firefly luciferase (FLux) *in vivo*, consistent with previously published data [107].

Whilst I found it relatively easy to detect reporter genes following gene transfer *in vivo* with GL67A, it has been difficult to demonstrate production of the therapeutic protein human α_1-antitrypsin. Importantly, I have shown that treatment with pCIK-sohAAT and pCIK-Lux leads to equivalent levels of vector-specific mRNA transcripts in murine lungs, proving that the lack of human α_1-antitrypsin protein is not due to failure of transcription. As reporter

gene expression following GL67A-mediated gene therapy is approximately three log orders above controls, it would be reasonable to assume that human α_1-antitrypsin should be detectable in the same order of magnitude. It is not clear why this is the case; whilst it was hypothesised that *Gaussia* luciferase has a longer half-life in murine serum than human α_1-antitrypsin, a literature search revealed that this may not be the case. hAAT has a half-life in human serum of three to five days [176] whilst *Gaussia* luciferase has been shown to have a half-life in murine serum of 20 minutes [177]. The half-life of human α_1-antitrypsin in murine serum is not known, yet the very short half-life of *Gaussia* luciferase *in vivo* not only rules out this hypothesis, but also implies that *Gaussia* luciferase is being continuously produced by transfected cells *in vivo*. Because *Gaussia* luciferase is measured by a luminescent reaction, there is a large signal amplification and therefore is is probable that the *Gaussia* luciferase assay is simply more sensitive than the ELISA kit used in this study.

A single dose of gene therapy with plasmids coding for hAAT did not lead to significant levels of expression in most experiments; in only one out of three experiments, expression at 48 hours post-transfection was significantly (but only three- to four-fold) higher than negative controls. A combined analysis of data from my three separate single-dose experiments did show statistically significant but very modest human α_1-antitrypsin expression in lung tissue homogenate, but this result must be interpreted with caution due to the limitations of combining data from different experiments. In particular, it should be noted that two different plasmids were used in these three experiments; in two experiments I used the plasmid pCIK-hAAT, and in the third experiment I used the codon-optimised plasmid pCIK-sohAAT, which may affect interpretation of the combined data analysis.

There have been several other reports of non-viral gene therapy for α_1-antitrypsin deficiency. Based on pre-clinical studies in rabbits [178] and cystic fibrosis bronchial epithelial cells [179], one group performed a trial in 5 patients with severe α1-antitrypsin deficiency [81]. In

this trial, CMV-hAAT was complexed to the cationic lipid DOTMA:DOPE and given to one nostril, with the untreated nostril acting as the control. Levels of hAAT in nasal lavage fluid from the treated nostril was double the levels in the untreated nostril at day 5 post-treatment; unfortunately, hAAT expression in nasal lavage fluid is reported in μg/mg protein, making it impossible to compare the data to broncho-alveolar lavage fluid in my preclinical experiments. Several other groups have delivered naked plasmid DNA [180], naked linear DNA [181] or lipid:plasmid complexes [182] carrying hAAT cDNA intravenously to mice, demonstrating high levels of hAAT expression in serum; however, none of these studies reported hAAT expression in the lung or broncho-alveolar lavage fluid, making it difficult to compare them to my studies.

Because only negligible amounts of human α_1-antitrypsin were produced following a single dose of gene therapy in my hands, a multi-dose experiment was performed. It is likely that gene therapy for chronic diseases (such as α_1-antitrypsin deficiency or cystic fibrosis) will require multiple administrations of gene therapy vectors; in the recent Phase IIb trial of gene therapy for cystic fibrosis, patients were given monthly doses of lipid:plasmid complexes for 12 months [105]. The multi-dose experiments presented in this chapter used a very similar product to the Phase IIb cystic fibrosis trial. The lipid vector, GL67A, was the same vector used in the clinical trial, and has previously been determined by the Consortium to be the most appropriate vector for gene delivery to the lung; in addition, the plasmid used (phCEFI-sohAAT) was identical to the trial plasmid with the exception of the transgene. This plasmid has some features of note; firstly, the promoter used, hCEFI, has been shown to lead to longer-lasting reporter gene expression compared to the more commonly used CMV promoter [183], which is advantageous for the treatment of chronic diseases. Secondly, the plasmid is entirely devoid of CpG-dinucleotides, which has been shown to reduce inflammation and sustain pulmonary gene expression [107]. These plasmids are also 'codon-

optimised', i.e. the DNA sequence has been modified to improve protein expression in mammalian cells.

In the first multi-dose experiment, the majority of mice treated with GL67A:pDNA complexes died shortly after the second dose (which was administered four days after the first dose) and the remaining mice appeared severely unwell. There were no signs of gross toxicity in mice receiving PBS. There are three components of the treatment which could have caused the toxic response: the anaesthetic, the lipid and the plasmid. I ruled out anaesthetic toxicity, as all the mice in the PBS group were alive and well after the second dose. A response to the plasmid DNA is possible, however the plasmid used in the hAAT group (hCEFI-sohAAT) is CpG-depleted, so is expected to be less inflammatory [107]. Because of a delay in preparing the plasmids for the experiment, the mice used were approximately 5 months old at the time of the experiment, which was felt by some (including the Named Animal Welfare and Care Officers) to be too old. Although I do not feel this is sufficient to explain the toxic effects of the lipid, in future experiments care was taken to ensure that mice were no more than two months old (ideally 6 weeks) at the time of treatment. Finally, it is feasible (although unlikely) that either (1) the lipid or gene therapy product could have become contaminated during preparation e.g. by bacteria or (2) a human error was made when preparing the GL67A:lipid plasmid complexes prior to treatment.

Before the Phase IIb trial of GL67A:hCEFI-CFTR in humans, an extensive toxicology study was performed in mice [108]; GL67A:pDNA complexes were well tolerated, even at 60-fold times the planned human dose. However, there are significant differences between the toxicology study and my experiments. Firstly, in the toxicology study mice were treated with aerosolised GL67A:pDNA complexes; in my experiments, mice were given a bolus of 100 µl GL67A:pDNA complexes by nasal sniffing. This simple difference may be enough to explain the toxicity I observed; pooling of the bolus of lipid in the small airways and alveoli could be

more toxic than inhaling aerosolised droplets, leading to failure of alveolar gas exchange and death. Aerosolisation experiments require a significantly larger volume of GL67A:pDNA complexes, as much of the material is lost in the environment of the treatment chamber; this would have made an aerosolisation experiment prohibitively expensive in this project.

Secondly, in the toxicology study mice received treatment at fortnightly intervals, whereas in my experiment a four- to five-day dosing interval was chosen because (1) I did not expect repeat doses of GL67A to be toxic, and (2) it would allow the experiment to be completed in a reasonable timeframe. However, it is apparent that mice were not able to tolerate boluses of GL67A:pDNA complexes at this dose interval. After much discussion and consultation with the Named Animal Care and Welfare Officers (NACWOs) at the animal house, a 10-day dose interval was decided for the second experiment as it was similar to the two-week dose interval that was tolerated in the toxicology study.

With the possible reasons for the toxic effects of GL67A:pDNA complexes in mind, the following improvements were made to the experimental design:

- The dose interval was increased to 10 days;
- To investigate whether or not this dose interval was appropriate, an initial cohort of three mice were treated with three doses at 10-day intervals; only once they had survived the third dose were the remaining animals treated;
- Mice were a maximum of two months old at the time of the first dose;
- Mice were observed before and after each dose by a NACWO for signs of toxicity;
- Lipid:plasmid complexes were prepared in a sterile microbiological safety cabinet to remove the possibility of bacterial contamination.

In the second multi-dose experiment, animals received either one or six doses of GL67A:hCEFI-sohAAT, or PBS at 10-day intervals. In the single dose group, there was no

significant human α_1-antitrypsin expression following a single dose, despite the fact that codon optimised plasmids were used; however, it is difficult to compare this observation to previous experiments because (1) a different strain of mice was used (C57Bl/6 in this multi-dose experiment, BALB/c mice in the previous single-dose experiments) and (2) the harvest timepoints were different (six days post-treatment in the multi-dose experiment, two days post-treatment in the single dose experiments).

Encouragingly, animals which received six doses of GL67A:hCEFI-sohAAT did show significant expression of human α_1-antitrypsin in lung tissue homogenate and epithelial lining fluid six days after the final dose, and no toxic effects were seen; all mice survived to the end of the experiment, and despite careful daily observation no gross toxic effects of treatment were seen. Although this is most likely due to the increased dose interval used (10 days instead of 5 days in the initial multi-dose experiment), I can't rule out the possibility that the toxicity seen in the first experiment was strain-specific (BALB/c mice were used in the initial experiment, and C57Bl/6 mice were used in the second). It would have been preferable to use the same strain of mice in both experiments, however it was necessary to use C57Bl/6 due to stock levels at the time of the experiment and in order to be compliant with the 3Rs.

The median level of hAAT in epithelial lining fluid was 331 (range 0.0-1107) ng/ml, compared to a therapeutic level of hAAT in human ELF of 70,000 ng/ml [13] based on data from patients with intermediate α_1-antitrypsin deficiency (Section 1.1.4) [21]. The median level of hAAT was therefore 200-fold below the therapeutic target level. In ELF, expression of hAAT following six doses of lipid:plasmid complexes was 7.5-fold higher than the single dose group, which suggests that giving more than six doses could increase hAAT expression further. However, as the level of hAAT obtained was so far off the therapeutic target, this would be neither appropriate nor feasible.

It was interesting to note that in the animals which expressed the highest levels of hAAT in lung and ELF, there was some spillover of hAAT into the serum. Whilst the levels of hAAT in serum were very low, it suggests that gene delivery to the lung can result in the production of proteins in the serum. This suggests that the lung could be used as a factory for the production of secreted proteins for the treatment systemic disease, an idea that will be explored further in future chapters.

It would have been interesting to perform a side-by-side comparison of the different plasmids available (four plasmids were available using two different promoters, pCMV and hCEFI, in both sequence optimised and non-sequence optimised forms). However, as I have shown that expression of human α_1-antitrypsin gene therapy with GL67A leads to modest levels of hAAT, it would have been of purely academic interest rather than of translational value. It was decided that it was therefore more appropriate to move on to studies using different gene therapy vectors.

The UK Cystic Fibrosis Gene Therapy Consortium has been developing lentiviral vectors pseudotyped with the F and HN surface proteins from Sendai virus for airway gene transfer. Pre-clinical data has been very encouraging, and in the rest of this thesis I will describe my experiments using rSIV.F/HN to produce α_1-antitrypsin *in vitro, in vivo* and in *ex vivo* human tissue culture models.

4 *In vitro* assessment of lentiviral vectors for the production of secreted proteins

4.1 Introduction

In Chapter 3 I described the development of non-viral vectors for α_1-antitrypsin gene therapy. As the optimal treatment strategy using the best non-viral vector for airway gene transfer failed to achieve therapeutic levels of α_1-antitrypsin in murine epithelial lining fluid, I moved on to investigate alternative gene transfer agents.

The UK Cystic Fibrosis Gene Therapy Consortium has developed lentiviral vectors pseudotyped with F and HN surface proteins from Sendai virus [134]. In this chapter, I will describe proof-of-concept experiments using these vectors *in vitro*; the development of the lentiviral vectors is discussed in more detail in Section 1.3.3.

Hypothesis: SIV vectors pseudotyped with Sendai virus F and HN proteins (rSIV.F/HN) can produce secreted proteins *in vitro*.

Aim: Transduce cells with rSIV.F/HN vectors carrying cDNA for the secreted reporter protein *Gaussia* luciferase or human α_1-antitrypsin, and demonstrate protein production.

Conclusion: It was not possible to completely control for pseudo-transduction of free protein left over from viral production, co-delivered with the viral vectors in these experiments. Therefore, *in vitro* transduction experiments are not the most sensitive method of assessing transduction efficiency of these vectors.

4.2 Materials & Methods

Recombinant SIV vectors pseudotyped with Sendai virus surface proteins F and HN (called rSIV.F/HN) were produced as described in General Material & Methods, Section 2.3.

4.2.1 Assessment of rSIV.F/HN in vitro

Transduction of HEK293T cells: human embryonic kidney (HEK) 293T cells were seeded onto 24-well culture plates (Corning, USA) at a cell density of 400,000 cells per well. Cells were cultured in 500 µl complete media (Dulbecco's Modified Eagle Medium (DMEM), 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (all regents from ThermoFisher, Waltham, USA)). Cells were incubated in a 37°C incubator at 5% CO_2.

On day 1 of the experiment, when approximately 80% confluent, cells were washed in cold Optimem (ThermoFisher, Waltham, USA) and then transduced with lentiviral vectors diluted in Optimem (250 µl total volume). Negative control cells were given 250 µl neat Optimem. Details of the group sizes and lentiviral vectors used are provided in the figure legends.

Cells were incubated overnight, and on day 2 of the experiment were supplemented with 250 µl media (DMEM plus 20% FCS). On day 3, the supernatant was removed and analysed for transgene expression as described in Section 2.6. Cells were washed in 1 ml PBS and lysed with 150 µl/well reporter lysis buffer (Promega, USA), and freeze-thawed three times. Stored lysates were analysed for complete protein using the Bio-Rad protein assay as described in Section 2.4.1.

Controlling for pseudotransduction: to control for pseudotransduction, an extra step was added. The supernatant removed on day 3 was discarded, and cells were then washed with PBS and given 500 µl fresh complete media. This was then collected after 24 hours (day 4) and subsequently analysed. Cells were lysed as before.

Transduction of A549 cells: lung adenocarcinoma A549 cells (ATCC, USA) were seeded onto 24-well culture plates (Corning, USA) at a cell density of 400,000 cells per well. Cells were cultured with complete media as before, and the experiments were performed as for HEK293 cell experiments above.

Further A549 cell transduction experiments: in later experiments A549 cells were seeded on 96-well culture plates (Corning, USA) at a density of 80,000 cells per well. This decision was made on the grounds that it would minimise the use of expensive lentiviral vectors, whilst also allowing extra dose groups to be included. Cells were cultured in 200 µl/well complete media (composition as above) and incubated as before.

Cells were transduced with lentiviral vectors rSIV.F/HN-hCEF-sohAAT or rSIV.F/HN-hCEF-soGLux when approximately 80% confluent. The viral titres could not be determined, as they were below the lower limit of detection of the TaqMan transduction assay. Therefore, the dose of virus given to each well of cells is reported as µl virus.

The lentiviral vectors were diluted in Optimem and cells transduced with 1, 10, 50 or 100 µl virus per well in 100 µl total volume (n=5 wells per dose group). Control cells (n=10 wells) were given 100 µl Optimem.

24 hours post-transduction, cells were supplemented with 100 µl/well DMEM plus 20% FCS. 48 hours post-transduction, supernatant was removed and discarded, and cells washed with PBS. Cells were supplemented with 200 µl complete media and incubated for a further 24 hours. At 72 hours post-transduction, supernatant was collected and analysed and cells lysed as described in Section 2.4.

4.3 Results

4.3.1 Assessment of rSIV.F/HN in HEK293T cells

The first viral prep available to be assessed was rSIV.F/HN-hCEF-soGLux, which is an SIV vector pseudotyped with F and HN proteins from Sendai virus [134]. In an initial experiment, human embryonic kidney (HEK) 293T cells (n=6 wells per group) were chosen as they are known to be easy to transduce (Uta Griesenbach, personal communication), and I wanted to make sure that lentiviral vectors produce secreted proteins before moving on to more in-depth experiments.

HEK293T cells were transduced with (a) unactivated virus, (b) activated virus, or (c) left untransduced. The F protein from Sendai virus is initially synthesised in an inactive form F_0 [184], and requires treatment with trypsin to be cleaved into active F_1 and F_2 subunits, which allow the virus to enter cells [185]. Therefore, I would not expect to see any protein expression when treating HEK293T cells with unactivated virus.

It was not possible to determine the viral titre used in the experiment, as the titre was below the lower limit of detection of the assay. The volume of virus used in the experiment was the maximum feasible volume that could be given to cells *in vitro*.

Interestingly, cells treated with unactivated virus showed approximately 3 log orders higher GLux expression than negative controls (Figure 4-1):

- *Unactivated rSIV.F/HN:* 2.6e7 (range 1.4e7-5.0e7) RLU/mg total protein;
- *Negative controls:* 1.4e4 (range 1.3e4-2.0e4) RLU/mg total protein, $p<0.001$).

There was no statistically significant difference in reporter gene expression between cells treated with unactivated and activated virus.

The most likely reason that high levels of GLux were observed in the cell culture supernatant is because of the transfer of free GLux protein made by the packaging cells during vector production, as a result of the transfection of these packaging cells with a plasmid carrying GLux cDNA. This process, called pseudotransduction, has previously been reported for retroviral vectors [186] [187] [188] and is clearly particularly problematic when studying secreted proteins. In future experiments, extra steps are added to control for the effect of pseudotransduction. Despite pseudotransduction, activated virus should still have led to more reporter gene expression than unactivated virus. It would appear therefore that the viral titre used in this experiment was too low, and it will be necessary to find a method to concentrate the virus in future *in vitro* experiments.

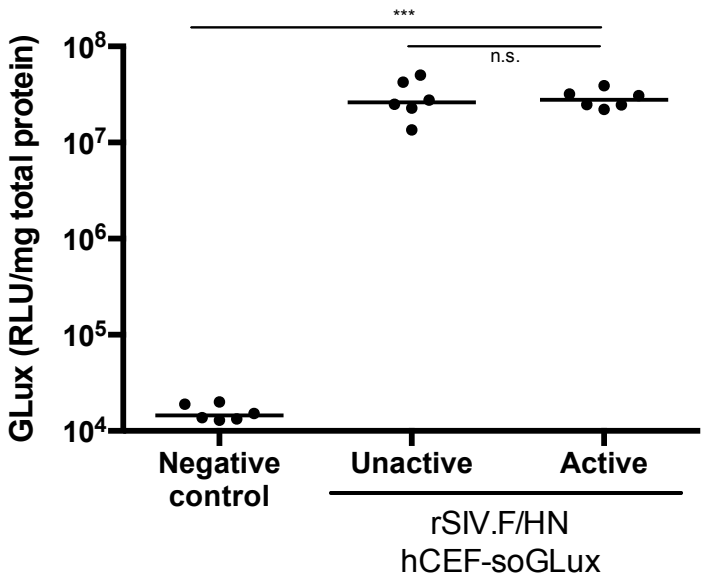


Figure 4-1 Expression of *Gaussia* luciferase in HEK293T cells transduced with rSIV.F/HN-hCEF-soGLux

Human embryonic kidney (HEK) 293T cells were transduced with equal volumes of unactivated or activated rSIV.F/HN-hCEF-soGLux (50 µl virus in 250 µl total volume; viral titre unknown), and *Gaussia* luciferase expression determined in the supernatant 48 hours post-transduction. Negative control cells were given 250 µl Optimem.

Each data point represents one well of cells. Horizontal bars represent group medians.

GLux = *Gaussia* luciferase, RLU = relative light units. ***=$p<0.001$, ns=not significant (Kruskal-Wallis test with Dunn's post-hoc analysis).

4.3.2 Concentration in Millipore spin columns increases particle titre approximately 10-fold

Because it was not possible to observe lentiviral-vector specific expression of a reporter protein in the above *in vitro* experiment, a method to concentrate the virus (i.e. improve the functional titre) needed to be found. One concentration method which has previously been used in the laboratory is treatment of vectors in Amicon Ultra-15 centrifugal filter units from Millipore. They were used as described in the General Materials and Methods chapter (section 2.3.2).

It was possible to compare the pre- and post-concentration viral particle titre in three virus preparations concentrated in Millipore spin columns. Concentration in Millipore spin columns improved particle titre (i.e. the number of viral particles) between 10- and 23-fold (Table 4-1) in the three virus preparations analysed.

It should be noted that it was not possible to perform the same comparison for *functional* titre, which is reported throughout this thesis. This is because the three batches of virus analysed were of low quality, and when unconcentrated, the functional titre was below the lower limit of detection of the TaqMan transduction assay.

Dividing the number of viral particles (particle titre, vp/ml) by the number of functional particles (functional titre, TTU/ml) gives the particle:infectivity ratio, a readout of how many viral particles need to be made by the producer cells to produce one active virus. In these three batches of virus, the P:I ratio ranges from 1:1989 to 1:8161. This is quite poor compared to other batches of virus produced by the Consortium, however it should be borne in mind that these three virus batches were unpurified; future improvements in viral production techniques have improved the P:I ratio to approximately 300 (Consortium data, unpublished).

Unfortunately analysis of both particle and functional titre could only be performed for three batches of virus, due to personnel and time constraints at the Consortium Core Facility in Oxford; this is why only functional titres are reported from here on. Nevertheless, it does seem that concentrating the virus in Millipore spin columns improves both particle and functional titre, justifying their use in future experiments.

Table 4-1 Concentration in Millipore spin columns increases particle titre 10- to 23-fold

Construct	Particle titre (vp/ml)		Fold increase in particle titre	Functional titre (TTU/ml)		Particle:infectivity ratio
	Pre-concentration	Post-concentration		Pre-concentration	Post-concentration	
rSIV.F/HN hCEF-soGLux	1.33e10	2.49e11	18	ND	1.02e8	2441
rSIV.F/HN hCEF-sohAAT	3.10e10	3.24e11	10	ND	3.97e7	8161
rSIV.F/HN CMV-FVIII	3.35e9	7.70e10	23	ND	3.87e7	1989

Concentration of three rSIV.F/HN batches in Millipore spin columns resulted in a 10- to 23-fold improvement in particle titre (viral particles (vp)/ml). All three constructs were produced in London in adherent cell cultures, and were not purified. In all three batches, the functional titre (TaqMan transducing units (TTU)/ml) of unconcentrated virus could not be determined (ND).

GLux=Gaussia luciferase. hAAT=human α_1-antitrypsin. FVIII=human Factor VIII. so=sequence optimised.

4.3.3 Controlling for pseudotransduction in cell culture experiments

By the time of the second *in vitro* transduction experiment, the first lentiviral vector carrying hAAT cDNA was available and this vector was therefore assessed *in vitro* to assess its activity before larger scale *in vivo* experiments were performed. HEK293T cells were transduced as before, with one major modification: to control for pseudotransduction, the cell culture supernatant was removed 48 hours after transduction, and the cells were washed and given 500 µl fresh complete medium. By doing this, it was hoped that protein produced during viral production would be mostly removed; only secreted protein produced following successful transcription of the transgene should be detected in the supernatant.

Two further modifications were included:

1. some cells were given activated virus concentrated in Millipore spin columns, which concentrate rSIV.F/HN approximately 10-fold (see above and General Material and Methods, section 2.3.2);
2. for each viral condition (unactivated, activated, or activated+concentrated), two dose groups were included, with cells given either 1 µl or 10 µl virus. The functional titre was not known at the time of the experiment (cells were again given the maximum feasible volume of virus at the time of the experiment); later, it was determined that in the "activated+concentrated" group 4e4 and 4e5 TTU/well virus had been used; it was not possible to determine the functional titre in the other groups as the titre was below the lower limit of detection of the assay.

72 hours post-transduction, the cell culture supernatant was harvested and hAAT expression determined. hAAT expression was significantly greater than untransduced controls in cells treated with 4e5 TTU/well concentrated virus ($p<0.01$, Figure 4-2). A viral titre of 4e4

TTU/well was not sufficient for transgene expression. There was no secretion of hAAT in response to treatment with an irrelevant virus (rSIV.F/HN-hCEF-soGLux).

In cells treated with unactivated virus, hAAT remained at background levels (approximately 2 ng hAAT per mg total protein), suggesting that the experimental modifications effectively controlled for pseudotransduction and confirming that the transgene expression seen in the highest dose group was a result of transcription and translation of the hAAT transgene in the treated cells.

This data represents proof-of-principle that a lentiviral vector can produce secreted proteins in cell culture models. However, as human embryonic kidney cells are not representative of human airway epithelial cells, it was decided to investigate lentiviral transduction in a lung-derived cell line in future experiments. In addition, a colleague who was simultaneously assessing other rSIV.F/HN vectors in HEK293T cells reported that they may not be the most appropriate cells in which to look for hCEF-driven transgene expression (Mario Chan, personal communication), which further justifies analysis in an alternative cell line.

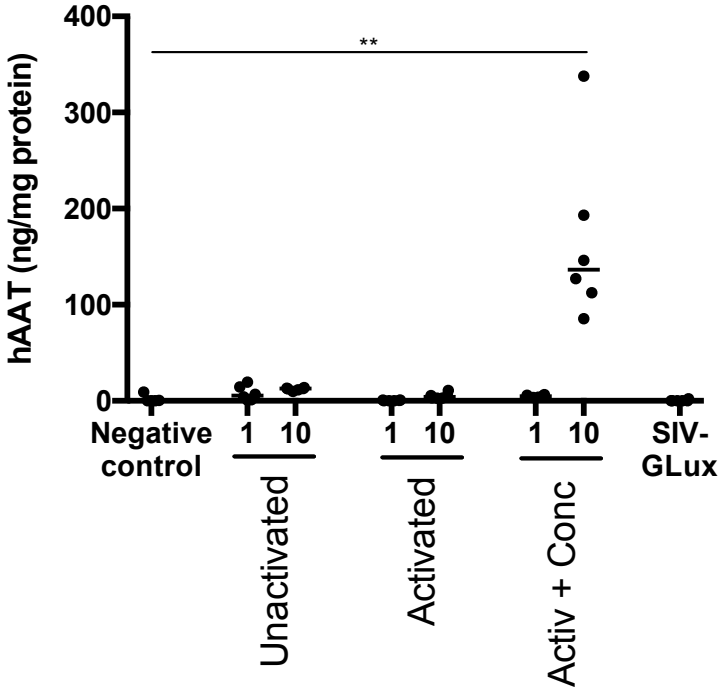


Figure 4-2 Expression of human α_1-antitrypsin in HEK293T cells transduced with rSIV.F/HN-hCEF-sohAAT

Human embryonic kidney (HEK) 293T cells were transduced with rSIV.F/HN-hCEF-sohAAT in 250 µl total volume at the doses shown (1 or 10 µl virus/well) and hAAT expression determined in cell culture supernatant 72 hours post-transduction.

Virus was either unactivated, activated, or activated and concentrated (activ + conc). In the concentrated group, 1 µl virus was later determined to be 4e4 TTU/ml; 10 µl virus was 4e5 TTU/ml. The titre of unactivated and unconcentrated virus was not able to be determined.

Negative control cells were given 250 µl Optimem. One group (SIV-GLux) was treated with rSIV.F/HN-hCEF-soGLux (10 µl/well in 250 µl total volume).

Each data point represents one well. Horizontal bars represent group medians.

**=$p<0.01$ (Kruskal-Wallis test with Dunn's post-hoc analysis).

4.3.4 Transduction of A549 cells with rSIV.F/HN lentiviral vectors

A549 cells are derived from a human alveolar cell adenocarcinoma, and show similar characteristics to type II alveolar epithelial cells [189]. Therefore, they are more relevant to airway gene transfer than HEK293T cells; in addition, parallel experiments in other projects implied that hCEF may work better in A549 cells (Mario Chan, personal communication).

To compare the ability of rSIV.F/HN to transduce HEK293T and A549 cells, both cell lines were given identical doses of virus. Cells were seeded at identical densities, and protein expression per well was normalised to the total protein content of that well to allow comparison between the two groups of cells. The maximum feasible dose at the time of the experiment (25 µl of activated, unconcentrated virus per well) was used. As before, I controlled for pseudotransduction by removing the transduction mix at 48 hours and replacing it with fresh media, which was subsequently analysed.

Interestingly, hAAT concentration in the supernatant of untransduced A549 cells was higher than that of HEK293T cells, with a median concentration of 3.0 (range 3.0-5.0) ng hAAT per mg total protein in untransduced A549 cells compared to 0.08 (range 0.06-0.28) ng hAAT per mg total protein ($p<0.01$). A literature search confirmed that unstimulated A549 cells secrete hAAT [7], whereas no reports that HEK293T cells secrete hAAT could be found.

In HEK293T cells, viral transduction did not lead to significant levels of hAAT production at the dose used; there was also no endogenous hAAT production in response to treatment with an irrelevant lentiviral vector. In A549 cells, there was significantly more hAAT produced following lentiviral transduction (Figure 4-3, $p<0.01$). There was also a suggestion of a trend towards increased hAAT expression in A549 cells following transduction with an irrelevant lentivirus; cells receiving rSIV.F/HN-hCEF-soGLux had a slightly higher median hAAT

concentration in supernatant compared to untransduced cells, although this did not reach statistical significance.

This preliminary experiment suggests that A549 cells are a better model for studying the transduction efficiency of rSIV.F/HN vectors carrying the hCEF promoter *in vitro* than HEK293T cells. In the next experiment, I will investigate lentiviral transduction in A549 cells further and explore the dose-response effects of treatment with lentiviral vectors.

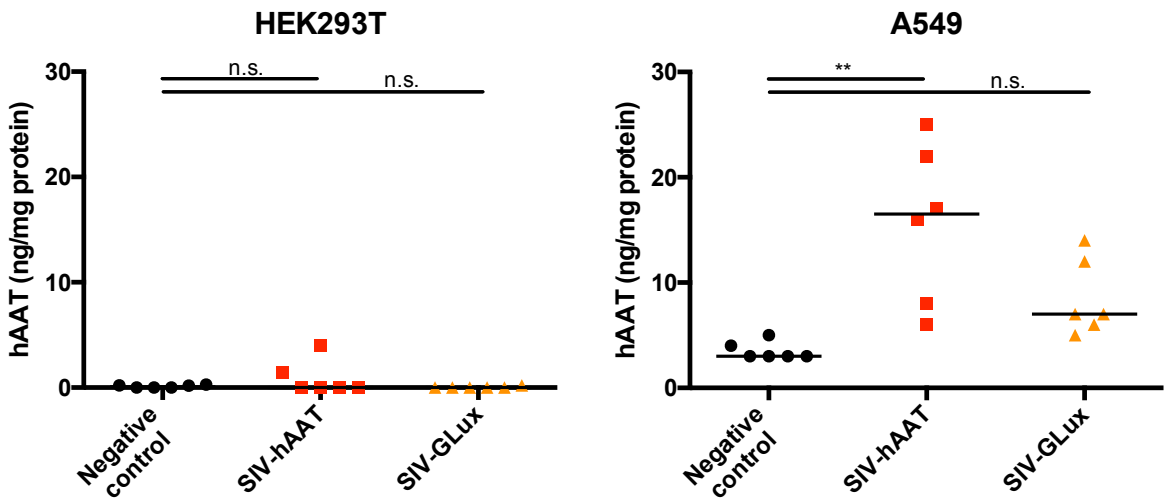


Figure 4-3 rSIV.F/HN transduces A549 cells with greater efficiency than HEK293T cells

Human embryonic kidney (HEK) 293T cells and A549 cells were seeded onto 24-well tissue culture plates at a density of 400,000 cells per well. Cells were treated with (a) rSIV.F/HN-hCEF-sohAAT (25 µl/well in 250 µl total volume), (b) rSIV.F/HN-hCEF-soGLux (25 µl/well in 250 µl total volume) or (c) untransduced (250 µl Optimem). hAAT expression in cell culture supernatant was determined 72 hours post transduction.

Each data point represents one well of cells. Horizontal bars represent group medians.

**=$p<0.01$, ns=not significant (Kruskal-Wallis test with Dunn's post-hoc analysis).

4.3.5 rSIV.F/HN leads to the production of secreted proteins in A549 cells in a dose-dependent manner

To assess another batch of rSIV.F/HN-hCEF-sohAAT, a further *in vitro* experiment was performed with A549 cells seeded onto 96-well plates. Cells were transduced with various doses of rSIV.F/HN vectors carrying hAAT or GLux cDNA, as I hypothesised that higher doses should lead to higher levels of transgene expression. The decision to use 96-well plates was made because (a) it would allow for the inclusion of more groups, and (b) it would allow for smaller volumes of expensive lentiviral vectors to be used. Otherwise, the transduction experiment was performed as before.

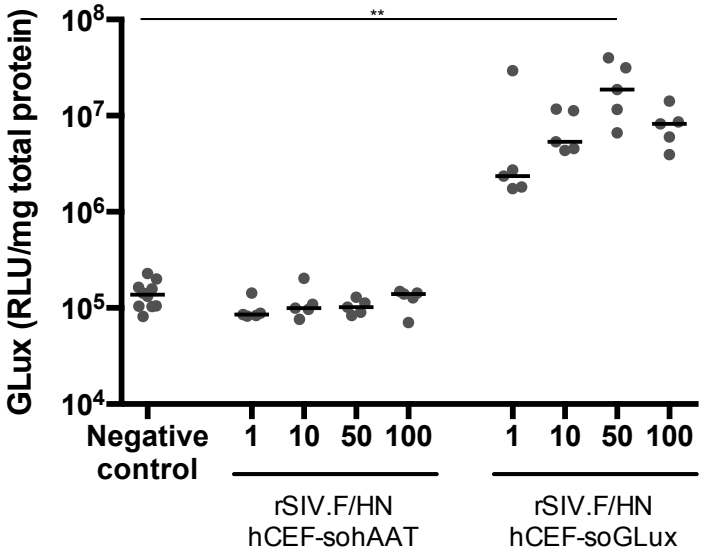
In this experiment the viral titre was not able to be determined, because the functional titre of both batches was below the lower limit of detection of the assay. Therefore, the dose of vector given is reported as µl virus per well of cells, in 100 µl total volume. Although it would have been desirable to know the titres used, this does not affect the interpretation of the data.

In all cells treated with rSIV.F/HN-hCEF-soGLux, *Gaussia* luciferase was detected above background levels. For cells given 50 µl virus in 100 µl total volume, expression was significantly greater than negative controls (Figure 4-4A: *treated:* median 1.9e7 (range 6.6e6-4.0e7) RLU per mg protein, *untreated:* 1.3e5 (8.2e4-2.3e5) RLU per mg protein, $p<0.01$). Interestingly, cells given 100 µl virus actually showed less GLux expression than those given 50 µl, although there was no statistically significant difference between the groups.

There was no dose-response correlation for GLux when all four dose groups were analysed (Spearman rank 0.41 (95% confidence interval (CI) -0.05 to 0.72), $p=0.072$). However, when the 100 µl dose group was removed from analysis, there was a significant dose-response correlation (Spearman rank 0.68 (95% CI 0.24 to 0.89), $p<0.01$). This suggests that there is a dose-response relationship, but that high titres of lentiviral vectors can be toxic to cells.

In cells treated with rSIV.F/HN-hCEF-sohAAT, hAAT was not detected above background levels with the lowest dose group (1 µl virus). However, all other doses of virus led to significant expression of hAAT compared to negative controls (Figure 4-4B). For cells treated with 50 µl virus, which showed the highest expression, median hAAT levels were 8.7 (range 6.0-15) ng hAAT per mg total protein, compared to 0.49 (range 0.34-1.4) ng hAAT per mg total in untransduced cells ($p<0.01$). Cells given 100 µl virus showed approximately equivalent expression to cells given 50 µl virus. The dose-response correlation for hAAT was highly significant (Spearman rank 0.81 (95% CI 0.57 to 0.93), $p<0.0001$). In this experiment, hAAT was *not* above background levels in cells treated with an irrelevant virus (rSIV.F/HN-hCEF-soGLux).

A.



B.

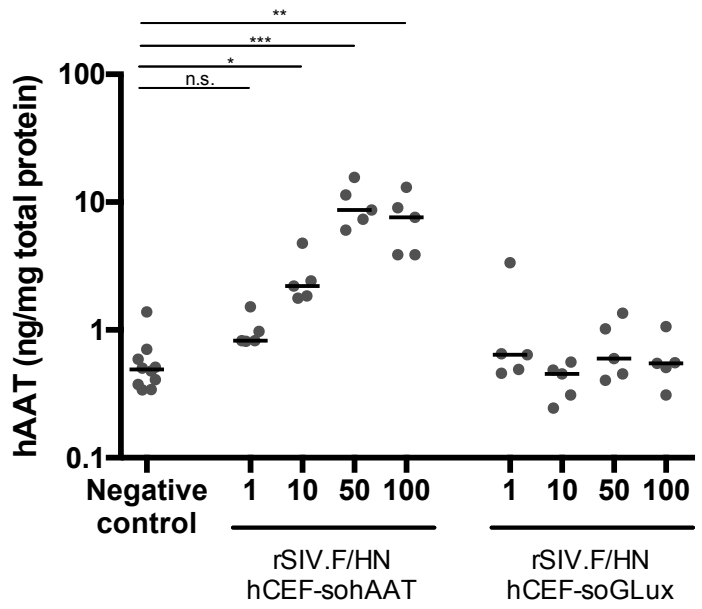


Figure 4-4 Transduction of A549 cells with rSIV.F/HN vectors

A549 cells were transduced with rSIV.F/HN carrying hAAT or GLux cDNA as shown. The x-axis shows the µl virus given per well of cells, in 100 µl total volume (n=5 per group). Control cells (n=10) were given 100 µl Optimem.

Expression of (a) *Gaussia* luciferase (GLux) and (b) human α_1-antitrypsin (hAAT) was determined in cell culture supernatant 72 hours after transduction.

Each data point represents one well. Horizontal bars represent group medians. RLU=relative light units

*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant (Kruskal-Wallis test with Dunn's post-hoc analysis).

4.4 Discussion

In this relatively short chapter, I have presented initial *in vitro* experiments to test the hypothesis that lentiviral vectors make secreted proteins. This is important before making large-scale viral batches and performing *in vivo* experiments. *In vitro* experiments are relatively inexpensive and represent a good initial model of transduction efficiency; however, their value is relatively limited compared to animal models and *ex vivo* human tissue models, which are presented in future chapters, as they generally can neither predict a vector's *in vivo* efficacy nor can they accurately determine whether therapeutic levels of protein are produced.

In the first experiment, it was not possible to detect any transgene expression because of the effect of pseudotransduction. This phenomenon is a result of the virus production process. To produce a lentiviral vector, HEK293T cells are co-transfected with several plasmids, coding for the various viral proteins, the F and HN pseudotype proteins, and the transgene of interest (here, GLux or hAAT) [121]. In earlier chapters I have demonstrated that plasmid transfection of HEK293T cells leads to significant protein expression; a similar process occurs here, as the eukaryotic promoter on the plasmid carrying the transgene of interest is inadvertently active in the producer cell line; these cells therefore synthesise the protein of interest and it is secreted into the vector supernatant along with the vector particles. When the vector supernatant is added to cells in transduction experiments, free protein remains in the culture media and can be detected in my assays, leading to false positives such as in Figure 4-1, where treatment of cells with unactivated virus seemed to lead to transgene expression. This may be particularly problematic when dealing with proteins with a long half-life, such as *Gaussia* luciferase, which has a long (~6 day) half-life in culture medium [175].

Pseudotransduction was first described by Liu and colleagues [186]. The authors used a retroviral vector (Maloney murine leukaemia virus) coding for β-galactosidase (β-gal) to transduce primary mouse hepatocytes, demonstrating that the β-gal protein was concentrated and co-purified during vector preparation, resulting in high rates of β-gal protein transfer. In a different study, Haas and colleagues used an HIV1-based lentiviral vector coding for green fluorescent protein (GFP) and transduced HEK293T cells [187]. They demonstrated that pseudotransduction occurs immediately at the time of vector administration, persisting for up to 72 hours. Pseudotransduction has also been demonstrated with HIV1-derived lentiviral vectors in primary neutrophils [188]. All these studies involved non-secreted reporter genes, suggesting that free protein from vector production can enter cells.

The effect of pseudotransduction should be reduced somewhat once extra viral production steps such as AKTA purification and concentration have been introduced. However, I was able to simply control for this by removing the media at 48 hours, washing the cells, and adding fresh media. The transduced cells then continued to produce secreted protein which was detected in the supernatant after a further 24 hour incubation. Despite this, I still cannot be completely sure that all secreted protein produced during vector production was removed from the cells, and therefore cannot conclusively determine that transgene expression had occurred.

Another method to demonstrate whether or not pseudotransduction had occurred would be to block various stages of the vector life-cycle, for example by inhibiting reverse transcriptase or protein biosynthesis [188]. Unfortunately, this was beyond the scope of this project. Difficulties controlling for pseudotransduction are one reason that rapid progression of lentiviral vectors into animal studies can be justified; animal studies can take place over a longer time-frame (7 days to 2 years), minimising the effect of pseudotransduction as free protein co-delivered with the lentiviral vector will gradually be removed.

Another limitation of the data in the chapter is that the viral titre was often unknown, as the batches were below the lower limit of detection of the functional titration assay, although I do not feel that this affects the interpretation of the data.

In my hands, it was relatively difficult to transduce human embryonic kidney (HEK) 293T cells with rSIV.F/HN vectors. HEK293 cells and their derivatives are commonly used for cell transduction experiments, as they are relatively easy to transduce; there are many reports of HEK293 cells being transduced by various lentiviral vectors in the literature [190] [191] [192]. My results could be explained by a lack of the receptors for the F and HN pseudotype proteins on the membrane of HEK293 cells; however, a more likely explanation, backed up by the results of other experiments performed in the laboratory at this time, is that the hCEF promoter is not active in HEK293 cells. I was able to demonstrate comparatively high transduction efficiency in A549 cells, which are lung adenocarcinoma cells derived from type II alveolar cells. It should be noted that A549 cells were observed to constitutively express human α_1-antitrypsin, an observation backed up by reports in the literature [7].

It is important to determine whether or not treatment with lentiviral vectors increases endogenous production of human α_1-antitrypsin; it is a protein involved in inflammatory processes [38], so it is reasonable to hypothesise that treatment with foreign agents (e.g. gene transfer agents such as GL67A or rSIV.F/HN) may stimulate local production. In HEK293T cells, two separate experiments showed that there was no endogenous production of hAAT following treatment with a lentiviral vector carrying *Gaussia* luciferase cDNA (Figure 4-2 and Figure 4-3). In A549 cells, there was a trend towards hAAT production in cells treated with an irrelevant lentivirus in the initial experiment (Figure 4-3), although this did not reach statistical significance. In a subsequent experiment in A549 cells, there was no endogenous production of hAAT in response to lentiviral vectors (Figure 4-4) and, as this experiment

consisted of multiple dose groups, I am confident in the conclusion that HEK293T and A549 cells do not produce endogenous hAAT in response to rSIV.F/HN.

In summary, the data in this chapter suggests that an rSIV lentiviral vector pseudotyped with Sendai virus F and HN proteins can produce secreted proteins *Gaussia* luciferase and human α_1-antitrypsin *in vitro*. However, pseudotransduction occurred and could not be completely controlled for, and therefore the data should be interpreted with caution. I will therefore move on to investigating the rSIV.F/HN vector *in vivo* in Chapter 5, and in human *ex vivo* tissue models in Chapter 6.

5 *In vivo* assessment of lentiviral vectors for the production of α_1-antitrypsin

5.1 Introduction

In Chapter 4 I described the assessment of the lentiviral vector rSIV.F/HN *in vitro*. In this chapter, I describe *in vivo* studies using this vector for the production of the secreted reporter protein *Gaussia* luciferase, and the secreted therapeutic protein human α_1-antitrypsin (hAAT).

As discussed previously, a therapeutic target for any treatment of α_1-antitrypsin deficiency is the level of hAAT seen in 'intermediate' deficiency, i.e. a Pi*SZ genotype (Section 1.1.4). These patients, who have one normal and one abnormal hAAT allele, do not tend to develop emphysema, and tend to have serum hAAT concentrations of at least 11 µM [21]. This has led to the hypothesis of a 'target' level of hAAT in serum of 11 µM, equivalent to 0.7 g/l. However, because most of the pathology due to α_1-antitrypsin deficiency is in the lung [1], and because the present study concerns the treatment of lung disease, it is more appropriate, in this context, to define target concentrations of hAAT in epithelial lining fluid (ELF). The concentration of hAAT in ELF is approximately 10% that of serum levels, so the target level of hAAT in ELF (or to describe it alternatively, the level of hAAT in ELF from Pi*SZ patients) is 1.1 µM, or 70 µg/ml.

Several other groups have attempted gene therapy for alpha-1-antitrypsin deficiency by delivering a viral vector to the lung. To the best of my knowledge, two other groups have achieved a therapeutic level of hAAT in the murine lung using a viral vector. Halbert *et al* administered an AAV6-hAAT vector dropwise to the nares of mice, achieving therapeutic levels of hAAT in murine epithelial lining fluid (50-200 µg/ml) for at least 7 months [92]. However, long-term gene expression was limited by an immune response to the vector

and/or transgene, and animals required immunosuppression in order for long-term hAAT expression to be observed. It appears therefore that gene therapy with an AAV6 vector would require re-administration, which may not be feasible given the immunogenicity of these vectors [193].

In a separate study, Wilson *et al* administered a VSV-G pseudotyped lentiviral vector by tracheal instillation, after mixing the vector 50:50 with Lipofectamine [94]. The group investigated different promoters, and observed hAAT levels of 140 μg/ml in epithelial lining fluid using a 1.2 kB EF1α promoter 6 weeks after gene transfer. Interestingly, Wilson and colleagues observed selective transduction of alveolar macrophages which persisted and expressed transferred genes for the lifetime of the mice in the study. Lentiviral vectors pseudotyped with F/HN proteins could be a better alternative as they transduce airway epithelial cells and can be repeatedly administered [134].

Hypothesis: rSIV.F/HN vectors can lead to therapeutic levels of secreted human α_1-antitrypsin in mice.

Aim: to transduce the murine lung with rSIV.F/HN, leading to the production of therapeutic levels of secreted human α_1-antitrypsin.

Conclusion: transduction of the murine lung with rSIV.F/HN carrying hAAT cDNA leads to (1) stable levels of protein expression for the lifetime of a mouse, and (2) can produce therapeutic levels of 70 μg/ml in epithelial lining fluid.

5.2 Materials & Methods

Recombinant SIV vectors pseudotyped with Sendai virus surface proteins F and HN (called rSIV.F/HN) were produced as described in General Materials & Methods, Section 2.3.

5.2.1 In vivo transduction experiments

Mice were anaesthetised using isoflurane (Section 2.5) and treated with 100 µl of gene therapy vector by nasal sniffing (Section 2.5.2).

Gaussia luciferase (GLux) short-term _in vivo_ studies:

Mice (n=5-6/group) were treated using a range of experimental procedures.

(a) four doses of unconcentrated rSIV.F/HN hCEF-soGLux (100 µl/dose) delivered on consecutive days. The titre of this virus batch could not be determined due to technical reasons, but this does not affect interpretation of the data.

(b) a single dose of concentrated rSIV.F/HN hCEF-soGLux (1e7 TTU/mouse in 100 µl). Negative control mice received four doses of virus diluent (100 µl DMEM-20) on consecutive days.

On day 7 after the first treatment, the mice were culled and tissues harvested as described in Section 2.5.3.

Gaussia luciferase long-term _in vivo_ studies:

Three further groups of mice were treated with a single dose of 100 µl concentrated rSIV.F/HN hCEF-soGLux (1e7 TTU/mouse), the same batch used in the single dose experiment described above. Mice (n=5-6 per group) were sacrificed 28, 102 and 365 days post-treatment. GLux expression was quantified in lung, BALF and serum.

hAAT short-term *in vivo* studies with low quality virus batch:

Three separate experiments were performed, as described in Table 5-2 (page 156). In the first experiment, 6 mice were treated with one dose of rSIV.F/HN-hCEF-sohAAT (2e7 TTU/mouse in 100 µl total volume) and 6 mice with five doses of virus on consecutive days (1e8 TTU/mouse total). Negative control mice (n=5) were treated with diluent (PBS, 100 µl/mouse). The animals were culled on day 7 and hAAT expression quantified in lung tissue homogenate, epithelial lining fluid and serum.

hAAT long-term *in vivo* studies with low quality virus batch:

Four further groups of mice (n=5 per group) were treated with rSIV.F/HN-hCEF-sohAAT (2e7 TTU/mouse in 100 µl) and harvested between 85 and 623 days post-treatment. For details on the treatment and harvest days, please refer to Table 5-3 (page 168).

hAAT short-term *in vivo* studies with high quality virus batches:

In the second short-term *in vivo* hAAT experiment, mice were treated with rSIV.F/HN-hCEF-sohAAT produced in adherent cultures purified through AKTA columns. 5 mice were given a total of 1.5e8 TU/mouse, in three doses 10 days apart, and sacrificed 10 days after the final dose as previously described. hAAT expression was compared to archived negative control samples.

In the third short-term *in vivo* hAAT experiment, mice (n=6 per group) were treated with (a) one dose of 1e8 TTU rSIV.F/HN-hCEF-sohAAT produced in suspension cultures and purified through AKTA columns, or (b) irrelevant control virus (rSIV.F/HN-hCEF-GFPLux) and sacrificed 10 days post-treatment.

Gaussia luciferase (GLux) repeat administration studies:

Animals (n=5-6 per group) were treated over a 12-day period with 100 µl rSIV.F/HN-hCEF-soGLux (titre unknown) or 100 µl PBS. Mice received either (a) 10 doses of virus, (b) 5 doses of PBS followed by 5 doses of virus, or (c) 9 doses of PBS followed by a single dose of lentivirus, as summarised on Table 5-1. It was decided *a priori* to compare the difference in gene expression between animals given one and five doses of rSIV.F/HN using a Mann Whitney *U* test (a non-parametric test was used due to the low *n* numbers in the experiment).

7 days after the final dose, animals were sacrificed and GLux expression quantified in lung, ELF and serum, and compared to GLux expression in negative controls.

Table 5-1 Dosing regimen used for Gaussia luciferase repeat administration studies

	Treatment day									
	1	2	3	4	5	6	7	8	9	10
10 dose group	SIV	SIV	SIV	SIV	SIV	SIV	SIV	SIV	SIV	SIV
5 dose group	PBS	PBS	PBS	PBS	PBS	SIV	SIV	SIV	SIV	SIV
1 dose group	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	SIV

Dosing regimen for *Gaussia* luciferase repeat administration studies. Animals received 100 µl total volume of SIV (rSIV.F/HN-hCEF-soGLux) or 100 µl PBS over a 12-day period.

5.2.2 Delivery of lentiviral vectors to the lungs of sheep

Sheep experiments were designed by myself in consultation with Dr. Gerry McLachlan at The Roslin Institute, University of Edinburgh, and subsequently performed by him.

The sheep were anaesthetised with gaseous isoflurane and intravenous propofol in a whole body negative pressure respirator [194].

14 days before gene transfer, baseline serum and BAL samples were collected. Two BAL washings were performed per lung, using 5 mls PBS delivered by a bronchoscope.

On day 0, animals were treated with lentiviral vectors. The virus was thawed and concentrated in Amicon spin columns (Millipore, Watford, UK) according to the manufacturer's recommendations (described in Section 2.3.2). For virus carrying hAAT cDNA, two virus batches (42 mls total) were thawed and then combined before concentration to approximately 4 mls (~10-fold concentration). 2.5 mls sterile filtrated PBS was added to the concentrated virus, giving a final volume of 6.5 mls virus which was divided into 3x2 ml aliquots (2 ml/sheep) For virus carrying GLux cDNA, one virus batch (22 mls total) was thawed and concentrated to 2 mls. 4.5 mls sterile filtrated PBS was added giving a final volume of 6.5 mls concentrated virus which was divided into 3x2 ml aliquots (2ml/sheep).

Sheep (n=3) were treated with 2 mls of rSIV.F/HN hCEF-sohAAT delivered to the left lung, and 2 mls of rSIV.F/HN hCEF-soGLux delivered to the right lung. This volume was chosen based on Dr. McLachlan's previous experience, which has shown that larger volumes tend to 'pool' in the airways rather than being evenly distributed throughout the lung.

2 mls of virus was sprayed into each lung using a Trudell AeroProbe catheter (Trudell Medical International, Ontario, Canada), a multi-lumen catheter which delivers sheared droplets of liquid to a specific area of the lung. The tip of the AeroProbe was positioned just

below the level of the bifurcation of the left or right 2nd right ventral diaphragmatic segment (Figure 5-12) and the aerosolised gene therapy vectors delivered at 50-psi air pressure to the left or right caudal diaphragmatic segment.

The remaining 0.5 mls of each concentrated virus was re-frozen to allow subsequent virus titration. However, due to various personnel and budgetary constraints, titration of these viruses could not be performed. Based on the pre-concentration titres, and the assumption that Millipore spin columns concentrate the virus 10-fold (discussed in Section 4.3.2), the approximate titres used in this experiment were 2.9e7 TTU per animal for GLux, and 2.8e8 TTU per animal for hAAT. BAL samples were collected on day 7 and 14 post-treatment.

At 28 days, the animals were sacrificed by lethal injection and the lungs removed for post-mortem analysis. Tissue samples were collected from different segments of the lung: left cardiac segment of the apicocardiac lobe (LC), left caudal diaphragmatic (LCD), left ventral diaphragmatic (LVD2), right cardiac (RC), right caudal diaphragmatic (RCD), right ventral diaphragmatic (RVD2). For histological analysis, a block of tissue from each segment was fixed in 10% formalin and paraffin embedded. Stained and sectioned slides were examined by a histopathologist at Edinburgh (Sionagh Smith).

5.2.3 Quantification of Gaussia luciferase and hAAT

Gaussia luciferase and hAAT were quantified as described in the General Materials & Methods Chapter (Section 2.6).

5.3 Results

The results presented in this chapter are described approximately in the chronological order in which the experiments were performed. During this time, the Consortium was developing and refining the techniques for producing the lentiviral vectors, meaning that different batches of virus were used in different experiments. These are summarised on Table 5-2.

Table 5-2 A summary of the lentiviral vector constructs and doses used in the experiments described in this chapter

Paragraph reference	Virus construct (rSIV.F/HN hCEF…)	Species treated	Production location	Culture	Media	AKTA Purified	Spin column concentrated	Doses	Overall titre given (TTU)	Negative control	Harvest days
GLux experiments											
5.3.1 5.3.2	soGLux	Mouse	London	Adherent	DMEM-20	No	No	4	Could not be determined	DMEM-20	7, 28, 102, 365
							Yes	1	1e8		
5.3.9	soGLux	Mouse	London	Adherent	PBS	Yes	No	1-10	Unknown	PBS	10
hAAT experiments											
5.3.3 5.3.4	sohAAT	Mouse	London	Adherent	PBS	No	Yes	1	2e7	PBS	7, 85, 365, 576, 623
							Yes	5	1e8	PBS	7
5.3.5	sohAAT	Mouse	London	Adherent	PBS	Yes	No	1	1e8	rSIV.F/HN hCEF GFPLux	10
5.3.6	sohAAT	Mouse	Oxford	Suspension	FS	Yes	Yes	3	1.5e8	PBS	30
Combined experiments											
5.3.11	sohAAT	Sheep	London	Adherent	PBS	Yes	Yes	1	2.8e8	soGLux	28
	soGLux		London	Adherent	PBS	Yes	Yes	1	2.9e7	sohAAT	

For more detail please refer to the text. 'Paragraph reference' refers to the paragraph in which the results of the experiment are described.
DMEM-20=Dulbecco's Modified Eagle Medium with 20% fetal calf serum; PBS=Phosphate-buffered saline; FS=Freestyle media; TTU=TaqMan transducing unit.

5.3.1 Lentiviral transduction of the murine airway leads to significant reporter gene expression at day 7

In the first *in vivo* experiment, mice were treated with 100 µl of rSIV.F/HN-hCEF-soGLux. The virus batch used was one of the earliest lentivirus batches produced in the Consortium's London laboratory. The comparatively low purity of this virus batch should be borne in mind when interpreting the results of the experiment.

Mice were treated with either (a) four doses of unconcentrated virus given on four consecutive days, or (b) one dose of concentrated virus given on the first day of the experiment, or (c) four doses of negative control (DMEM-20). It was not possible to determine the titre of the unconcentrated virus, as it was below the detection limit of the assay; the concentrated virus had a titre of 1e8 TTU/ml, i.e. 1e7 TTU/dose. The viral titre was not known at the start of the experiment; the volume given (100 µl) is the maximum volume that can be administered to the murine airway at one time, and therefore represented the maximum feasible dose at the time of the experiment.

Mice were harvested 7 days post transduction; therefore, this experiment was a short-term experiment to test the principle that lentiviral vectors can produce secreted proteins. Some experiments in this chapter were designed to assess the duration of gene expression, and are described as long-term (i.e. longer than 7 days).

Significant levels of GLux in lung and epithelial lining fluid

Animals were harvested 7 days after the first dose, and GLux expression determined in lung tissue homogenate, epithelial lining fluid and serum. Animals treated with 1e7 TTU of virus

showed high levels of GLux expression. In lung and epithelial lining fluid, GLux expression was ~5 log orders higher than controls (Figure 5-1):

- **Lung:**

  Treated: 4044 (1454-6806) RLU/mg protein, *untreated:* 0.13 (0.07-0.27) RLU/mg protein, $p<0.001$, n=6 per group

- **ELF:**

  Treated: 6.0e7 (1.4e7-1.1e8) RLU/µl, *untreated:* 3490 (3015-5002) RLU/µl untreated, $p<0.01$, n=5-6 per group

- Data expressed as median (range)

Animals treated with unconcentrated virus (titre undetermined) showed lower levels of protein expression in lung tissue and ELF.

This is the first proof-of-concept data that lentiviral vectors administered to the lung can make secreted proteins in lung tissue and epithelial lining fluid. It will also be interesting to look at GLux expression in serum.

Significant levels of GLux in serum

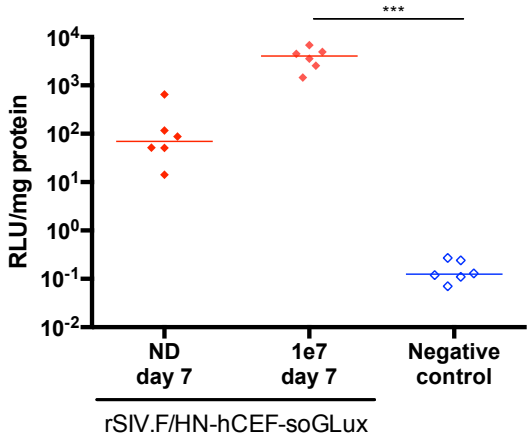
There are two reasons to look at protein expression in serum following lung gene transfer: (1) assuming that there is a positive correlation between protein levels in the target organ and serum, a blood test offers a quick, cheap and relatively non-invasive method to estimate protein levels in the target organ in a clinical trials; and (2) if gene therapy vectors administered to the lung result in serum protein expression, it opens up the possibility for treating systemic diseases, such as haemophilia, using airway gene transfer.

In the current experiment, GLux expression in serum was approximately three-fold above controls when animals were treated with concentrated 1e7 TTU (*treated:* 286 (114-411) RLU/µl, *untreated:* 40 (34-93) RLU/µl untreated, $p<0.05$, n=4-6 per group; Figure 5-1C). In

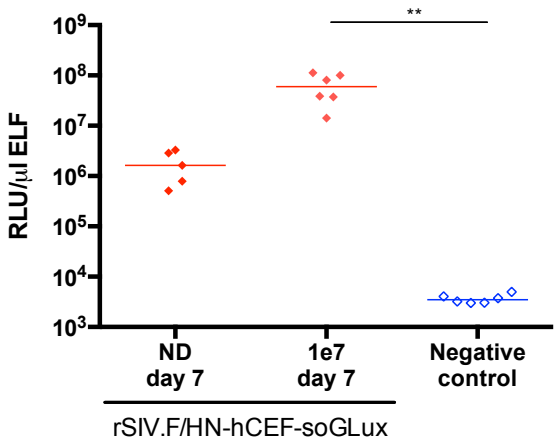
contrast, animals treated with unconcentrated virus showed no detectable GLux expression in serum.

Taken together, the data from this experiment are the first proof-of-concept that lentiviral gene therapy can produce a secreted reporter protein (GLux) in the lung, and gene delivery to the lung results in reporter protein expression in the serum. In a continuation of this experiment, I will next analyse the duration of gene expression in animals treated with the high dose (concentrated) lentivirus.

A. Lung tissue homogenate



B. Epithelial lining fluid



C. Serum

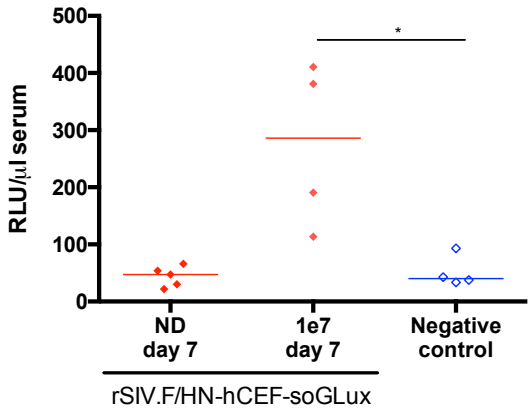


Figure 5-1 Expression of Gaussia luciferase in mice treated with rSIV.F/HN-hCEF-soGLux

Mice (n=6 per group) were transduced with rSIV.F/HN carrying *Gaussia* luciferase (soGLux) or negative control (PBS) by nasal sniffing, and sacrificed at day 7 post-treatment. Treated mice were given either 4 doses of 100 µl unconcentrated virus, the titre of which could not be determined (ND), or one dose of 100 µl concentrated virus (1e7 TTU/mouse). *Gaussia* luciferase expression was determined in (a) lung tissue homogenate, (b) epithelial lining fluid (ELF) and (c) serum.

Each data point represents one animal. Horizontal bars represent group medians. RLU=relative light units.

*=$p<0.05$, **=$p<0.01$, *** $p<0.001$ (Kruskal-Wallis test with Dunn's post-hoc analysis).

5.3.2 Transduction with a single dose of lentivirus leads to GLux expression for at least one year

Encouraged by the results from the short-term GLux experiment, a further 16 animals were treated with one dose of concentrated virus (1e7 TTU/dose), and harvested at day 28, 102 and 365 post-transduction to determine the length of reporter gene expression following lentiviral transduction. It was decided that the dose of 1e7 TTU would be most appropriate because in the previous experiment, the lower dose was not sufficient for reporter gene expression in the serum. Expression was stable for at least 12 months in both lung tissue homogenate and ELF; whilst serum expression was more modest, GLux levels were still stable for 365 days:

- **Lung tissue homogenate** (Figure 5-2A):

  treated: 2959 (1197-5512) RLU/mg protein at day 365

  untreated: (0.13 (0.07-0.27) RLU/mg protein, $p<0.01$

- **ELF** (Figure 5-2B):

  treated: 1.16e8 (1.06e8-1.63 e8) RLU/µl at day 365

  untreated: 4.85e3 (4.26e3-5.33e3) RLU/µl, $p<0.01$

- **Serum** (Figure 5-2C):

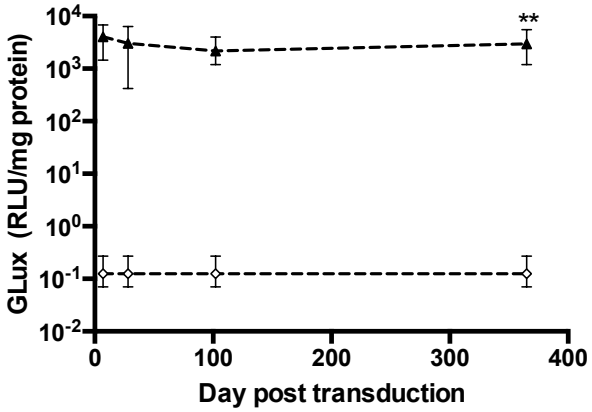
  treated: 286 (114-411) RLU/µl at day 365

  untreated: 38 (23-61) RLU/µl, $p<0.05$

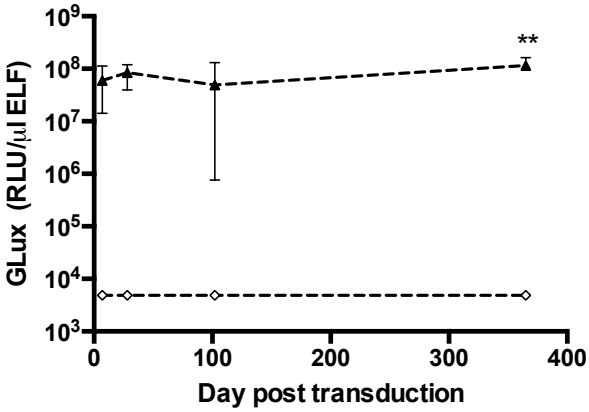
  Data expressed as median (range)

This data shows that a single dose of lentiviral gene therapy to the murine lung results in high levels of secreted reporter gene expression for at least one year. This provides a suitable platform for the investigation of viral vectors carrying the therapeutic transgene, α_1-antitrypsin.

A. Lung tissue homogenate



B. Epithelial lining fluid



C. Serum

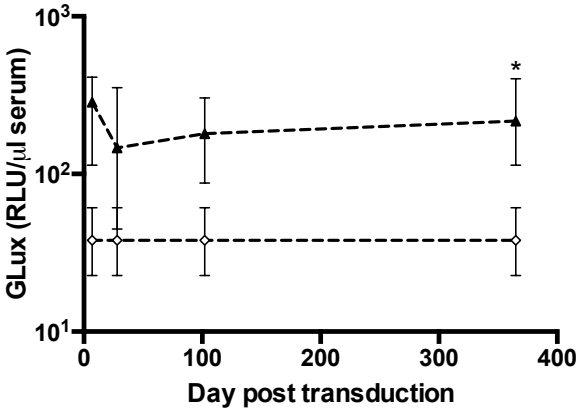


Figure 5-2 Sustained expression of Gaussia luciferase for at least one year following lentiviral transduction

Mice (n=5-6 per group) were transduced with rSIV.F/HN carrying *Gaussia* luciferase (1e7 TTU/mouse, closed symbols) or negative control (PBS, open symbols) by nasal sniffing, and sacrificed at the indicated timepoints. *Gaussia* luciferase expression was determined in (a) lung tissue homogenate, (b) epithelial lining fluid (ELF) and (c) serum. Symbols represent group medians (n=5-6 per group), error bars represent range.

RLU, relative light units.

*=$p<0.05$, **=$p<0.01$ compared to negative control at day 365 (Mann Whitney *U* test).

5.3.3 Lentiviral transduction of the murine airway leads to significant levels of hAAT expression at day 7

Having shown that delivery of the lentiviral vector rSIV.F/HN to the lung results in significant expression of the secreted reporter protein *Gaussia* luciferase, a short-term *in vivo* experiment using a virus carrying the human α_1-antitrypsin gene was performed.

Because the unconcentrated virus used in the short-term GLux experiment led to relatively low expression levels, it was decided to only use concentrated virus in this experiment. Mice (n=4-6 per group) were treated with either (a) a single dose of rSIV.F/HN-hCEF-sohAAT, (b) five doses of rSIV.F/HN-hCEF-sohAAT on consecutive days, or (c) diluent (negative control). The two different doses were chosen (1) to see if a dose-response relationship could be observed, and (2) because I wanted to use the maximum feasible dose based on virus stocks available at the time of the experiment. The virus batch used was still unpurified, so only modest expression was expected. The virus titre was 2e7 TTU/dose, so animals receiving 5 doses received 1e8 TTU virus.

Animals were harvested seven days after the first dose, and expression of hAAT determined in lung tissue homogenate, epithelial lining fluid and serum (Figure 5-3). When compared to the negative control group, animals in the 2e7 TTU (single dose) group expressed significant hAAT in all three tissues analysed:

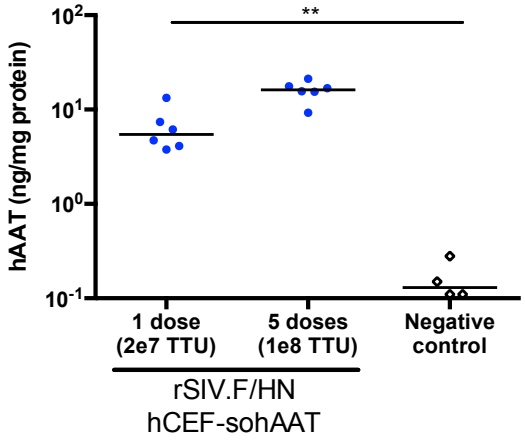
- **Lung:** *treated:* 5.4 (3.7-13) ng hAAT per mg total protein; *untreated:* 0.13 (0.11-0.28) ng hAAT per mg total protein; $p<0.01$ (Mann-Whitney *U* test)
- **ELF:** *treated*: 3249 (2149-4508) ng/ml; *untreated:* 182 (115-343) ng/ml; $p<0.05$
- **Serum**: *treated:* 30 (22-62) ng/ml; *untreated:* 1.8 (0.78-3.1) ng/ml; $p<0.01$
- Data expressed as median (range)

It was also notable that animals receiving 5 doses (1e8 TTU) showed 2.4-4.5 fold increases in protein expression compared to animals receiving a single dose:

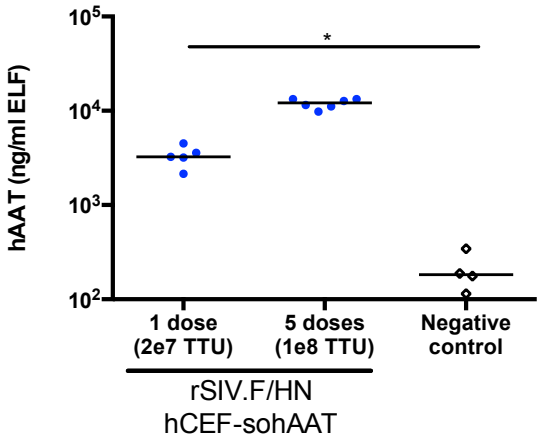
- **Lung:** 3-fold increase
    - *multiple dose group:* 16 (9.3-21) ng hAAT per mg total protein
    - *single dose group:* 5.4 (3.7-13) ng hAAT per mg protein
- **ELF**: 3.7-fold increase
    - *multiple dose group:* 12,098 (9793-13, 296) ng/ml
    - *single dose group:* 3249 (2149-4508) ng/ml
- **Serum**: 5.7-fold increase
    - *multiple dose group:* 172 (112-201) ng/ml
    - *single dose group:* 30 (22-62) ng/ml

These data show that lentiviral gene therapy can produce significant levels of a therapeutic protein, α_1-antitrypsin, in the murine lung for at least 7 days. In the next experiment the duration of gene expression will be analysed in greater detail.

A. Lung tissue homogenate



B. Epithelial lining fluid



C. Serum

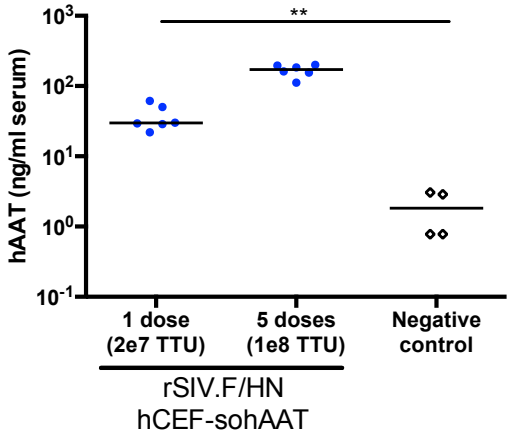


Figure 5-3 Expression of α_1-antitrypsin in mice treated with rSIV.F/HN-hCEF-sohAAT

Mice (n=4-6 per group) were treated with rSIV.F/HN carrying hAAT or negative control. Mice received either one dose (2e7 TTU/mouse) or five doses (1e8 TTU/mouse) of concentrated virus and were harvested 7 days after the first dose. hAAT expression was quantified in (a) lung tissue homogenate, (b) epithelial lining fluid (ELF) and (c) serum.

Each data point represents one animal. Horizontal bars represent group medians.

*=$p<0.05$, **=$p<0.01$ (Kruskal-Wallis test with Dunn's post-hoc analysis).

5.3.4 A single dose of lentivirus leads to hAAT expression for at least 20 months in murine airway

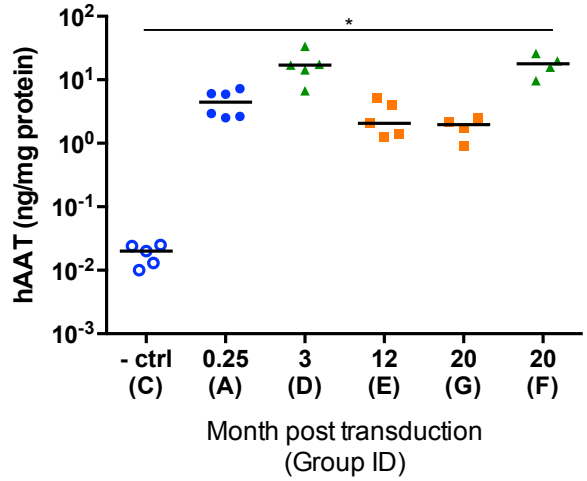
Having observed short-term expression of α_1-antitrypsin 7 days after lentiviral-mediated gene transfer, I next analysed the long-term duration of gene expression in mice.

A further cohort of mice was treated with rSIV.F/HN-hCEF-sohAAT. The same batch of unpurified lentivirus used in the short-term experiment described above was used in this experiment, and all experimental variables (apart from harvest date) were kept identical to allow direct comparison between the two experiments.

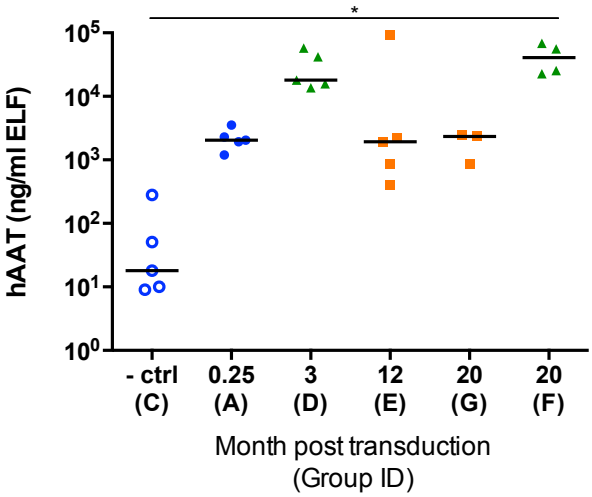
Limited quantities of the virus batch were available due to high production costs, so animals were treated with the "low" dose used in the initial experiment (2e7 TTU in 100 µl total volume). In total, 20 additional mice were treated in two groups of 10, six weeks apart, due to the mice stocks available at the time of the experiment.

The overall results of the experiment are presented on Figure 5-4.

A. Lung tissue homogenate



B. Epithelial lining fluid



C. Serum

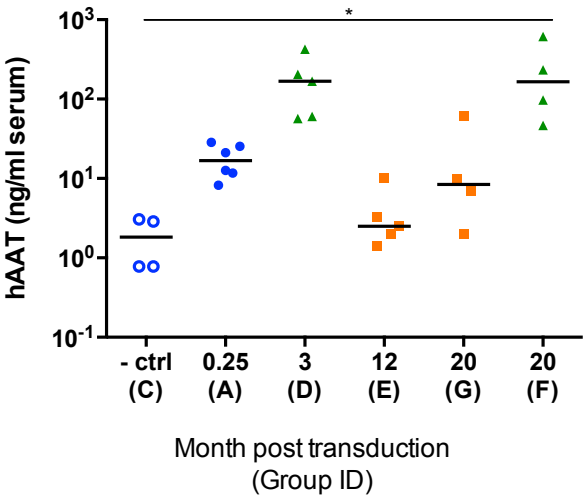


Figure 5-4 Sustained expression of α_1-antitrypsin for 20 months following lentivirus transduction

Mice (n=4-6 per group) were treated with a single dose of rSIV.F/HN-hCEF sohAAT (2e7 TTU/mouse) or negative control by nasal sniffing, and sacrificed at the indicated timepoints (shown on the x-axis, along with the group identifier in brackets). hAAT expression was determined in (a) lung tissue homogenate, (b) epithelial lining fluid (ELF) and (c) serum. All samples for each tissue were analysed on the same hAAT ELISA kit.

Animals received the same batch and dose of virus, but were treated on different days. Group A (blue dots) were treated on 11th November 2013. Groups D and F (green triangles) were treated on 28th November 2013. Groups E and G (orange squares) were treated on 11th January 2014.

Please note that Group B is not represented on this graph, because these animals received a higher dose.

Each data point represents one animal. Horizontal bars represent group medians. *=$p<0.05$ (comparing month 20 samples (Group F) with the negative control samples; Mann Whitney *U* test).

Table 5-3 Treatment groups in the long-term hAAT *in vivo* experiment (Figure 5-3 and Figure 5-4)

Group	Treated with	Date treated	Titre given (TTU/dose)	*n* treated	*n* survived to harvest *(%)*	Harvest (*x* days after dose 1)
A	rSIV.F/HN hCEF so hAAT batch #1	11-Nov-2013	2e7	6	6 *(100)*	7
B			1e8 (5 doses)	6	6 *(100)*	7
C	DMEM-20		0	4	4 *(100)*	7
D	rSIV.F/HN hCEF so hAAT batch #1	28-Nov-2013	2e7	5	5 *(100)*	85 (3 months)
E		14-Jan-2014		5	5 *(100)*	365 (12 months)
F		28-Nov-2013		5	4 *(80)*	623 (20 months)
G		14-Jan-2014		5	4 *(80)*	576 (20 months)

Groups A, B and C were the first cohort. They were treated on 11th November 2013 and were harvested on day 7 (data reported on Figure 5-3).

The experiment was extended when 20 further mice were treated with the same batch and dose of virus. These 20 mice were treated on two separate days, as described in the table. All experimental variables other than harvest date were kept identical, allowing joint analyses of hAAT expression data.

In Group F, one animal was found dead by staff at the animal house. This animal was 463 days (15 months) old. In Group G, one animal was found dead by staff at the animal house. This animal was 502 days (15 months) old.

TTU=TaqMan Transducing Units

Expression of α_1-antrypsin in mice 20 months post-gene transfer

The oldest animals examined (group F, n=4) were culled 20 months post-gene transfer. In these animals, hAAT expression was higher than negative controls in all tissues analysed:

- **Lung** (Figure 5-4A):

  treated: 17.84 (9.6-26) ng hAAT per mg protein

  untreated: 0.02 (0.01-0.03) ng hAAT per mg protein, $p<0.05$ (Mann-Whitney *U* Test)

- **ELF** (Figure 5-4B):

  treated: 40,700 (22,723-68,777) ng/ml

  untreated: 18 (9-280) ng/ml, $p<0.05$

- **Serum** (Figure 5-4C)

  treated: 166 (47-613) ng/ml

  untreated: 1.8 (0.78-3.1) ng/ml, $p<0.05$

- Data expressed as median (range)

This encouraging data shows that one dose of rSIV.F/HN can produce a secreted protein for 20 months, which is close to the lifetime expectancy of a mouse.

Subgroup analysis of long-term hAAT experiment

As discussed above, the long-term experiment was a continuation of the short-term experiment, rather than being a completely separate experiment. For logistical reasons, the additional mice were treated in two sets of ten, 6 weeks apart (for details of the treatment dates, please refer to Table 5-3). At the conclusion of the experiment, it became apparent that there was a difference between these two sets of mice. Mice treated on 28th November 2013 (Groups D and F, represented by green triangles on Figure 5-4) showed approximately one log higher hAAT expression compared to those treated on 11th January 2014 (Groups E and G, orange squares on Figure 5-4).

The virus batch used was the first preparation of rSIV.F/HN-hCEF-sohAAT, which had been aliquoted at the time of production and immediately frozen and stored. Each aliquot was thawed one hour before use, and had not been thawed in the interim period. This suggests that the viral particles which had been frozen for a longer period of time had degraded; this may be because this was a low quality preparation, which had not been purified or concentrated. Nevertheless, animals treated with virus always showed significantly higher hAAT expression in lung tissue homogenate and ELF compared to negative controls (Figure 5-5). In mice treated with 'older' virus (Groups E and G), serum hAAT was at background levels.

Importantly, when the expression of hAAT was compared between groups of animals *treated* on the same day but *harvested* on different days, there was no significant difference between the group medians. This suggests that rSIV.F/HN leads to very stable levels of secreted protein in murine lung.

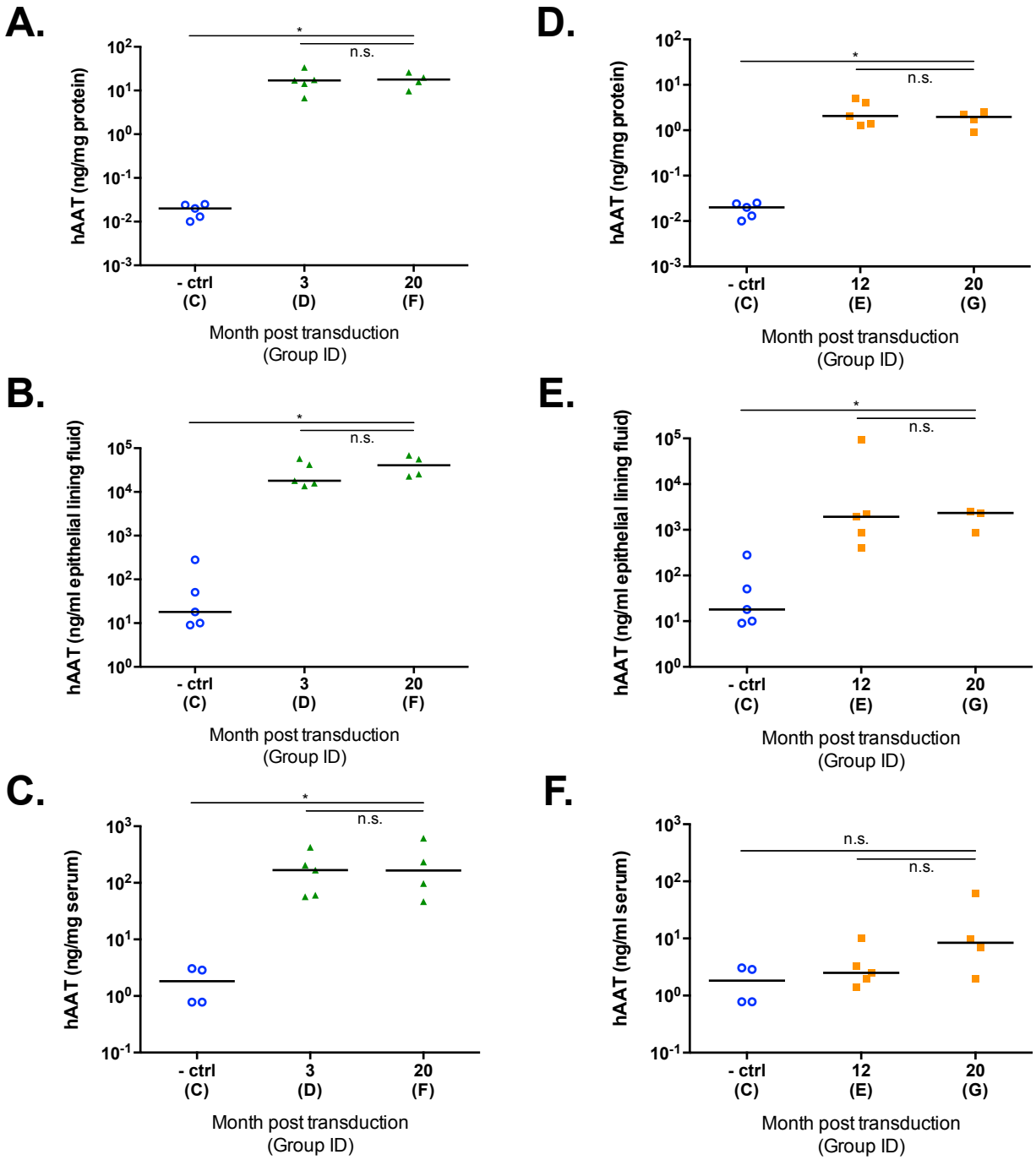


Figure 5-5 Analysis of the effect of virus storage on efficacy of gene transfer

Here, data from Figure 5-4 are separated according to the treatment day.
(**A-C**) Mice (n=4-6 per group) were treated with a single dose of rSIV.F/HN-hCEF-sohAAT (2e7 TTU/mouse) on 28th November 2013 and harvested 3 or 20 months post-transduction. hAAT expression was determined in (a) lung tissue homogenate, (b) epithelial lining fluid and (c) serum and compared to archived negative control samples.
(**D-F**) Mice (n=4-6 per group) were treated with a single dose of rSIV.F/HN-hCEF-sohAAT (2e7 TTU/mouse) on 11th January 2014 and harvested 12 or 20 months post-transduction. hAAT expression was determined in (d) lung tissue homogenate, (e) epithelial lining fluid and (f) serum and compared to archived negative control samples.
Each data point represents one animal. Horizontal bars represent group medians. *=$p<0.05$, ns=not significant (Kruskal-Wallis test with Dunn's post-hoc analysis).

Conclusions from the long-term hAAT experiment

These data show that a single treatment with rSIV.F/HN-hCEF-sohAAT generates sustained hAAT expression for 20 months. However, the mean level of hAAT observed in the epithelial lining fluid (~3000 ng/ml for animals given 2e7 TTU virus, ~12,000 ng/ml for animals given 1e8 TTU virus) is sub-therapeutic and does not meet the target hAAT threshold of 70,000 ng/ml. Further modifications to the virus preparation will therefore be necessary to drive therapeutic levels. An increased titre of viral vector would be desirable, however improvements in the viral production technique to purify and concentrate the virus may themselves lead to higher hAAT expression.

5.3.5 A purified lentivirus leads to higher expression of hAAT

In the hAAT experiments presented so far in this chapter, a low quality, unpurified lentiviral vector was used. Throughout the course of this project the UK Cystic Fibrosis Gene Therapy Consortium was improving the viral production methods, with the first significant improvement being the availability of AKTA purification. In the next *in* vivo experiment, a highly purified batch of rSIV.F/HN-hCEF-sohAAT was therefore used.

A purified batch yields about 20 mls of virus, which was then concentrated approximately 10-fold in Millipore spin columns and delivered to 5 mice (3 doses approximately 10 days apart), with a total of 1.5e8 TTU given to each animal. The mice were harvested 10 days after the final dose, and hAAT expression quantified and compared to archived negative control tissues.

Expression of hAAT was significantly higher in animals treated with lentivirus than negative controls:

- **Lung** (Figure 5-6A):

treated: 20 (12-61) ng hAAT per mg total protein

untreated: 0.018 (0.012-0.053) ng hAAT per mg protein, $p<0.01$

- **ELF (**Figure 5-6B)*:*

  treated: 70,024 (52,752-174,738) ng/ml

  untreated: 188 (115-343) ng/ml, $p<0.05$

- **Serum (**Figure 5-6B)

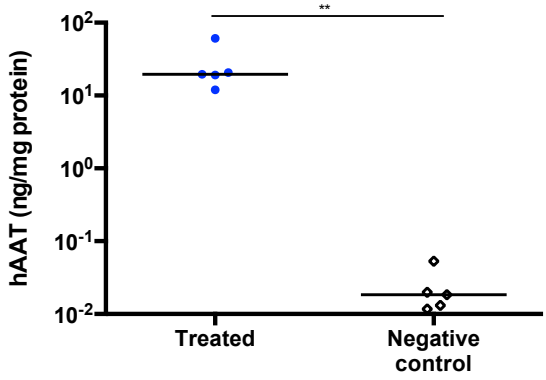
  treated: 385 (158-465) ng/ml

  untreated: 6.5 (4.8-9.7) ng/ml, $p<0.01$

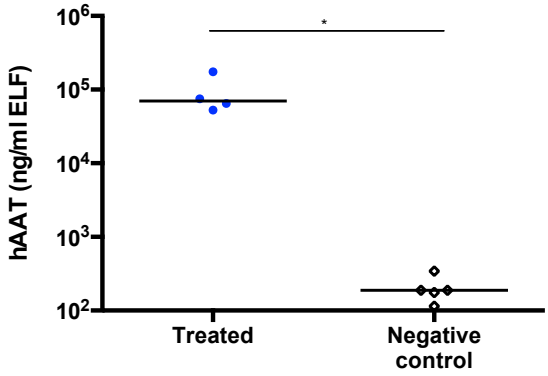
- Data expressed as median (range)

Encouragingly, the median hAAT expression in ELF (~70,000 ng/ml) is equivalent to the therapeutic threshold in human ELF of 70,000 ng/ml, providing proof-of-concept that hAAT gene therapy with highly purified lentiviral vectors can produce therapeutic levels of hAAT in an animal model. Further improvements in the lentivirus production techniques may produce still higher levels of hAAT and will be considered next.

A. Lung tissue homogenate



B. Epithelial lining fluid



C. Serum

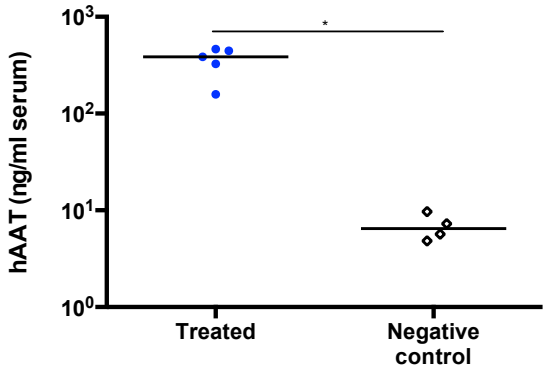


Figure 5-6 Expression of hAAT following administration of purified lentivirus produced in adherent cell cultures to the murine lung

Purified rSIV.F/HN-hCEF-sohAAT was administered to the lungs of n=5 mice by nasal sniffing (3 doses at 10-day intervals, total dose 1.5e8 TTU/mouse). Animals were sacrificed 10 days after the final dose and hAAT expression determined in (a) lung tissue homogenate, (b) epithelial lining fluid (ELF) and (c) serum, compared to archived negative control samples.

Each data point represents one animal. Horizontal bars represent group medians.

*=$p<0.05$, **=$p<0.01$ (Mann Whitney *U* test).

5.3.6 Lentiviral vectors produced in suspension cultures transduce murine airway

The two virus preparations used in the above experiments were produced in the Consortium's London laboratory, using adherent cultures of HEK293T cells. An alternative method of production is to use suspension cultures, which allow for higher volumes to be handled; this leads to increased viral yield. In the Consortium's Oxford laboratory, investigators have successfully scaled up viral production to 5 litre batches and, importantly, have developed a method that is serum-free and GMP-compliant and is therefore suitable to use for a first-in-man trial of lentiviral gene therapy for cystic fibrosis (manuscript submitted).

A preparation of rSIV.F/HN-hCEF-sohAAT produced in a 5 litre suspension culture was assessed *in vivo*. Mice (n=6 per group) were treated with (a) 1e8 TTU in 100 µl total volume or (b) an irrelevant lentivirus (rSIV.F/HN-hCEF-GFPLux) and harvested 10 days post-gene transfer.

Expression of hAAT was significantly higher in animals treated with a lentivirus coding for hAAT compared to an irrelevant gene in all three tissues:

- **Lung** (Figure 5-7A):

  treated: 23 (2.9-37) ng hAAT per mg total protein

  untreated: 0.018 (0.012-0.053) ng hAAT per mg protein, $p<0.01$
- **ELF** (Figure 5-7B):

  treated: 67,470 (445-207,830) ng/ml

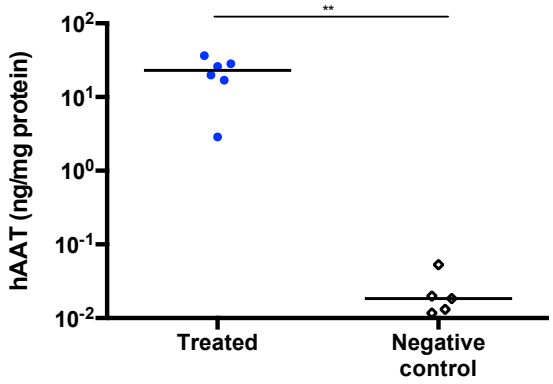
  untreated: 104 (75-136) ng/ml, $p<0.01$
- **Serum** (Figure 5-7C):

  treated: 398 (86-676) ng/ml

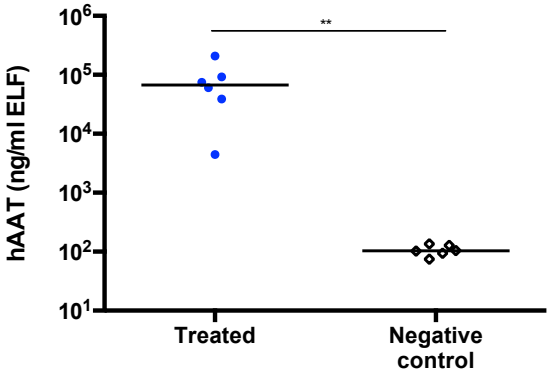
  untreated: 5.4 (4.6-8.2) ng/ml, $p<0.01$
- Data expressed as median (range)

The level of human α_1-antitrypsin seen in murine ELF following treatment with virus produced in GMP-compliant serum-free cell suspension cultures was similar to the therapeutic target level in ELF of 70,000 ng/ml.

A. Lung tissue homogenate



B. Epithelial lining fluid



C. Serum

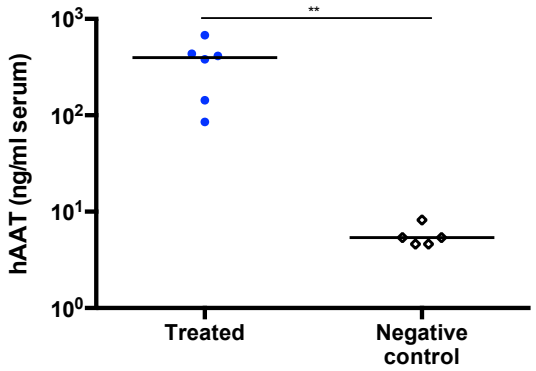


Figure 5-7 Expression of hAAT following administration of purified lentivirus produced in suspension cell cultures to the murine lung

Purified virus manufactured in 5 litre suspension cultures was administered to the lungs of n=6 mice by nasal sniffing (one dose, 1e8 TTU virus/mouse). Six negative control mice were given an irrelevant lentivirus (rSIV.F/HN hCEF-GFP-Lux). Animals were sacrificed 10 days after the final dose and hAAT expression determined in (a) lung tissue homogenate, (b) epithelial lining fluid (ELF) and (c) serum.

Each data point represents one animal. Horizontal bars represent group medians.

**=$p<0.01$ (Mann Whitney *U* test).

5.3.7 Improvements in vector production method result in improved transgene expression

Having performed three separate experiments where mice were treated with rSIV.F/HN-hCEF-sohAAT, I compared the relative hAAT expression between experiments. As all mice received the same volume (100 µl) of the same vector construct by the same delivery method (nasal sniffing), the following samples could justifiably be compared together:

- From Figure 5-3, animals treated with 2e7 TTU and 1e8 TTU virus (1 to 5 doses, harvested 7 days after the first dose; animals represented by black squares on Figure 5-8);
- From Figure 5-6, animals treated with 1.5e8 TTU virus (3 doses, harvested 10 days after the final dose; animals represented by black triangles on Figure 5-8);
- From Figure 5-7, animals treated with 1e8 TTU virus (1 dose, harvested 10 days after the final dose; animals represented by black dots on Figure 5-8).

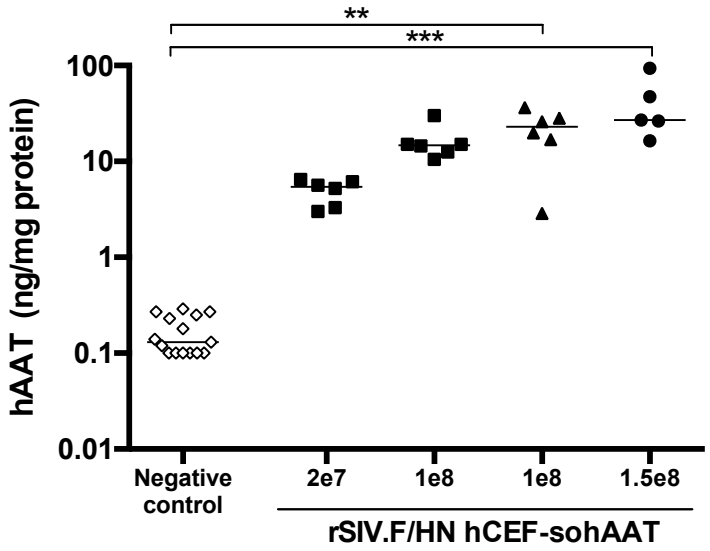
The selected data are displayed together on Figure 5-8, with groups arranged by increasing viral titre. With an increased viral titre, improved expression of hAAT was generally observed. Of particular interest is the level of hAAT expression in epithelial lining fluid (ELF, Figure 5-8B). As discussed previously, the target level of hAAT expression in ELF is 70 µg/ml (70,000 ng/ml), a level of hAAT that has been shown to be protective in patients with Pi*SZ (intermediate hAAT deficiency). All animals treated with 1.5e8 TTU virus showed hAAT expression comparable to this therapeutic target level, as did the majority of animals treated with 1e8 TTU virus produced in suspension cultures (black triangles on Figure 5-8).

It is also interesting to compare the hAAT expression between the two groups treated with 1e8 TTU of unpurified virus produced in adherent cultures (black squares) and 1e8 TTU purified virus produced in serum-free suspension cultures (black triangles). Although there was no statistically significant difference in hAAT expression between these two groups of

animals in the three tissues examined, there was a trend towards improved hAAT expression for animals treated with purified virus, which suggests that the improved viral production techniques improve transgene expression.

The *in vivo* data presented here show that treating wild-type mice with rSIV.F/HN-hCEF-sohAAT results in significant expression of hAAT that is similar to the therapeutic target level in human ELF. This justifies progression towards large animal studies, which will be considered in detail towards the end of the current chapter.

A. Lung tissue homogenate



B. Epithelial lining fluid

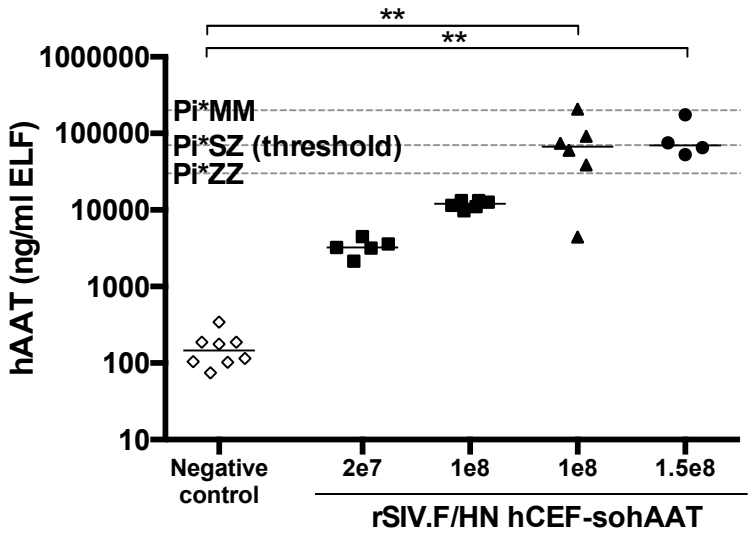


Figure 5-8 Dose-dependent expression of hAAT following lentiviral transduction *(continues on next page)*

For details please refer to legend on next page

C. Serum

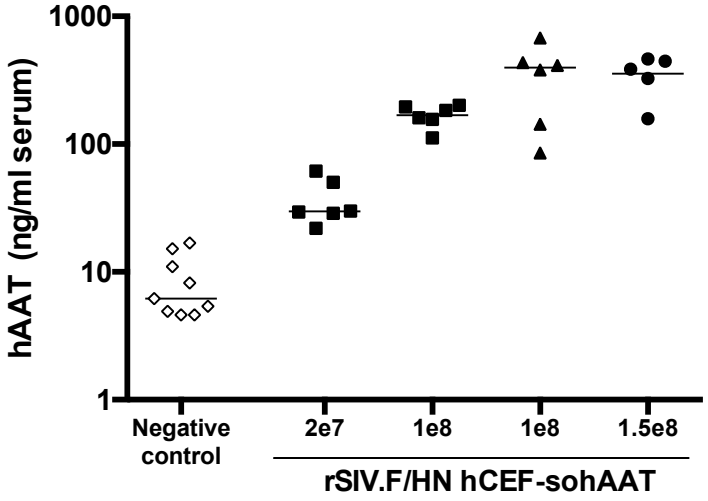


Figure 5-8 Dose-dependent expression of hAAT following lentiviral transduction *(continued from previous page)*

In three independent experiments described in this chapter, mice were transduced with four different doses of rSIV.F/HN-hCEF-sohAAT or negative control (PBS) by nasal sniffing. Mice were given doses between 2e7 to 1.5e8 as indicated in the figure (n=4-6 per group). Mice were sacrificed 7-10 days after the final dose and hAAT expression determined in (a) lung tissue homogenate, (b) epithelial lining fluid (ELF) and (c) serum. Each data point represents an individual animal, horizontal bars indicate the group medians.

In (b), dashed lines represent the normal levels of hAAT in epithelial lining fluid for adult humans with the indicated alpha-1-antitrypsin genotypes, Pi*MM (normal), Pi*SZ (intermediate deficiency) and Pi*ZZ (severe deficiency). The serum level of hAAT in Pi*SZ patients is considered a therapeutic target level [21].

**=$p<0.01$, ***$p<0.001$ (Kruskal Wallis test with Dunn's post-hoc analysis).

5.3.8 Does the level of hAAT in serum predict the level of hAAT in lung and ELF post gene therapy?

The ultimate goal of the current work is to perform an α_1-antitrypsin gene therapy trial in humans. In such a trial, it will not be feasible to quantify hAAT in lung tissue; and whilst it would be possible to sample hAAT in epithelial lining fluid, bronco-alveolar lavage is a very invasive procedure. It is therefore important to assess whether serum hAAT levels can predict the level of hAAT in the lung tissue and ELF, so I compared levels of serum hAAT with ELF/lung hAAT in all treated animals from the previous experiments.

As shown in Figure 5-9, there was a significant correlation between serum hAAT and both ELF hAAT (Spearman rank 0.88, $p<0.0001$) and lung tissue homogenate hAAT (Spearman rank 0.86, $p<0.0001$). These results raise the possibility of using serum hAAT as a predictor of lung and ELF hAAT in future pre-clinical or clinical studies; of note, serum could be collected repeatedly to assess the duration of gene expression in clinical trial patients.

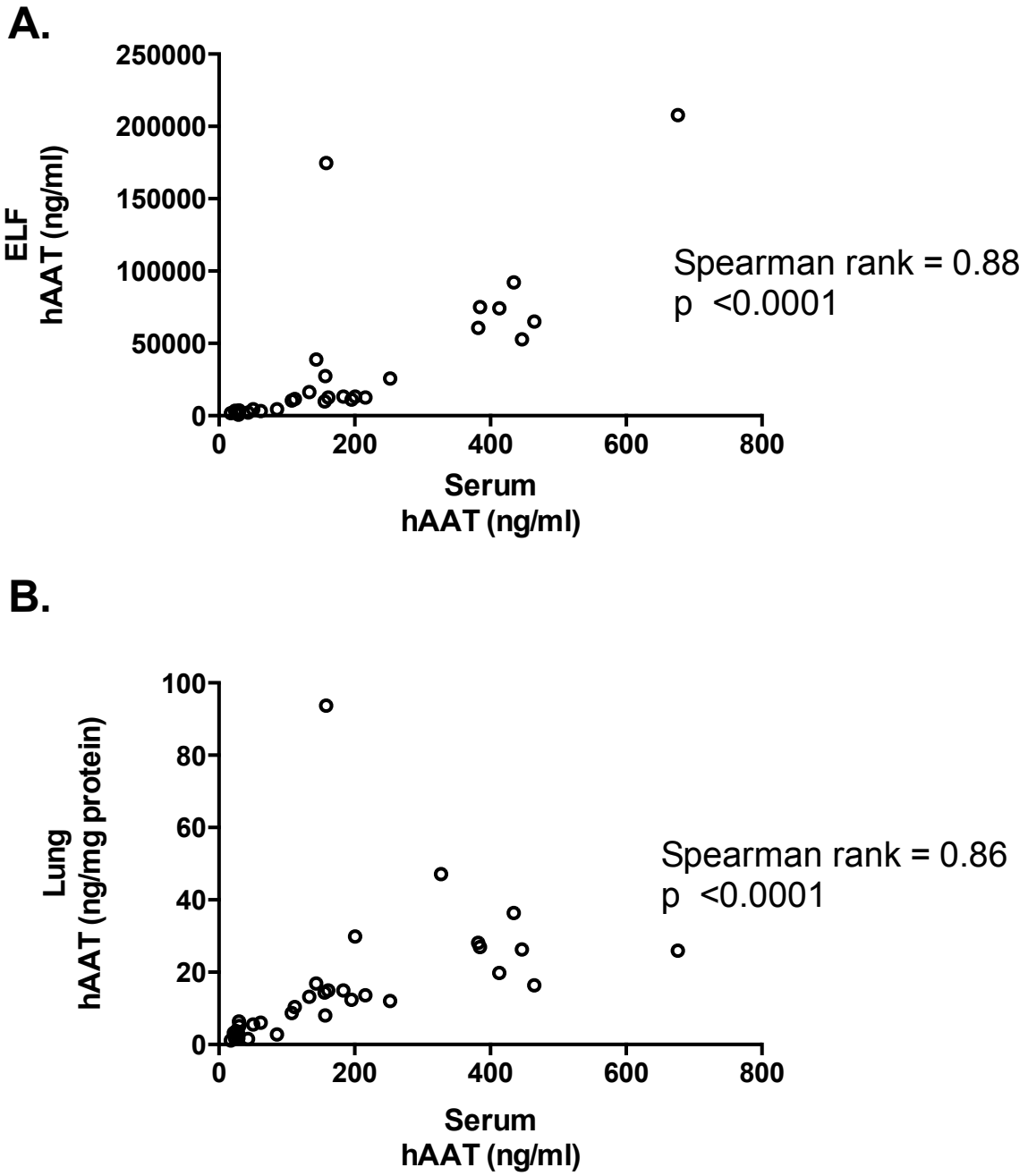


Figure 5-9 Correlation between level of hAAT in serum and airways following gene transfer to the lung

Mice were treated with rSIV.F/HN-hCEF-sohAAT in three separate experiments (for details see Figure 5-3, Figure 5-6 and Figure 5-7.) hAAT expression was quantified in serum, lung tissue homogenate and epithelial lining fluid (ELF). I compared the relationship between hAAT expression in (a) serum vs. ELF and (b) serum vs. lung tissue homogenate. A strong positive correlation was found (Spearman rank for non-parametric data). Each data point represents one animal.

5.3.9 rSIV.F/HN can be given repeatedly to the airway without loss of efficacy

A major concern when using a virus for gene therapy is a loss of efficacy on repeat dosing, due to a immune response to the virus. Furthermore, when treating chronic diseases such as α_1-antitrypsin deficiency, a single dose of gene therapy may not be sufficient to obtain therapeutic protein levels; therefore, it may be necessary to treat patients with multiple doses of gene transfer agents over subsequent days. To see whether there was a loss of efficacy of the rSIV.F/HN vector on repeat administration, a multi-dose experiment using rSIV.F/HN-hCEF-soGLux was planned.

It was not possible to titrate the virus prior to the experiment due to financial and personnel constraints, so a simple initial experiment was performed to ensure that the batch was functional *in vivo*. Two mice were treated with two different doses of virus, to select the smallest feasible dose of virus to use in the main repeat administration experiment. 7 days post-treatment, GLux expression in BAL was higher in the animal treated with undiluted virus (1.2e6 RLU/µl) than the animal treated with 1:10 diluted virus (4.2e5 RLU/µl) or an animal treated with PBS (1324 RLU/µl) (data not shown). For the main experiment, it was therefore decided to use 1:2 diluted virus.

An experiment was then performed in which mice received either (a) 10 doses of lentivirus carrying GLux, (b) 5 doses of PBS and then 5 doses of lentivirus, or (c) 9 doses of PBS followed by a single dose of lentivirus. The GLux expression in these mice was then compared to archived negative control samples, in which animals had received 6 doses of PBS (the decision not to include a new negative control group in this experiment was made on ethical grounds, as discussed above).

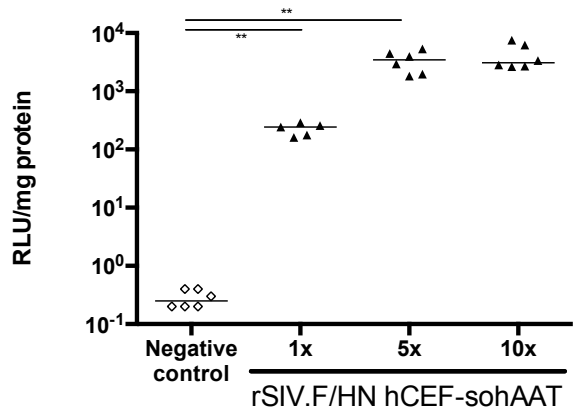
When compared with animals treated with a single dose of virus, animals given 10 doses showed approximately 10-fold higher GLux expression in all tissues (Figure 5-10). There was

a significant increase in gene expression between animals given one dose and five doses of lentiviral vector:

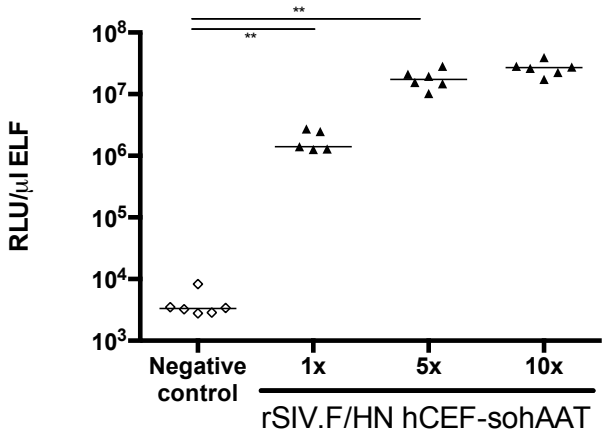
- **Lung:**
    - 1 dose: 243 (160-290) RLU/mg protein
    - 5 doses: 3457 (1822-5333) RLU/ml, $p<0.01$
- **BALF**
    - 1 dose: 1.4e6 (1.3e6-2.7e6) RLU/µl
    - 5 doses: 1.7e7 (1.0e7-2.8e7) RLU/µl, $p<0.01$
- **Serum:**
    - 1 dose: 37 (30-53) RLU/µl
    - 5 doses: 79 (49-115) RLU/µl, $p<0.01$
- Data expressed as median (range)

This data suggests that rSIV.F/HN can be repeatedly administered on subsequent days with no loss of efficacy; this could be important in clinical trials, if multiple doses are needed to deliver the necessary titre of viral vector.

A. Lung tissue homogenate



B. Epithelial lining fluid



C. Serum

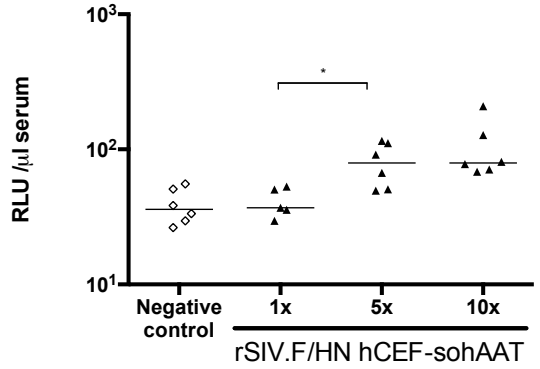


Figure 5-10 There is no loss of efficacy on repeat administration of rSIV.F/HN-hCEF-soGLux

Mice were transduced with 1, 5 or 10 doses of rSIV.F/HN carrying *Gaussia* luciferase (100 µl total volume, titre unknown) and given PBS on other days. Control animals were treated with 10 doses of PBS. Animals were culled 7 days after the final dose and *Gaussia* luciferase expression was determined in (a) lung tissue homogenate, (b) epithelial lining fluid (ELF) and (c) serum.

Each data point represents one animal. Horizontal bars represent group medians.

*=$p<0.05$, **=$p<0.01$ (Mann Whitney *U* test).

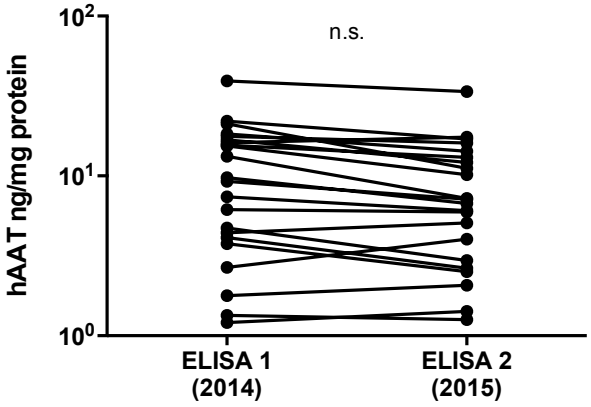
5.3.10 Long-term stability of hAAT in frozen tissue samples

Human proteins may not be stable in frozen mouse tissue samples, and although I was unable to find any literature suggesting that α_1-antitrypin degrades over time in frozen samples, it remains a theoretical risk.

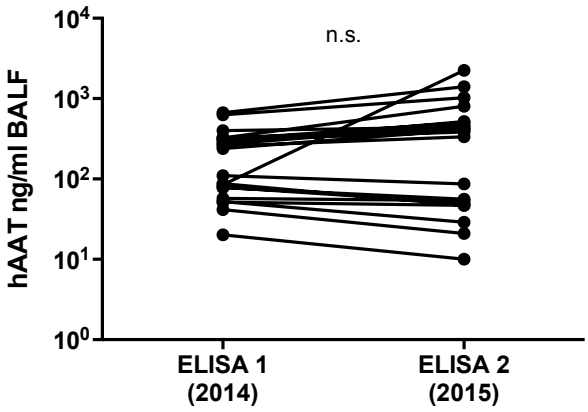
I therefore compared α_1-antitrypsin expression in lung tissue homogenate, broncho-alveolar lavage fluid (BALF) and serum samples from a subset of stored samples, which were anaylsed on an ELISA in 2014 and then again in 12 months later, having been frozen in the interim. Note that broncho-alveolar lavage fluid is collected after performing a lavage, and this is what is analysed on an ELISA and stored. Therefore, it is not appropriate to discuss epithelial lining fluid (ELF) in this context.

In all three tissues, there was no statistically significant difference in the group medians between the two ELISAs performed on the same stored samples in 2014 and 2015 (Figure 5-11). This suggests that human α_1-antitrypsin produced following gene transfer is stable in frozen murine tissues for at least one year.

A. Lung tissue homogenate



B. Broncho-alveolar lavage fluid



C. Serum

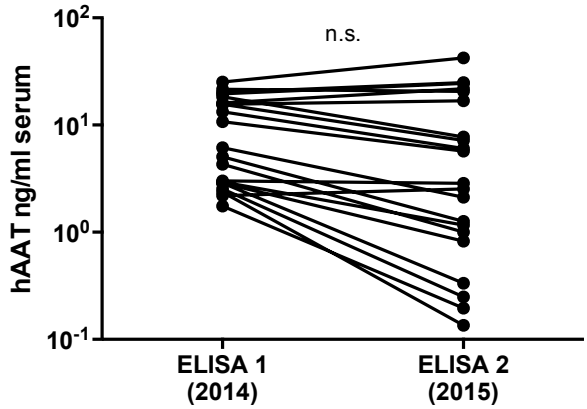


Figure 5-11 Stability of human α_1-antitrypsin in frozen lung tissue homogenate, broncho-alveolar lavage fluid and serum samples

(**A**) 22 lung samples, (**B**) 21 broncho-alveolar lavage (BALF) samples and (**C**) 21 serum samples from Figure 5-4 and
Figure 5-5 were analysed twice, approximately one year apart, having been stored in the interim at -80°C. Each paired data point represents the human α_1-antitrypsin (hAAT) expression in the same sample analysed at two different time points.
ns=no statistically significant difference between group means (Mann-Whitney *U* Test).

5.3.11 Transduction of ovine lung with rSIV.F/HN

Having demonstrated that I can achieve therapeutic levels of hAAT in the murine epithelial lining fluid, a logical progression is to treat a larger animal with the lentiviral vector.

Three sheep were treated with approximately 2.9e7 TTU of rSIV.F/HN-hCEF-soGLux given to the right lung, and approximately 2.8e8 TTU of rSIV.F/HN-hCEF-sohAAT given to the left lung. A Trudell AeroProbe catheter was positioned at the level of the 2nd ventral diaphragmatic segment on the left or the right side, and 2 mls of aerosolised vector delivered towards the left or right caudal diaphragmatic segment (Figure 5-12).

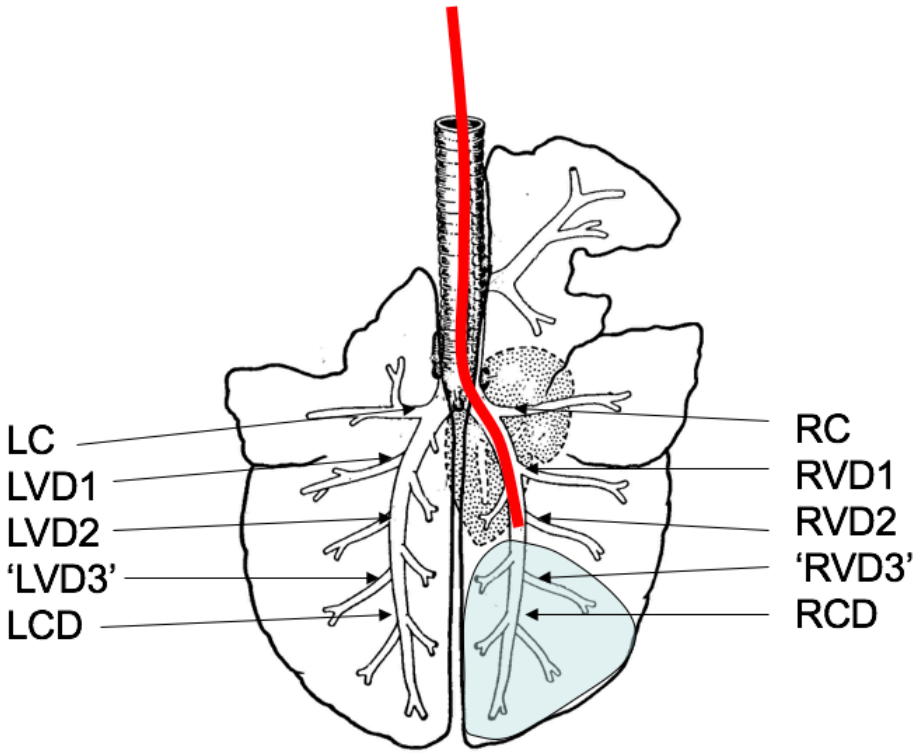


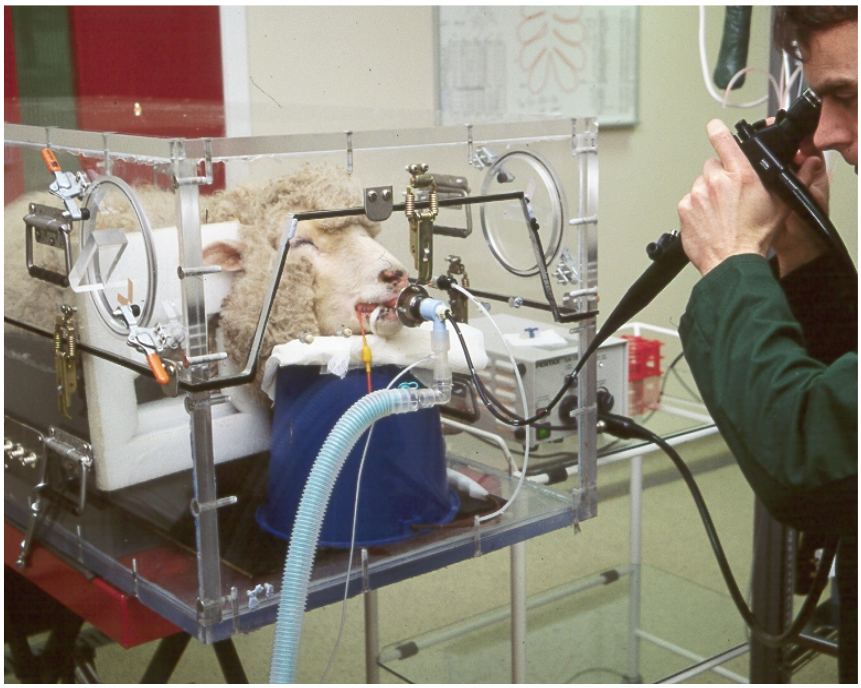
Figure 5-12 Delivery of lentiviral vectors to the ovine lung using the Trudell AeroProbe

Sheep were given lentiviral vectors by a Trudell AeroProbe catheter, which was positioned at the level of the 2nd ventral diaphragmatic segment. The diagram shows the positioning for the right lung; for delivery to the left lung, the catheter would be positioned at the same level on the left side.

Shaded in blue is the area likely to be exposed to aerosol. LC, left cardiac segment of the apicocardiac lobe; LCD, left caudal diaphragmatic; LVD, left ventral diaphragmatic (1st/2nd/3rd segment); RC, right cardiac; RCD, right caudal diaphragmatic; RVD, right ventral diaphragmatic (1st/2nd/3rd segment).

Picture courtesy of Dr. Gerry McLachlan.

A.



B.

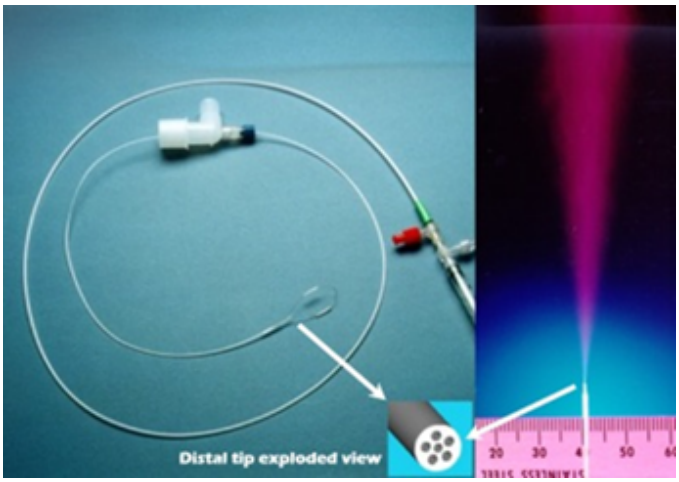


Figure 5-13 Equipment used in the sheep experiment

(**A**) Sheep were placed in a whole body negative pressure ventilator and anaesthetised with gaseous halothane. A bronchoscope was passed, allowing visualisation of the trachea and bronchioles.
(**B**) The Trudell AeroProbe multi-lumen catheter was passed down the bronchoscope. This catheter delivers sheared liquid droplets to the directed area of the lung.

Photos courtesy of Dr. Gerry McLachlan.

BALF samples were collected 14 days pre-treatment, and 7 and 14-days post-treatment. BALF samples from each lung at each time point were analysed for GLux expression, and compared to the pre-treatment samples. There was no difference between the post-treatment and the pre-treatment samples for either the first BAL washing (Figure 5-14A) or the second washing (Figure 5-14B). In contrast, a 0.1 mg/ml sample of recombinant *Gaussia* luciferase analysed alongside these samples gave the expected RLU reading of 2e4 RLU/µl, meaning that failure of the GLux assay can be ruled out as a cause for lack of protein detection.

BALF samples from day 7 only were then analysed for hAAT expression and compared to pre-treatment samples. hAAT was barely detectable above the lower limit of detection of the assay in all BALF samples, whether pre- or post-treatment (Figure 5-15). I therefore conclude that at this titre, rSIV.F/HN vectors carrying the hCEFI promoter cannot produce significant levels of transgene in the lungs of sheep.

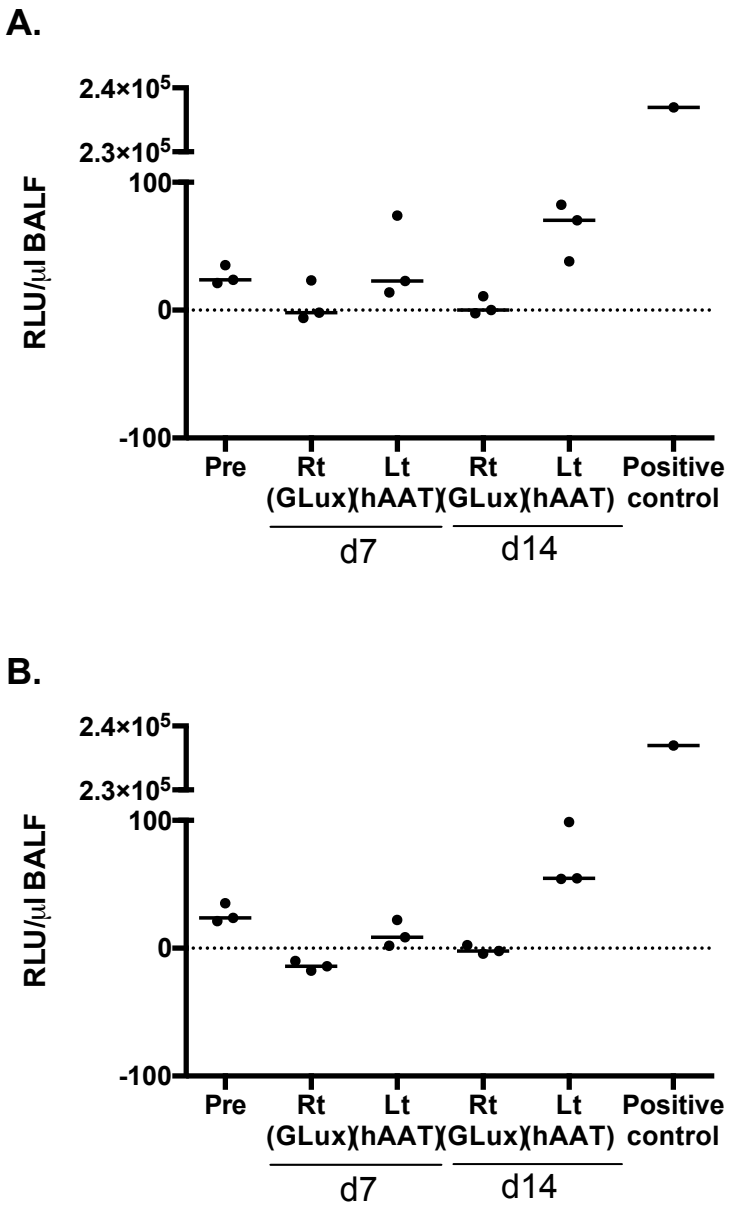


Figure 5-14 Expression of Gaussia luciferase in broncho-alveolar lavage fluid samples from sheep given lentivirus carrying GLux (right lung) or hAAT (left lung)

Three sheep were given approximately 2.9e7 TTU rSIV.F/HN-hCEF-soGLux to the right lung, and approximately 2.8e8 TTU rSIV.F/HN-hCEF-sohAAT to the left lung. Broncho-alveolar lavage was performed 14 days pre-treatment and at 7 and 14 days post-treatment, and samples from the first wash (**A**) and second wash (**B**) analysed for GLux expression (given as RLU [relative light units] per µl of sample, standardised to an appropriate blank (PBS), compared to a positive control (recombinant *Gaussia* luciferase, 0.1 mg/ml). There was no statistically significant difference between the pre-treatment samples and any treated samples (Kruskal-Wallis test with Dunn's post-hoc analysis).

Each point represents one sample from one animal. Horizontal bars represent group medians.

Abbreviations used: Pre=pre-treatment samples (day -14). BALF = broncho-alveolar lavage fluid.

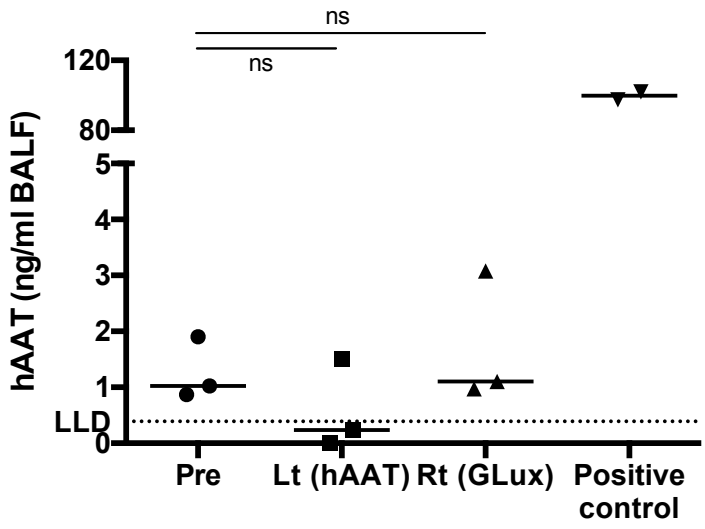


Figure 5-15 Expression of human α_1-antitrypsin in broncho-alveolar lavage fluid samples from sheep given lentivirus carrying GLux (right lung) or hAAT (left lung)

Three sheep were given approximately 2.9e7 TTU rSIV.F/HN-hCEF-soGLux to the right lung, and approximately 2.8e8 TTU rSIV.F/HN-hCEF-sohAAT to the left lung. Broncho-alveolar lavage was performed 14 days pre-treatment and at 7 days post-treatment, and samples from the first wash were analysed for human α_1-antitrpsyin (hAAT) expression and compared to a positive control (purified human α_1-antitrypsin standard, diluted to 100 ng/ml). There was no statistically significant difference (ns) between the pre-treatment samples and any treated samples (Kruskal-Wallis test with Dunn's post-hoc analysis).

Each point represents one sample from one animal. Horizontal bars represent group medians.

Abbreviations used: Pre=pre-treatment samples (day 14). BALF=broncho-alveolar lavage fluid. LLD=lower limit of detection of the ELISA.

5.4 Discussion

In this chapter, I describe the administration of lentiviral vectors to the lungs of mice and sheep, with the aim of producing secreted proteins. I have shown that the application of rSIV.F/HN to the murine airway leads to significant, sustained expression of the secreted proteins *Gaussia* luciferase and hAAT even when very modest viral titres were used. Importantly, a single dose of rSIV.F/HN is shown to lead to sustained expression of secreted proteins at the furthest time-points examined: for GLux, animals were treated with 1e7 TTU virus and protein expression at 12 months was equivalent to that at day 7. For hAAT, animals were treated with 2e7 TTU virus and protein expression at 20 months was not significantly different to that at 7 days. As mice have a lifespan of approximately two years, I can say with some confidence that administration of lentivirus to the murine airway leads to gene expression for the lifetime of the mouse. This is consistent with previous Consortium data, which has shown stable expression of the non-secreted reporter genes GFP for one year [148] and Firefly luciferase for 22 months [134]. On the one hand, such long term gene expression would seem to imply that progenitor cells have been transduced; however, it has been reported that the half-life of terminally differentiated murine airway epithelial cells (AECs) is 17 months [195], suggesting that the long-term gene expression seen in these experiments is simply due to transduction of AECs.

Previously published data from the Consortium has shown neither any evidence of toxic effects of rSIV.F/HN, nor any effect of rSIV.F/HN on survival in mouse experiments [134]. In this chapter, I treated 88 mice with rSIV.F/HN and also did not see any toxic effects of the lentivirus. Two mice died before the end of the planned experiments; these mice were approximately 15 months post-treatment (i.e. 17 months old), and towards the end of their natural life-cycles, and their deaths were therefore not thought to be treatment-related, although it was not possible to perform histological analysis. Formal, regulatory compliant

toxicology assessments of rSIV.F/HN are currently being performed prior to a first-in-man clinical trial of lentiviral gene therapy for cystic fibrosis.

The implications of the loss of efficacy of lentiviral vectors following long-term storage has implications for future work. In

Figure 5-5, the effect of storing the virus for an extra 6 weeks was clearly demonstrated by the reduced protein expression in animals treated later. To some extent, this may be due to the low quality of the virus preparation used – it was an early batch of virus which had not been purified using AKTA columns and tangential flow filtration. The impurities in the batch of virus used may be sufficient to explain the loss of efficacy on later dosing; but future experiments are necessary to determine whether or not this is the case.

In the case of hAAT, I was able to achieve a level of protein expression in epithelial lining fluid that would be considered therapeutic in humans. Other data from the Consortium has demonstrated that when mice are given 8e8 TTU virus (in 100 µl total volume) by nasal sniffing, approximately 14% of airway epithelial cells were transduced (manuscript submitted). The maximum feasible dose I was able to use in hAAT experiments was 1e8 to 1.5e8 TTU virus; by extrapolation it would appear that the therapeutic level of hAAT was obtained in these experiments following transduction of a very modest 2% of airway epithelial cells. This would imply that, with an increased viral titre, it would be possible to achieve even higher levels of hAAT.

Whilst two other groups have reported therapeutic levels of hAAT in murine ELF following airway transduction with a viral vector, both studies have limitations. Halbert *et al* used an AAV6 vector; it was unsuitable for repeated application, and animals needed to be treated with immuno-suppressants for therapeutic protein production to be observed [92]. In my experiments, no immunosuppression was necessary for therapeutic levels of hAAT to be

observed. In their paper, Halbert and colleagues showed that hAAT expression in serum of immuno-competent mice gradually declined over the course of 7 months, whereas in my hands there was no decline in hAAT serum levels over the course of 20 months.

In the other previous study, Wilson *et al* used an invasive delivery method (tracheal instillation), and the viral vector also needed to be co-administered with Lipofectamine, which may not be suitable in humans [94]. The lentivirus I used in these mouse experiments required neither an invasive delivery method nor combination with other gene therapy vectors, representing an improvement on the previous work. Furthermore, the VSV-G pseudotyped lentivirus used by Wilson *et al* preferentially transduced alveolar macrophages, whereas rSIV.F/HN transduces most airway cells (AECs, type I and II pneumocytes, club cells and goblet cells, manuscript submitted). As rSIV.F/HN transduces a greater number of cells, it could be a more efficient method of gene therapy for α_1-antitrypsin deficiency.

In this chapter and throughout this thesis, I have referred to therapeutic target levels of hAAT in epithelial lining fluid. However, the best tissue for measuring hAAT would be the lung interstitium, which is the site of action of both neutrophil elastase and hAAT. It is extremely difficult to isolate murine lung interstitium [59] as it contains blood vessels and therefore serum, interfering with results. I have observed and reported significant hAAT expression in lung tissue homogenate (which contains cellular material, blood vessels and interstitium) and anticipate that levels of hAAT in epithelial lining fluid post-gene transfer would closely reflect levels of hAAT in the lung interstitium. I therefore do not feel that reporting hAAT levels in the 'second-best' tissue affects the interpretation of the data; however, in future large animal studies, it would be ideal to analyse lymph for human α_1-antitrypsin as this is a more reliable measure of interstitial deposition (Rob Stockley, personal communication).

One problem frequently encountered when attempting gene transfer to the lung is the development of an immune response to either the gene transfer agent (e.g. virus) or the

transgene (e.g. *Gaussia* luciferase or human α_1-antritrypsin). This is of relevance because the treatment of chronic diseases such as α_1-antritypsin deficiency may well require either (a) split delivery of gene transfer agents over several doses, if the necessary titre to achieve therapeutic benefit cannot be achieved in a single dose, or (b) repeated treatment, perhaps at yearly intervals for the lifetime of a patient. Importantly, it is believed that the half-life of human airway epithelial cells is 3 months [196] so it is likely that for gene therapy to be effective in the treatment of chronic lung diseases, it will be necessary to either transduce progenitor cells or to administer treatment at regular intervals.

To address the first point, a daily repeat administration experiment was performed where mice were treated with rSIV.F/HN-hCEF-soGLux daily for 5 or 10 days, with reporter gene expression compared to animals treated with one dose of the same batch of virus. The results showed that daily repeat administration was feasible, and significantly increased transduction efficiency.

Due to limited virus availability it was not possible to perform the daily repeat administration experiment with the therapeutic gene α_1-antitrypsin, and nor was it possible to perform a repeat administration experiment with a larger dose interval. An ideal experiment would have been to administer rSIV.F/HN on a monthly basis, to see whether an immune response developed to the vector. However, previous data from the Consortium has shown that monthly administration of rSIV.F/HN coding for the non-secreted reporter protein firefly luciferase is feasible [134], with no difference in reporter gene expression between (a) animals treated with one dose of SIV-Lux and (b) animals treated with two doses of SIV-GFP (day 0, day 28) and one dose of SIV-Lux (day 56).

It is currently unclear why lentiviral vectors can be repeatedly administered to the airways whereas adenoviral and adeno-associated viral vectors cannot. Possible reasons include (a) low inherent immunogenicity of lentiviral vectors, (b) rapid cell entry of lentiviral vectors

which prevents a neutralising antibody response or (c) other causes. Importantly, the Consortium's data is consistent with data published by another group who also were able to repeatedly administer a lentiviral vector to the murine airway [122].

In this chapter, many of the experiments have been performed over a long time period (up to two years) which has necessitated storage of frozen animal samples for long periods of time. I have shown in this chapter that hAAT is stable in frozen murine serum, BALF and lung tissue homogenate samples. This is encouraging not only because it allows further long-term experiments to be designed, but also because it generates pilot data to support the long-term storage of clinical samples, which may be necessary in large trials.

In most of the later experiments, GLux and hAAT were observed in serum after administration to the murine lung. Serum expression was generally about 1-2% of the levels observed in ELF. This raises the intriguing possibility of using the airway as a factory to produce proteins to be secreted into the serum, which could be of relevance for the treatment diseases such as haemophilia A or B. I will discuss the possibilities of other uses of the lentiviral vector platform in Chapter 10.

It was also interesting to observe the strong positive correlation between serum hAAT and the hAAT concentration in ELF and lung following gene therapy. In pre-clinical and clinical studies, it would be ideal to have a non-invasive test to assess the success of gene transfer. Blood tests are easier, cheaper to perform and significantly less invasive than broncho-alveolar lavage, and the fact that serum levels of hAAT were predictive in this small animal model is encouraging and opens up the possibility of using serum hAAT as a surrogate marker for lung/ELF hAAT in future clinical trials.

The experiments presented in this chapter were studies in laboratory mouse strains, and not disease models of emphysema or α_1-antitrypsin deficiency. I will discuss mouse models in the Future Work section of this thesis (Chapter 10).

Having observed high levels of gene expression in a small animal, I sought to investigate lentiviral-mediated gene therapy in a more clinically relevant large animal model. The Consortium has previously investigated gene transfer in sheep, as they have a similar lung structure to man and similar delivery methods are possible [106]. The sheep model has been validated for both non-viral gene transfer [106] and viral gene transfer with Sendai virus, which has the same F and HN envelope proteins as our pseudotyped rSIV.F/HN vector [116]. Different delivery devices have been extensively studied, including the Trudell AeroProbe, which generates an aerosol at the tip of the catheter, which can be placed in the trachea, left or right main bronchus, or in a particular lung segment.

The Consortium has previously shown that when gene transfer agents are delivered into one lung, there is no spillover of non-secreted reporter protein β-gal into the contralateral lung [116], proving that the viral particles only enter airway cells on the side to which they are delivered. Given that the high cost of breeding, housing and treating sheep meant that a maximum of three sheep could be used in this experiment, I felt that it would therefore be an acceptable compromise to treat each animal with two different viruses, one to each lung. I would expect both *Gaussia* luciferase and α_1-antitrypsin to be secreted, but I would not expect them to be found in the epithelial lining fluid of the contralateral lung; therefore, I am confident that the delivery of two different viruses will not affect interpretation of the data in this experiment.

It should be noted that the same work showed that when the AeroProbe is directed to a single lung segment, there is some spillover into adjacent segments, likely due to pooling of the aerosol. For this reason, multiple tissues were collected for analysis.

No evidence of gene expression was seen in these preliminary sheep experiments, and it is likely that a higher titre of virus will need to be delivered to the lungs of large animals for successful gene transfer to occur. Very modest titres (2.9e7 TTU of virus carrying GLux, 2.8e8 TTU of virus carrying hAAT) were delivered in this experiment, which may be sufficient to explain the lack of gene expression in this experiment; modest titres were used due to the expense of producing lentiviral vectors (see below). Interestingly, whilst the Consortium has demonstrated gene expression in sheep with non-viral agents driven by the cytomegalovirus immediate/early promoter (pCIK) [106], it has been suggested that the hCEF promoter, used in the lentiviral vectors in the sheep experiment presented in this chapter, may not be active in sheep (Ian Pringle, personal communication). Importantly, the hCEF promoter worked in the recent trial of non-viral gene therapy for cystic fibrosis [105], indicating that sheep may not be the most appropriate large animal model for studies using this promoter. In addition, we cannot rule out the possibility that sheep may have mounted an immune response to foreign proteins produced following gene therapy, explaining the lack of detection; in future experiments, it may be preferable to analyse samples earlier (e.g. 24/48 hours post-transduction) to rule this out.

It is necessary to consider some of the limitations of the experiments presented in this chapter. Firstly, it is unfortunate that the viral titre used in some experiments is not known. At my laboratory we do not have the in-house facilities to determine viral titre, instead outsourcing this work to the Consortium's Core Facility in Oxford. Constraints of budget, time and personnel meant that in some situations it was not possible for viral titration experiments to be performed. However, I do not feel that this hinders the interpretation of the results presented; for example, in Figure 5-10 it is quite clear that there was GLux expression even though the viral titre was not known.

Furthermore, some of the experimental design in this chapter is sub-optimal due to the high costs of manufacturing the virus, with a single 600 ml preparation in adherent cells costing around £3000 to manufacture. After purification and concentration, this gives approximately 2 mls of lentiviral vector, sufficient for about 20 doses in mice. The 5 litre preparations in serum-free suspension cultures cost £15,000, with a similar output of 2 mls of highly concentrated lentiviral vectors. This means that in most experiments, a compromise had to be made with either (a) the number of animals treated and/or (b) the titre of virus used being lower than desirable. Although I do not feel that the interpretation of the data in this chapter has been negatively affected by this, many of the experiments would have been strengthened by having larger group numbers to aid statistical analysis.

I have presented dose-response data that show higher transgene expression with increased viral doses. Ideally a dose-response experiment would have been performed using a single batch of virus; however, this was not possible due to the relatively small volume of virus produced in each production run, so the data presented is a summary of three separate experiments. However, I do not feel that this distracts from the key message, which is that with higher titres of rSIV.F/HN, I can achieve therapeutic levels of the transgene α_1-antitrypsin in murine lung.

In summary, the experimental data in this chapter show that a lentivirus can successfully deliver therapeutic levels of hAAT to the murine lung; however, sheep may not be a suitable large animal model for studying this vector. In Chapter 7, I will assess the functionality of the recombinant hAAT produced by gene therapy *in vitro* and *in vivo*; in the next Chapter, I will investigate lentiviral-mediated gene transfer in various *ex vivo* human tissue culture models.

6 Viral-mediated gene therapy for the production of secreted proteins in *ex vivo* human tissue models

6.1 Introduction

In Chapter 5, I described the production of therapeutically relevant levels of human α_1-antitrypsin following lentiviral-mediated gene transfer in mice. Further evidence for the potential clinical translation of rSIV.F/HN is sought by attempting to transduce *ex vivo* human tissue. In this chapter, I describe experiments in three different *ex vivo* models of human respiratory epithelium: (a) fully differentiated human air-liquid interface cultures, (b) precision-cut lung slices and (c) primary human nasal epithelial brushings. The different models have different strengths and weaknesses; for example, they have different cell compositions and different lifespans. It is also useful to study different models as the same gene transfer agent can behave differently in various models [197].

Air-liquid interface (ALI) cultures are polarised models of human respiratory epithelium, reconstituted from human donor tissue. They are histologically and functionally similar to respiratory epithelium *in situ*, and have previously been used by the Consortium to investigate gene transfer with the non-viral agent GL67A [164] and the lentiviral vector rSIV.F/HN [134] for the production of non-secreted proteins.

Precision-cut human lung slices are a useful tool for studying gene transfer in the lung, as they are obtained from human donors undergoing lung surgery, and lung tissue architecture is therefore well preserved [198]. Nasal brushings, on the other hand, are obtained from a different anatomical region and therefore allow for studies of gene transfer in an alternative target organ.

Hypothesis: I hypothesise that rSIV.F/HN vectors can transduce human tissue *ex vivo*.

Aim: to investigate gene expression following transduction of air-liquid interface cultures, lung slices and primary nasal epithelial cells from human donors *ex vivo*.

Conclusion: I report successful transduction of ALIs with a lentiviral vector coding for the reporter protein *Gaussia* luciferase. High levels of background human α_1-antitrypsin make ALIs an unsuitable model for hAAT gene therapy. However, successful transduction of human lung slices and primary nasal epithelial cells support the progression of rSIV.F/HN to further pre-clinical and clinical studies.

6.2 Materials & Methods

6.2.1 Gene transfer into human air-liquid interface cultures

Fully differentiated MucilAir™ human airway epithelial cells from healthy donors, grown as air liquid interface (ALI) cultures, were purchased from Epithelix SÀRL (Geneva, Switzerland). These ALIs are grown on 6.5 mm inserts on a microporous insert, with a cell density of approximately 500,000 cells per well. ALIs were cultured at 37°C and 5% CO_2 in a tissue culture incubator as per the manufacturer's recommendations. The basolateral culture medium (MucilAir culture medium, Epithelix) was replaced every 2-3 days, as per the manufacturer's recommendations.

ALIs were transduced with viral vectors at the titres indicated in the figure legends. At the time of transduction, ALIs were less than 80 days old as it has previously been shown that older ALIs are more difficult to transduce [164]. Viral vectors were diluted in PBS and applied to the apical surface of the ALIs in 15 to 100 µl total volume, as shown in the figure legends. Negative control ALIs were treated with (a) an equivalent volume of PBS *and/or* (b) an irrelevant lentiviral vector, as described. After 4 hours, the virus or PBS was removed and ALIs incubated for 5 to 80 days, as indicated in the figure legends.

At the indicated timepoints, the apical surface of the ALIs were washed by applying 100 µl PBS, incubating them at 37°C for one hour, and then removing the PBS with a laboratory pipette. These apical washing samples were stored at -80°C and subsequently analysed for transgene expression as appropriate.

6.2.2 Gene Transfer into precision-cut human lung slices

Resected human lung tissue was collected after clinically-indicated surgery performed at the Royal Brompton and Harefield NHS Foundation Trust, London, UK. All studies were approved by the relevant Ethics Committees. Precision cut lung slices were prepared using a method previously described by Moreno *et al* [199]. Briefly, the airways were instilled with low melting point agarose solution, which was allowed to cool to 4°C to stabilise the tissue for subsequent cutting. After cooling, tissue cores were prepared using a rotating sharpened metal tube. From these cores 250 μm thick tissue slices were prepared using a Krumdieck tissue slicer.

After cutting, slices were placed in 12-well tissue culture plates (1 slice/well) and washed eight times (with 1 ml DMEM containing 1% penicillin/streptomycin, 200 mM L-glutamine, 10 mg/ml gentamicin, 0.5% amphotericin and 50 μg/ml vancomycin) to remove the low melting point agarose.

Plates were incubated at 37°C and 5% CO_2 and slowly rotated (1 rpm) at approximately a 40° angle, in 1 ml tissue culture medium described above. The slices were approximately one week old at the time of transduction. Lentiviral vectors (rSIV.F/HN-hCEF-soGLux and rSIV.F/HN-hCEF-sohAAT) were diluted in Optimem and applied to the lung slices in 1 ml total volume. Titres used in the experiment were:

- *rSIV.F/HN-hCEF-soGLux:* 2e6, 1e7, 5e7 TTU/slice (n=6 per group)
- *rSIV.F/HN-hCEF-sohAAT:* 8e5, 4e6, 2e7 TTU/slice (n=6 per group)

Lung slices were transduced for 6 hours, after which the vector solution was removed, and lung slices washed three times with 1 ml of PBS. Cells were given 1 ml culture media (as above) which was removed and replaced every day, and stored at -20° for further analysis.

6.2.3 Gene transfer into human nasal brushings

Nasal brushings from the inferior turbinate of 5 healthy volunteers were collected based on a method from Rutland and Cole [200]. Collection was approved by the Royal Brompton and Harefield NHS Foundation Trust Research Ethics Committee and all subjects gave informed written consent. Each nostril was brushed once with a 3 mm cytology brush (Diagmed, Thirsk, UK) [201]. Cells were collected into D-MEM (Sigma-Aldrich) containing 1% penicillin/streptomycin, pooled, centrifuged (1600 *g* for 5 min) and counted.

Approximately 2e5 cells were transduced with lentiviral vectors rSIV.F/HN-hCEF-soGLux or rSIV.F/HN-hCEF-sohAAT (1e6 or 1e7 TTU/dose diluted in 50 µl total volume Optimem, n=6 per group). Control samples received Optimem alone. Cells were transduced for 4 hours at 37°C, after which 50 µl DMEM containing 20% fetal calf serum (FCS) (Sigma-Aldrich) and 2% penicillin/streptomycin were added to each sample and cells incubated for a further 20 hours at 37°C in 5% CO_2. Subsequently cells were harvested, centrifuged at 1500 g_{av} for 5 min, re-suspended in reporter lysis buffer (Promega, UK) and subjected to three freeze-thaw cycles. Saved supernatants were then tested for the presence of GLux or hAAT as appropriate.

6.3 Results

6.3.1 Fully differentiated human air-liquid interface (ALI) cultures express secreted reporter protein following transduction with rSIV.F/HN-hCEF-soGLux

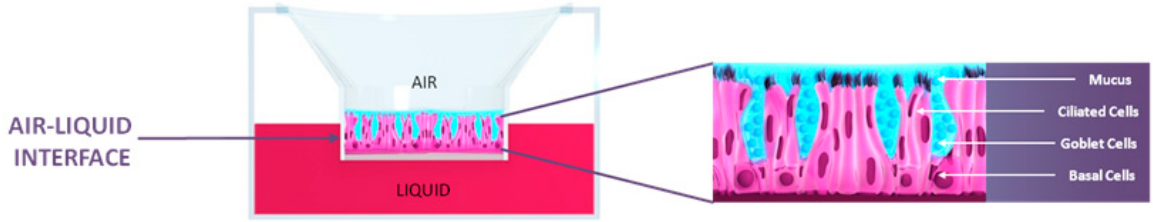
Human air-liquid interfaces (ALIs) are a highly relevant model of the human airway epithelium, as they contain an epithelial layer (consisting of epithelial cells, goblet cells and basal cells) which is exposed to air on the apical surface; nutrients are provided by basolateral culture medium (Figure 6-1A). Therefore, I investigated the ability of rSIV.F/HN vectors to transduce ALIs.

In an initial experiment, ALIs (n=7 per group) were treated with 1e7 TTU/ALI rSIV.F/HN-hCEF-soGLux in 100 µl total volume (resulting in a multiplicity of infection (MOI) of ~20), or given an equivalent volume of irrelevant control lentivirus. After 4 hours the lentiviral vectors were removed. *Gaussia* luciferase expression was determined in apical washes 3, 6, 14, 21 and 80 days post-transduction. The dose was chosen as it was the maximum feasible dose based on vector availability at the time of the experiment.

At all time-points analysed, *Gaussia* luciferase expression was significantly higher in ALIs treated with rSIV.F/HN-hCEF-soGLux (Figure 6-1B). Reporter gene expression peaked at day 14 (*treated:* median GLux expression 9.3e5 RLU/µl (range 6.7e5-4.1e6), *untreated:* median GLux expression 11 RLU/µl (range 8-13), $p<0.001$) and persisted until at least day 80, the end of the experiment.

This provides proof-of-concept that fully differentiated human airway epithelial cells produce secreted proteins after transduction with rSIV.F/HN and supports further *ex vivo* and *in vivo* studies.

A



B

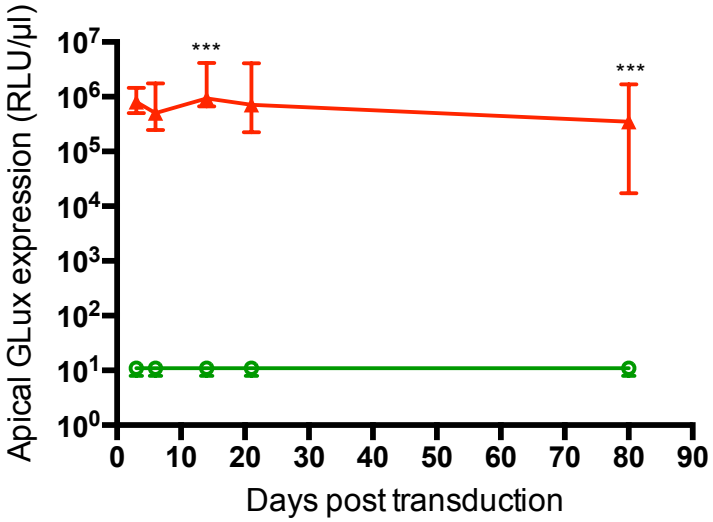


Figure 6-1 Expression of *Gaussia* luciferase for 80 days in air-liquid interface (ALI) cultures following lentiviral-mediated gene transfer

(A) Schematic of MucilAir™ air-liquid interface cultures. ALIs are reconstituted using primary human cells and contain ciliated epithelial cells, goblet cells and basal cells. Cells are joined by tight junctions and cilia can been seen beating by light microscopy. Schematic reproduced from www.epithelix.com.

(B) Fully differentiated human air-liquid interface (ALI) cultures were transduced with 100 µl rSIV.F/HN-hCEF-soGLux (1e7 TTU/ALI, MOI 20) (filled triangles) or 100 µl of an irrelevant lentivirus (open circles). Expression was determined in apical washes at the indicated timepoints.

Each data point represents the group median (n=7 ALIs per group). Error bars represent the range. RLU = relative light units.

***=$p<0.001$ compared to negative controls at indicated timepoints (Mann Whitney *U* test).

6.3.2 Treatment with rSIV.F/HN did not increase human α_1-antitrypsin above background levels in human air-liquid interface cultures

Having demonstrated expression of a secreted reporter protein in human ALIs, I next investigated production of the therapeutic gene α_1-antitrypsin following a similar treatment strategy. On day 0, fully differentiated ALIs (n=6 per group) were transduced with 7.4e6 TTU rSIV.F/HN-hCEF-sohAAT in 15 µl total volume (a multiplicity of infection (MOI) of 15), or treated with an irrelevant control lentivirus. The dose given was the maximum feasible dose (based on vector availability at the time); the virus was applied in 15 µl volume to maintain the maximum feasible concentration. Importantly, before the experiment I determined that 15 µl PBS totally covered the apical surface of ALIs, meaning that all cells should have been exposed to virus.

ALIs were treated for 6 hours, before the viral vectors were removed. Apical washings were then performed at the timepoints indicated in Figure 6-2. Expression of hAAT in ALIs treated with rSIV.F/HN-hCEF-sohAAT was not significantly greater than ALIs treated with an irrelevant lentivirus at day 1, 4 or 8 post-transduction. To see if a further dose of rSIV.F/HN could boost hAAT expression, ALIs were given a second dose on day 11. At day 18, expression of hAAT in treated ALIs was still not above background levels in control cells.

The results of this initial experiment suggest that ALIs might produce a high level of endogenous hAAT, which will make it difficult to observe hAAT expression following gene transfer. This highlights the need for an untransduced control group, which will be included in future experiments described below.

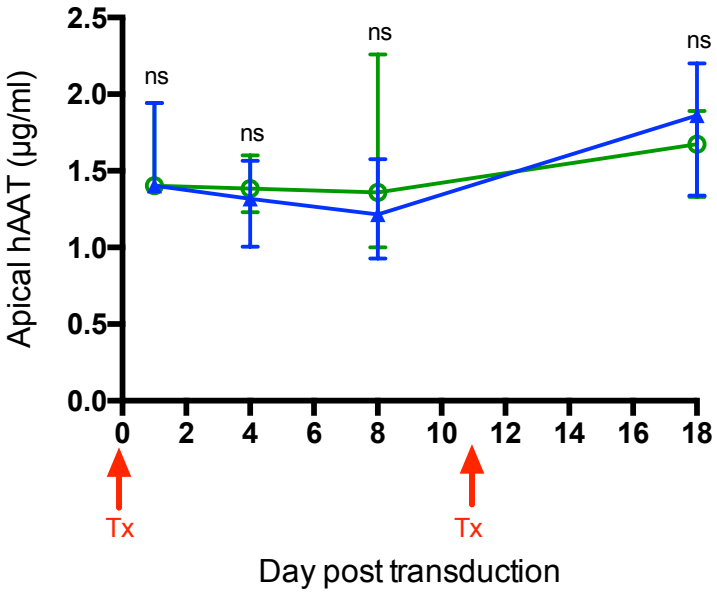


Figure 6-2 Transduction of human air-liquid interface cultures with lentivirus carrying human α_1-antitrypsin cDNA

On day 0 of the experiment, human ALIs were treated with 15 µl total volume of rSIV.F/HN-hCEF-sohAAT (7.4e6 TTU/ALI, MOI 15) (closed blue triangles) or an irrelevant control virus (open green circles). hAAT expression was determined in apical surface washes at the indicated timepoints.

On day 11, ALIs were given a second dose as indicated by the red arrows.

Each data point represents group medians (n=4-6 per group). Error bars represent the range. ns=not significant (Mann Whitney *U* test between treated and untreated group at each time-point).

6.3.3 Refinements in the experimental technique were not able to boost hAAT levels above background levels in human ALIs

As I was not able to see hAAT above background levels in human ALIs, I performed a further experiment with several modifications.

ALIs (n=4-6 per group) were treated with (a) rSIV.F/HN-hCEF-sohAAT (1e6, 1e7 or 1e8 TTU/ALI in 100 µl total volume, equivalent to MOI of approximately 2, 20 or 200), (b) rSIV.F/HN-hCEF-soGLux (1e8 TTU/ALI in 100 µl total volume, equivalent to MOI 200) or (c) 100 µl PBS.

Modifications in the experimental procedure of note were:

- the PBS group was included to determine how much hAAT is secreted by untreated ALIs (PBS groups were not included in previous experiments due to high costs (ALIs cost approximately 100€ per insert); but the results obtained highlighted the need for addition of these controls);
- ALIs were given 100 µl total volume of viral vectors, to deliver the maximum feasible dose (as determined by the maximum volume that can be added to the apical surface of the ALI);
- the lentiviral vectors were produced by the Consortium Core Facility in Oxford, using 5 litre cell suspension cultures and as such, they represent the highest dose and best quality lentiviral vectors currently available;
- for hAAT, three dose groups were included to give the best possible chance of seeing a positive result; i.e., higher titres would presumably lead to more hAAT expression but conversely they could also be toxic to ALIs.

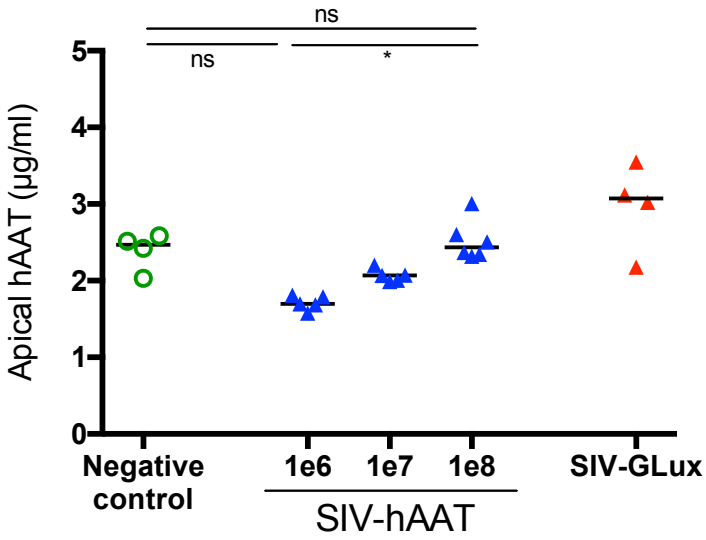
Despite these modifications, it was still not possible to detect hAAT above background levels (Figure 6-3A); at day 5 post transduction, hAAT expression was no greater than either ALIs

treated with PBS, or ALIs treated with a lentiviral vector coding for GLux. Surprisingly, there was a hint of a dose-response in the ALIs treated with rSIV.F/HN-hCEF-sohAAT; ALIs treated with 1e8 TTU expressed significantly more hAAT than ALIs treated with 1e6 TTU (*1e8 TTU group:* median 2.4 (range 2.3-3.0) ng/ml, *1e6 TTU group:* median 1.7 (range 1.6-1.8) ng/ml, $p<0.05$ (Kruskal-Wallis test with Dunn's post-hoc analysis)); however, these levels were no higher than untransduced controls (median 2.4 (range 2.0-2.6) ng/ml).

To confirm that gene transfer had occurred in this experiment and that ALIs were viable, I measured the *Gaussia* luciferase expression in ALIs treated with rSIV.F/HN-hCEF-soGLux. At day 3 and 21 post-transduction, expression of *Gaussia* luciferase was significantly higher in treated ALIs compared to (a) untreated ALIs or (b) ALIs treated with rSIV.F/HN-hCEF-sohAAT (Figure 6-3B).

In summary, this data shows that rSIV.F/HN can produce secreted reporter protein in human ALIs. However, high background levels of hAAT in human ALIs from healthy donors (between 1.5 and 3 μg/ml) make it impossible to see hAAT expression following gene transfer with the maximum feasible titre of rSIV.F/HN.

A. hAAT expression



B. GLux expression

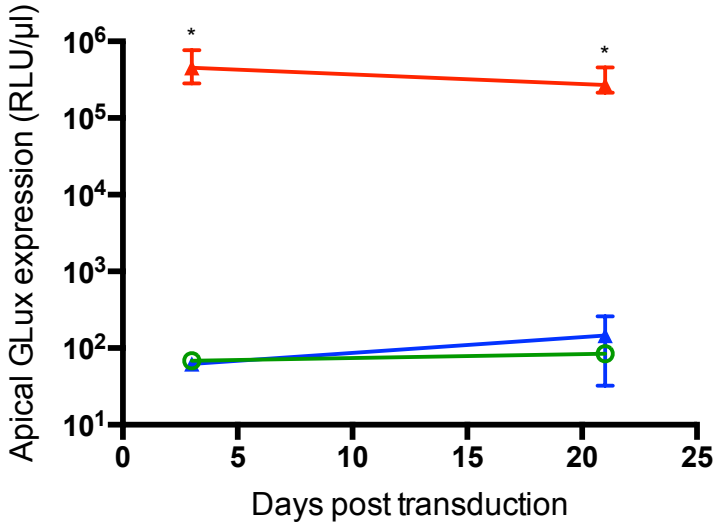


Figure 6-3 Transduction of human air-liquid interface cultures with lentiviral vectors carrying human α_1-antitrypsin cDNA

Human ALIs (n=4-6 per group) were treated with 100 µl total volume of rSIV.F/HN-hCEF-sohAAT (1e6, 1e7 or 1e8 TTU/ALI, MOI 2, 20 or 200) (closed blue triangles), rSIV.F/HN-hCEF-soGLux (1e8 TTU/ALI, MOI 200) (closed red triangles) or PBS (open blue circles).

(A) hAAT expression was determined in apical surface washes 5 days post transduction. *=$p<0.05$, ns=not significant (Kruskal-Wallis test with Dunn's post-hoc analysis).

Each data point represents one well. Horizontal bars represent group medians.

(B) *Gaussia* luciferase expression was determined in apical surface washes day 3 and 21 post transduction. RLU = relative light units. *=$p<0.05$ (treated compared to negative control; Mann Whitney *U* test).

Each data point represents group median. Horizontal bars represent range. Error bars represent range.

6.3.4 Production of secreted proteins following lentiviral transduction of precision-cut human lung slices

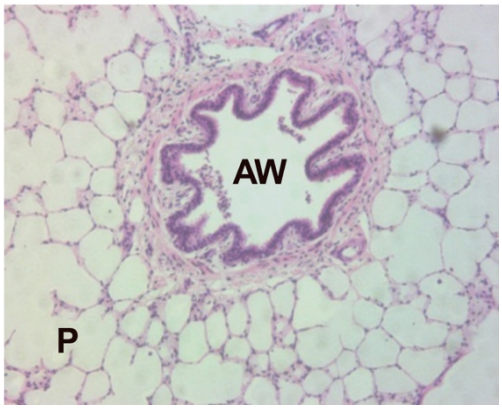
Because of the difficulties encountered in observing hAAT production in human air-liquid interface cultures, I next examined an alternative *ex vivo* human tissue culture model. Primary human airway epithelial cells were obtained from precision-cut human lung slices (Figure 6-4A) generated from lung resection tissue and transduced with rSIV.F/HN-hCEF-sohAAT (8e5, 4e6 or 2e7 TTU/slice, n=6 per group) or rSIV.F/HN-hCEF-soGLux (2e6, 1e7, 5e7 TTU/slice, n=6 per group). GLux and hAAT expression was determined for up to 8 days post-transduction, when the tissue ceased to be viable.

24 hours post-transduction, GLux expression in supernatant was higher in the low and medium dose groups; although GLux expression was detected in the high dose group, it was not significantly greater than controls, likely due to toxicity of the virus at this high dose:

- Gaussia *luciferase expression 24 hours post transduction:*
    - *2e6 TTU:* 4.1e6 (3.8e6-4.1e6) RLU/µl ($p<0.001$)
    - *1e7 TTU:* 3.3e6 (2.3e6-4.0e6) RLU/µl ($p<0.01$)
    - *5e7 TTU:* 7.5e5 (4.0e5-8.7e5) RLU/µl (not significant)
    - *Control:* 300 (287-315) RLU/µl
- *Data expressed as median (interquartile range)*

Reporter gene expression decreased after day 3 and by day 8, there was no statistically significant difference between expression in treated and untreated lung slices. As stated above, the lung slices were one week old by the time of treatment and two weeks old by the end of the experiment, and were becoming increasingly non-viable.

A



B

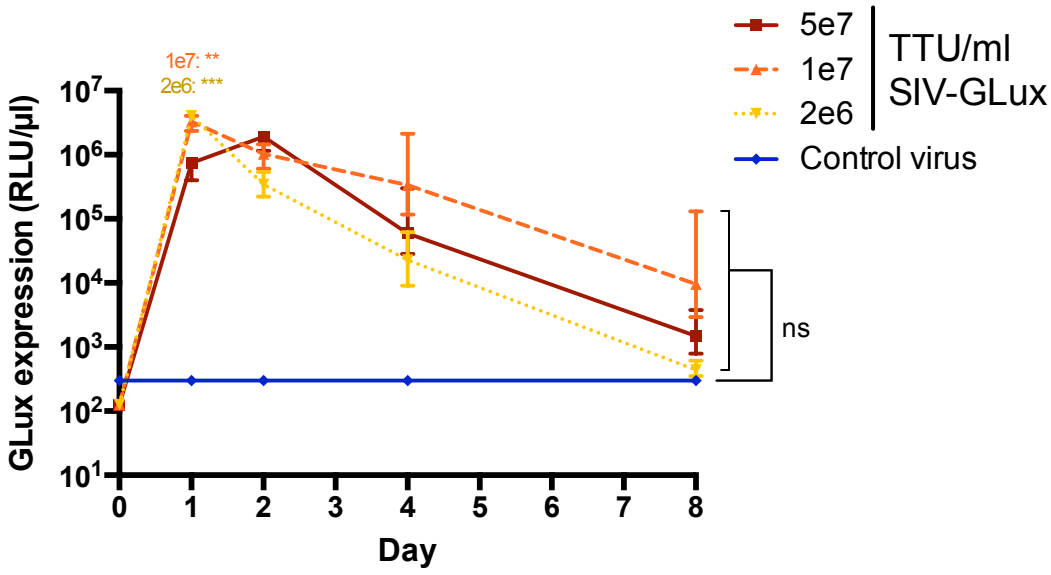


Figure 6-4 Expression of *Gaussia* luciferase following lentiviral transduction of precision-cut human lung slices

(A) Lung slices are primary human pulmonary cells, obtained from lobar or whole-lung resections. Lung tissue is fixed in low-melting point agarose, and cut into 250 µm thick discs (8 mm in diameter). Observed is an airway (AW) and parenchyma (P). Reproduced with permission from [134].

(B) On day 0, lung slices (n=6 per group) were treated with 3 different doses of rSIV.F/HN-hCEF-soGLux or 3 different doses of rSIV.F/HN-hCEF-sohAAT. *Gaussia* luciferase expression was determined in supernatant at the indicated timepoints. For clarity, only one group of lung slices treated with irrelevant control virus is represented here.

Each data point represents the group median (n=6 per group). Error bars represent the interquartile range.
RLU=relative light units.

**=p<0.01 (1e7 TTU dose group at day 1 compared to negative control); ***=p<0.001 (2e6 TTU dose group at day 1 compared to negative control); ns=not significant (for all dose groups at day 8 compared to negative control) (Kruskal-Wallis with Dunn's post-hoc analysis).

Expression of hAAT in human lung slices

Having observed significant levels of reporter gene expression in lung slices, I next examined hAAT expression. As the hAAT ELISA is significantly more expensive and labour-intensive than the *Gaussia* luciferase assay, I only examined hAAT expression at day 0, 2 and 8.

At 2 days post-transduction, hAAT expression in supernatant was significantly higher in the 2e7 dose group compared to controls. hAAT was expressed above background levels in other dose groups, but this difference did not reach statistical significance:

- *hAAT expression 48 hours post transduction:*
    - *8e5 TTU:* 198 (47.6-588) ng/ml (not significant)
    - *4e6 TTU:* 168 (112-559) ng/ml (not significant)
    - *2e7 TTU:* 320 (207-1307) ng/ml ($p<0.01$)
    - *Control:* 9.3 (7.5-10.0) ng/ml
- *Data expressed as median (interquartile range)*

At 8 days post-transduction the lung slices were deteriorating and although levels of hAAT expression were higher in treated slices than controls, there was no statistical significance (Figure 6-5).

These data show that the lentiviral vector rSIV.F/HN can successfully transduce human lung slices *ex vivo*, which help to place the vector on a translational route into the clinic. In the final experiment in this chapter, I will attempt to transduce another *ex vivo* human tissue – primary nasal brushings.

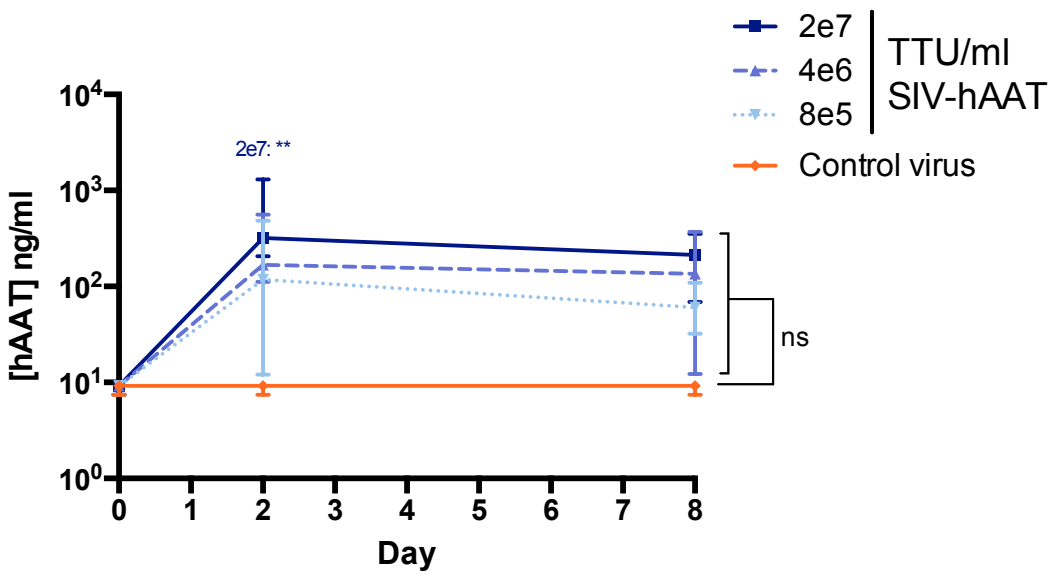


Figure 6-5 Expression of human α_1-antitrypsin following lentiviral transduction of precision-cut human lung slices

On day 0, lung slices (n=6 per group) were treated with 3 different doses of rSIV.F/HN-hCEF-sohAAT or 3 different doses of rSIV.F/HN-hCEF-soGLux. Human α_1-antitrypsin (hAAT) expression was determined in supernatant at the indicated timepoints. For clarity, only one group of lung slices treated with irrelevant control virus is represented here.

Each data point represents the group median (n=6 per group). Error bars represent the interquartile range. RLU, relative light units.

**=$p<0.01$ (2e7 TTU dose group at day 1 compared to negative control); ns=not significant (for all dose groups compared to negative control at day 8) (Kruskal-Wallis test with Dunn's post-hoc analysis).

6.3.5 Production of secreted protein following lentiviral transduction of nasal brushings

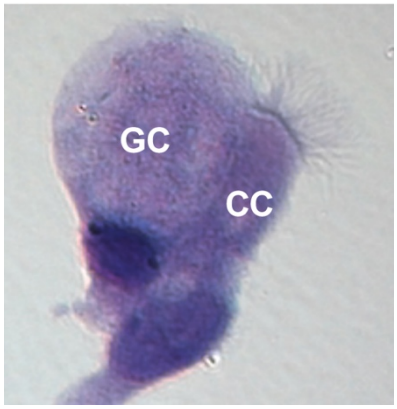
The nose represents an alternative target organ for gene therapy for secreted proteins, and nasal epithelial cells are similar to lung epithelial cells [202]; therefore, the nose could be used as a 'factory' to produce proteins such as α_1-antitrypsin and Factor VIII. Therefore, it is important to assess the ability of rSIV.F/HN to transduce nasal tissue.

Freshly obtained nasal brushings were taken from healthy volunteers and transduced with rSIV.F/HN carrying hAAT or GLux (1e6 or 1e7 TTU/reaction). These doses represent an approximate MOI of 5 and 50, respectively.

High levels of pseudo-transduction following treatment with rSIV.F/HN-hCEF-soGLux prevent further analysis

I first wanted to control for pseudo-transduction (described in Section 4.3.1), by analysing the vector solution for free GLux. This is relevant in nasal brushings because unlike ALIs and lung slices, they cannot be washed; furthermore, nasal brushings have a short lifespan and long-term experiments are not possible. I spiked tissue culture media from untreated cells with an identical volume of rSIV.F/HN-hCEF-soGLux to that given to the nasal brushing cells in the highest dose group (1e7 TTU/reaction), and also spiked tissue culture medium with a 10-fold higher dose (Figure 6-6B). Unfortunately, GLux expression in spiked samples was 3- to 4-log orders higher than unspiked samples ($p<0.01$), which means that I cannot reliably analyse nasal brushings for GLux expression following gene transfer.

A



B

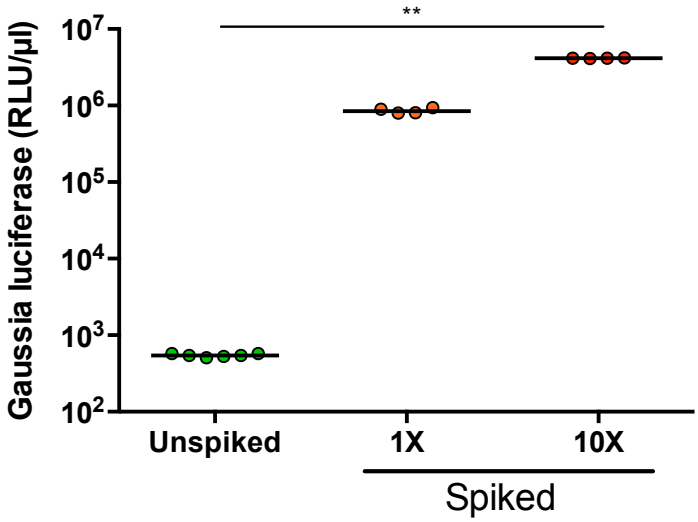


Figure 6-6 Pseudo-transduction prevents further analysis of rSIV.F/HN-hCEF-soGLux transduction in the nasal brushing experiment

(A) Microscopic section of human airway epithelial cells obtained from nasal brushings. A goblet cell (GC) and ciliated airway epithelial cell (CC) are visible. Reproduced with permission from [134].

(B) Tissue culture media from untreated cells was spiked with rSIV.F/HN-hCEF-soGLux to mimic the conditions in the nasal brushing experiment (1X) or with 10 times the amount of lentiviral vector used in the experiment (10X). Gaussia luciferase expression was compared to unspiked culture medium.

Each data point represents one sample analysed on the *Gaussia* luciferase assay. Horizontal bars represent group medians. RLU=relative light units.

**=$p<0.01$ compared to negative control (Kruskal-Wallis test with Dunn's post-hoc analysis).

Expression of hAAT in human nasal brushings

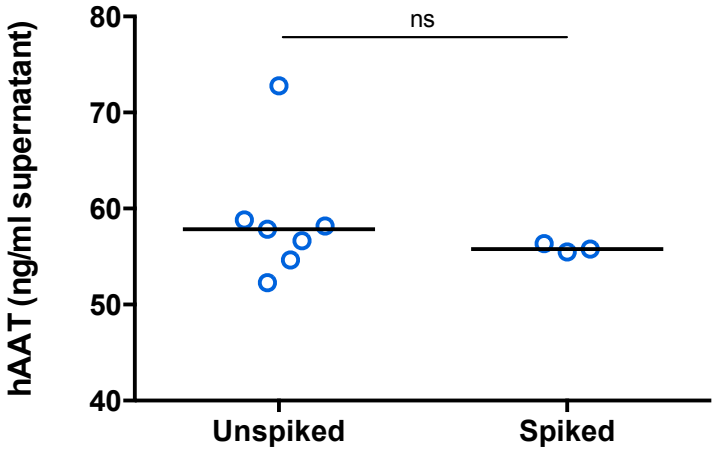
I then spiked tissue culture media with an equivalent amount of rSIV.F/HN-hCEF-sohAAT as used in the top dose group in the experiment to quantify pseudotransduction of hAAT Unfortunately, I was not able to analyse 10-fold spiked samples, due to limited availability of the virus. Unlike in the *Gaussia* luciferase spiked samples, there was no significant difference between hAAT levels in tissue culture media from untreated cells, and media spiked with an equivalent amount of lentiviral vector coding for hAAT used in the nasal brushings experiment (Figure 6-7A).

I subsequently showed that transduction of human nasal brushing cells with rSIV.F/HN-hCEF-sohAAT at a multiplicity of infection (MOI) of 5 led to significant ($p<0.0001$) levels of hAAT in supernatant compared to untransduced cells (Figure 6-7B). At a higher MOI of 50, expression of hAAT was lower, presumably due to toxic effects of the lentiviral vector.

- *Treated with rSIV.F/HN-hCEF-sohAAT:*
    - *MOI 5 (1e6 TTU/reaction):* 389 (339-502) ng hAAT per ml supernatant ($p<0.001$ compared to untreated and SIV-GLux)
    - *MOI 50 (1e7 TTU/reaction):* 206 (151-375) ng hAAT per ml supernatant
- *Treated with rSIV.F/HN-hCEF-soGLux:* 146 (102-185) ng hAAT per ml supernatant
- *Untreated:* 140 (103-176) ng hAAT per ml supernatant
- *Data expressed as median (range)*

This data shows that primary human nasal tissue can be successfully transduced with lentiviral vectors coding for human α_1-antitrypsin, suggesting that the human nasal epithelium could be used as a target organ for hAAT gene therapy.

A



B

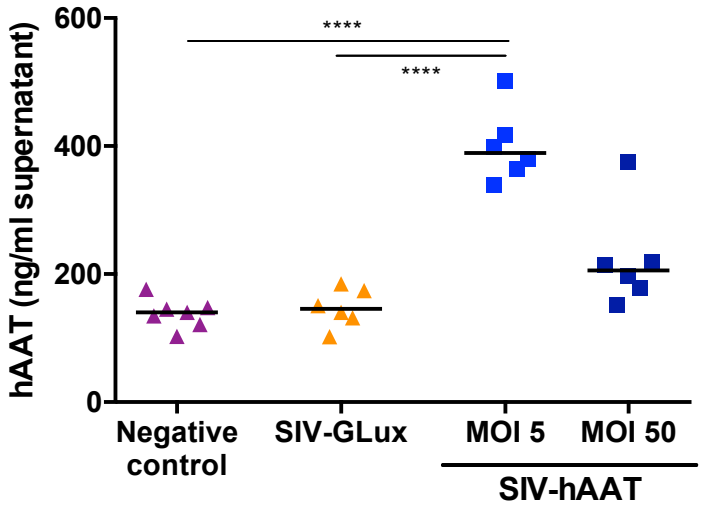


Figure 6-7 Expression of hAAT in human nasal brushings treated with rSIV.F/HN-hCEF-sohAAT

(A) Tissue culture media from untreated cells was spiked with rSIV.F/HN-hCEF-sohAAT to mimic the conditions in the nasal brushing experiment. Human α_1-antitrypsin (hAAT) expression was compared to unspiked culture medium.

Each data point represents one sample analysed on the hAAT ELISA. Horizontal bars represent group medians. Ns=not significant.

(B) Nasal brushings (2e5 cells per reaction) were treated with (a) rSIV.F/HN-hCEF-sohAAT (1e6 or 1e7 TTU/reaction, equivalent to MOI of 5 or 50 respectively), (b) rSIV.F/HN-hCEF-soGLux (1e7 TTU/reaction=MOI 50) or (c) left untreated (untx). hAAT expression in supernatant was determined 24 hours post-transduction.

Each data point represents one Eppendorf tube of primary human nasal epithelial cells. Horizontal bars represent group medians. MOI=multiplicity of infection. ****=$p<0.0001$ (Kruskal-Wallis test with Dunn's post-hoc analysis).

6.4 Discussion

In this chapter, I transduced three different *ex vivo* tissue culture models with lentiviral vectors. I have shown that (1) ALIs can produce high levels of a secreted reporter protein for at least three months, (2) ALIs express high levels of endogenous hAAT and are therefore less useful for studies of hAAT gene therapy, and (3) rSIV.F/HN can successfully transduce precision-cut human lung slices and primary human nasal epithelial cells.

Air-liquid interface (ALI) cultures are a valuable and highly relevant *ex vivo* model. MucilAir™ inserts are reconstituted from primary human cells following pulmonary lobectomies, and contain ciliated airway epithelial cells, goblet cells, basal cells, and tight junctions [203] [204]. ALIs are fully differentiated and have a slow rate of mitosis [205], which make them a more relevant model of pulmonary gene transfer than immortalised cell lines such as HEK293T cells or A549 cells (which have a high rate of mitosis, facilitating entry of plasmid or viral DNA into the nucleus). Other features which make ALIs a relevant model for human airways are (a) mucus production and (b) ciliary clearance (cilia can be seen to be beating under light microscopy). ALIs therefore represent a good model of the difficult-to-transduce human airway epithelium, i.e. the target cells for gene therapy for α_1-antitrypsin deficiency and other pulmonary diseases such as cystic fibrosis. They can also be kept for many months (up to one year), which allows for the long-term study of gene expression.

The UK Cystic Fibrosis Gene Therapy Consortium has previously successfully transduced ALIs with rSIV.F/HN lentiviral vectors coding for firefly luciferase. At MOIs of 25 to 250, high levels of reporter gene were produced at day 5 [148]. In a further experiment, reporter gene expression was seen to be stable for at least three months [134]. rSIV.F/HN was directly compared to the non-viral vector GL67A, which has recently been shown to be of benefit to patients with cystic fibrosis in a Phase IIb trial [105]. Despite the success of GL67A in a

clinical trial, it was not possible to detect firefly luciferase expression above background levels in ALIs after GL67A transfection, implying that ALIs are difficult to transduce. In a separate study, it was shown that by co-treating ALIs with the nuclear pore dilating agent trans-cyclohexane-1,2-diol, levels of *Gaussia* luciferase increased from 0 to 20-fold above background levels following gene transfer, although this was associated with significant cell damage [206]. The fact that a gene transfer agent which produced a positive result in a clinical trial produced a negative result in ALI experiments provides encouragement in light of the data presented in this chapter, where it was not possible to produce hAAT following treatment of ALIs with rSIV.F/HN.

In this chapter, I have demonstrated significant expression of the secreted reporter protein *Gaussia* luciferase in two separate ALI experiments. It has previously been shown that gene therapy with the non-viral gene transfer agent GL67A can produce significant levels of *Gaussia* luciferase in ALIs [164], but this is the first report of GLux expression using the rSIV.F/HN lentiviral vector. In the first experiment, a very modest titre of 1e7 TTU (MOI 20) was used; however, it still led to expression of *Gaussia* luciferase approximately 5 log orders higher than negative controls. *Gaussia* luciferase expression also lasted for at least 80 days, the furthest time-point analysed. This strengthens the *in vivo* data from Chapter 5 showing that rSIV.F/HN can stably transduce non-dividing cells and produce secreted transgenes for many months post-gene transfer. Interestingly, when a higher MOI of 200 was used in a further experiment, the level of *Gaussia* luciferase seen was approximately the same. However, as these experiments used different batches of lentiviral vectors, and were performed on different batches of ALIs more than one year apart, it is not appropriate to draw any firm conclusions from this observation.

I do not anticipate a problem with pseudo-transduction in ALI experiments, as observed in Chapter 4 for *in vitro* experiments in HEK293T and A549 cells. In ALIs the treatment

solution (applied apically) was removed 6 hours after transduction (not 48 hours, as for earlier *in vitro* experiments) and the apical surface of the ALIs was thoroughly washed. In addition, the basolateral medium was changed every 2-3 days, which should remove any free protein from vector production.

Despite the positive results in the GLux experiments, it was not possible to see significant levels of human α_1-antityrypsin above background levels in ALIs following gene transfer with rSIV.F/HN. It appears from my data that untreated ALIs express α_1-antitrypsin at a concentration of between 1.5 to 3 μg/ml. This is not surprising as it has previously been demonstrated that bronchial epithelial cells secrete α_1-antritypsin [6]. However, it does make it difficult to demonstrate α1-antitrypsin expression following gene transfer to these cells, as very high levels of transgene need to be made in order to observe expression above background levels. I am confident that the negative data presented is not due to a problem with the vector used, because experiments in mice with the same batches of lentiviral vectors resulted in significant transgene expression (for example, the batch of lentiviral vector used for the ALI experiment in Figure 6-3 lead to significant ($p<0.01$) expression of hAAT in murine lung tissue homogenate, ELF and serum (Figure 5-7)).

There are a number of patient groups to whom hAAT gene therapy could be useful. Some groups, such as patients with α_1-antitrypsin deficiency (AATD), have little or no circulating hAAT present; other groups, such as patients with COPD or cystic fibrosis, have a normal level of hAAT but a protease:antiprotease imbalance leads to the destruction of lung tissue. In this chapter, I have described experiments using ALIs from healthy donors; however, ALIs from COPD and cystic fibrosis patients are available and theoretically it would be possible to grow ALIs from donors with α_1-antitrypsin deficiency. It would be useful to perform gene transfer experiments in ALIs from patients with AATD, particularly because it would be easier to observe hAAT production in cells that don't produce endogenous hAAT.

It is important to note that the process of collecting samples from ALIs (apical washing) involves dilution of the apical surface liquid (ASL). The surface area of an ALI grown on a 6.5 mm transwell is 33.2 mm^2; assuming that the height of airway surface liquid is 7 μm [207], the dilution of ASL when 100 μl of PBS is added is approximately 430-fold. I do not feel that this affects interpretation of the data.

The viral titre used in my final ALI experiment, 1e8 TTU/ALI, is the maximum feasible titre that can be given to ALIs with current production methods; the only way of improving the titre further would be to treat ALIs with multiple doses of virus on different days, however the costs involved in purchasing ALIs and producing enough virus were prohibitive as part of this PhD and require further grant funding. Therefore, I moved on to study two alternative *ex vivo* tissue culture models – precision-cut lung slices and nasal brushings.

Lung slices are another highly relevant model of lung gene transfer; like ALIs, they are obtained from human primary cells and retain tissue architecture better than most other *ex vivo* tissue culture models [208]. rSIV.F/HN has previously been used in human lung slices, with vectors coding for the non-secreted reporter gene firefly luciferase (FLux) at a titre of 2e7 TTU/slice [134]. 2 days post gene transfer, FLux expression was 3 logs order above controls; expression gradually decreased until the experiment was terminated 14 days post gene transfer.

The results of my experiments in human lung slices were consistent with this previously published data. In my hands, transduction of lung slices with similar titres (1e7 and 5e7 TTU/slice) of a lentiviral vector coding for the secreted reporter gene *Gaussia* luciferase led to expression approximately 4 log orders above controls. Expression followed a similar time-course, although it should be noted that in my experiment the lung slices only remained viable for 7 days post-transduction, consistent with them being over a week old by the time I was able to treat them.

Treatment with a lentiviral vector coding for α_1-antitrypsin was slightly less efficient, with expression of hAAT tending to be around 1 to 2 log orders above controls at day 2. This is consistent with data presented earlier in this thesis, where I have shown that assays for *Gaussia* luciferase are more sensitive than for hAAT.

The only way I was able to control for pseudotransduction in this experiment was to thoroughly wash the lung slices after viral vectors had been removed. The fact that protein was still detectable 8 days following transduction (i.e. after the slices had been washed on 8 consecutive days) confirmed that protein expression was due to gene transfer rather than pseudotransduction.

Finally in this chapter, I report successful transduction of primary human nasal brushings with lentiviral vectors coding for human α_1-antitrypsin. Whilst I was able to show that pseudotransduction was not an issue in nasal brushing experiments for hAAT, the same was not true for GLux. The viral vector supernatant expressed significant levels of *Gaussia* luciferase (3- to 4-log orders higher than controls), which meant that I couldn't reliably analyse GLux expression in nasal brushings.

The nose represents an alternative target site for gene transfer, as it is more accessible than the lung and possibly an easier tissue in which to monitor gene expression clinically. The Consortium has previously reported data in human nasal brushings, showing that the non-secreted reporter protein Firefly luciferase was expressed at similar levels after being treated with rSIV.F/HN at multiplicities of infection (MOI) of 25 and 250. Interestingly, whilst I saw significant levels of hAAT in my experiments with an MOI of 5, an MOI of 50 resulted in approximately 2-fold less hAAT expression. This raises the possibility that larger amounts of virus were toxic in my experiments. Importantly, in my nasal brushing experiments highly purified and concentrated lentiviral vectors produced in suspension cell cultures were used, whereas earlier experiments used vectors produced in adherent cell cultures.

It is also not clear why pseudotransduction was such a problem in nasal brushing cells treated with vectors coding for GLux, but not for hAAT. One possible explanation is that the relatively small (19 kDa), stable *Gaussia* luciferase protein survived the anion exchange and tangential flow filtration steps involved in lentiviral production, whereas hAAT (52 kDa) did not. This issue warrants further research, which was not possible with the resources available in this project. Importantly, it should be noted that the lentiviral vectors used in the first ALI experiment (Figure 6-1) and the lung slice experiment reported in this chapter were produced using different methods and were not purified; the differences in the vectors used in the different experiments mean that they cannot be directly compared. Furthermore, the viral vector used in the second ALI experiment (Figure 6-3) was the same as used in the nasal brushing experiment. In the ALI experiment, GLux expression was seen for at least 21 days (the furthest time-point studied); by this time, the ALIs had been washed and media changed at least 12 times, implying that the effects of pseudo-transduction should have been removed. This shows that pseudo-transduction does not necessarily limit the use of certain viral vector preparations in experiments.

In summary, the data presented in this chapter lead to a number of important conclusions. There is enough positive data to prove that rSIV.F/HN vectors can produce secreted proteins in *ex vivo* models of human pulmonary tissue. hAAT could be successfully produced in lung slices and nasal brushings, although high levels of endogenous hAAT expression in air-liquid interface cultures limit their use in studies of hAAT gene therapy. I have also shown that ALIs can produce secreted reporter protein for approximately three months. In the next chapter, I will assess whether hAAT protein produced following gene therapy *in vivo*, *in vitro* and *ex vivo* is functional and carries out its primary function, the inhibition of the serine protease neutrophil elastase.

7 Assessment of the functionality of α_1-antitrypsin protein produced by gene therapy

7.1 Introduction

Having shown that gene therapy with a lentiviral vector can produce secreted human α_1-antitrypsin in mice and various *ex vivo* tissue culture models, I next sought to determine if the protein produced following gene therapy is functional.

Although hAAT has a number of roles in the body [38], its most important role is the inhibition of serine proteases such as neutrophil elastase. Unopposed activity of neutrophil elastase (NE) and other proteases leads to proteolytic destruction of lung tissue, the main cause of morbidity and mortality in α_1-antitrypsin deficiency [1].

Therefore, it is important to investigate the ability of hAAT produced by gene therapy to inhibit neutrophil elastase. A literature search revealed several reports of the investigation of neutrophil elastase inhibition, most of which were based on the measurement of the rate of neutrophil elastase-mediated cleavage of a specific substrate, MeOSuc-Ala-Ala-Pro-Val-pNA or similar [209]. Although some of the details differ between publications, the main principle of the NE rate assay is that cleavage of a specific substrate by NE results in a colour change that can be measured due to liberation of the chromophore pNA (p-nitroaniline); if NE is inhibited (e.g. by α_1-antitrypsin), the substrate cannot bind, is therefore not cleaved, and no colour change occurs (Figure 7-1).

Several commercially-available assays that operate on this principle were found from a variety of suppliers. In the first instance, a chromogenic assay from Enzo Life Sciences was selected based on the grounds that (a) the substrates and methods used in the kit were similar

to many reports found in the literature, and (b) unlike the other assays identified, the Enzo assay was compatible with the Appliskan plate reader used in the laboratory.

Hypothesis: secreted human α_1-antitrypsin produced following gene transfer is functional and inhibits neutrophil elastase.

Aim: perform a neutrophil elastase activity assay on samples from *in vitro, ex vivo* and *in vivo* experiments, to demonstrate functionality of the recombinant protein.

Conclusion: Molecules in tissue culture medium introduce sample scatter, making it difficult to observe NE inhibition after gene transfer *in vitro*. However, I show that hAAT produced *in vivo* effectively inhibits neutrophil elastase, supporting progression of rSIV.F/HN to further pre-clinical and clinical studies.

Much of the work presented in this chapter was performed by Lidia Cammack as part of a six-month Master's project. As the day-to-day supervisor of this project, I was responsible for the design and interpretation of the experiments and assisting Lidia in performing them. Experiments that were performed by Lidia are clearly identified in the figure legends.

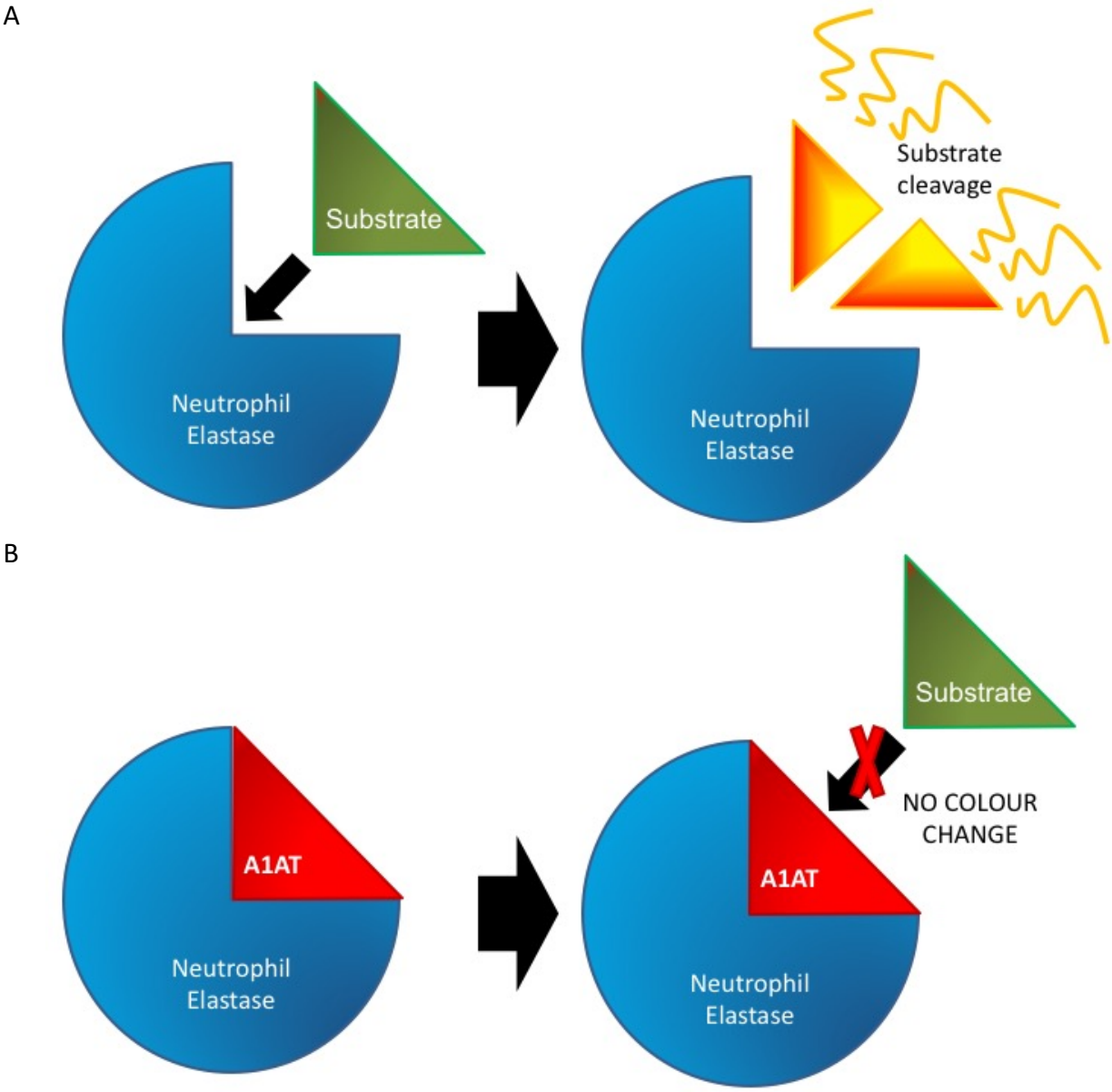


Figure 7-1 Principle of rate assays to measure neutrophil elastase activity

(a) A specific, labelled substrate for neutrophil elastase (usually MeOSuc-Ala-Ala-Pro-Val-pNA) is cleaved by neutrophil elastase, which results in a measureable colour change.

(b) Inhibitors of neutrophil elastase, such as α_1-antitrypsin or the test inhibitor elastatinal, irreversibly bind neutrophil elastase. This prevents cleavage of the substrate, and no colour change occurs.

Schematic courtesy of Lidia Cammack

7.2 Materials & Methods

A commercially available neutrophil elastase (NE) colorimetric drug discovery kit was purchased from Enzo Life Sciences (Exeter, UK) and performed according to the manufacturer's instructions.

20 µl of sample was incubated with 95 µl assay buffer (100 mM HEPES, 500mM NaCl, 0.05% Tween-20, pH 7.25) containing 0.22 µM per well neutrophil elastase. *Blank* (no NE and no sample) and *control* (no sample) wells were included each time the assay was performed. To validate the assay, the activity of either 100 µM elastatinal (a test inhibitor supplied with the kit [210]) or purified human α_1-antitrypsin (Sigma, UK), diluted to the concentrations stated in the figure legends, was measured. All samples were diluted in assay buffer as appropriate. All samples were incubated for 30 minutes at 37°C. Each sample, blank and control was analysed in duplicate.

The samples analysed are described in the figure legends. "Spiked' samples were spiked with the appropriate amount of purified human α_1-antitrypsin (Sigma, UK).

The reaction was started with the addition of 5 µl of synthetic NE substrate MeOSuc-Ala-Ala-Pro-Val-pNA (2 mM). After addition of 5 µl substrate to each well, the plate was immediately placed in a plate reader and absorbance read at 405 nm at one-minute intervals, for 10 minutes.

For each sample analysed in duplicate, the mean optical density was plotted against time in GraphPad Prism (version 6). The reaction velocity was calculated by using the software to calculate the slope of the line of best fit (**V**). Data was expressed as percentage remaining neutrophil elastase activity which was calculated using the following formula, which anchors *control* samples to 100% neutrophil elastase activity:

$$Sample\ percentage\ remaining\ NE\ activity = \left(\frac{\mathbf{V}\text{sample}}{\mathbf{V}\text{control}}\right) x\ 100$$

7.2.1 Calculating molarity of human α_1-antitrypsin

To aid interpretation of the neutrophil elastase assay, it was occasionally necessarily to calculate the molarity of hAAT from the concentration in ng/ml or µg/ml. This was done using the equation:

$$C = \frac{m}{v} x \frac{1}{MV}$$

where *C* is the molar concentration in moles, *m* is the mass of the solute in grams, *v* is the volume of solution in litres, and *MV* is the molecular weight in Daltons. Human α_1-antitrypsin has a molecular weight of 52,000 Daltons [38].

7.3 Results

7.3.1 Assay validation and interpretation of reaction velocities

Before investigating the ability of hAAT produced following gene transfer to inhibit neutrophil elastase, it was necessary to validate the assay. In the first stage of validation, the assay was performed with *blank, control* and *test inhibitor* samples as recommended by the manufacturer. *Blank* refers to a well with neither NE nor sample; *control* refers to a well with no sample, and *test inhibitor* refers to a well with elastatinal, an inhibitor of neutrophil elastase supplied with the kit.

In the assay, mean optical density (mOD) readings are taken every minute for 10 minutes. The mOD is a measure of the release of the chromophore pNA from MeOSuc-Ala-Ala-Pro-Val-pNA, the highly specific substrate for neutrophil elastase. If neutrophil elastase has not been inhibited (for example in the *control* wells), the substrate is broken down and pNA gradually released, leading to gradually higher mOD readings over time. If there is no neutrophil elastase present (i.e. in the *blank*), no substrate breakdown will occur, and the mOD readings over time will remain constant. If neutrophil elastase has been partially inhibited (e.g. by the test inhibitor elastatinal), the rate of change in mOD over time will be somewhere between the control and blank values.

In our hands, the mean optical density (mOD) readings and reaction velocities of blank, control and test inhibitor samples were similar to those reported in the manufacturer's data sheet (Figure 7-2A and Figure 7-2B). When the percentage of NE activity was calculated from the reaction velocities and compared to the control (anchored to 100% neutrophil elastase activity), 100 μM of test inhibitor elastatinal was shown to reduce NE activity to 57% (Figure 7-3A), comparable to the amount of inhibition of neutrophil elastase reported in the manufacturer's datasheet (approximately 60%, Figure 7-3B).

This validation process was repeated for each new assay kit purchased from Enzo Life Sciences. For two out of the seven kits purchased, the kit could not be validated despite proper storage and handling of the kit reagents according to the manufacturer's recommendations. Representative reaction velocities are shown on Figure 7-4, where it can be seen that there is no activity of the neutrophil elastase enzyme. On both occasions the manufacturer replaced the kit, and the replacement kit was successfully validated.

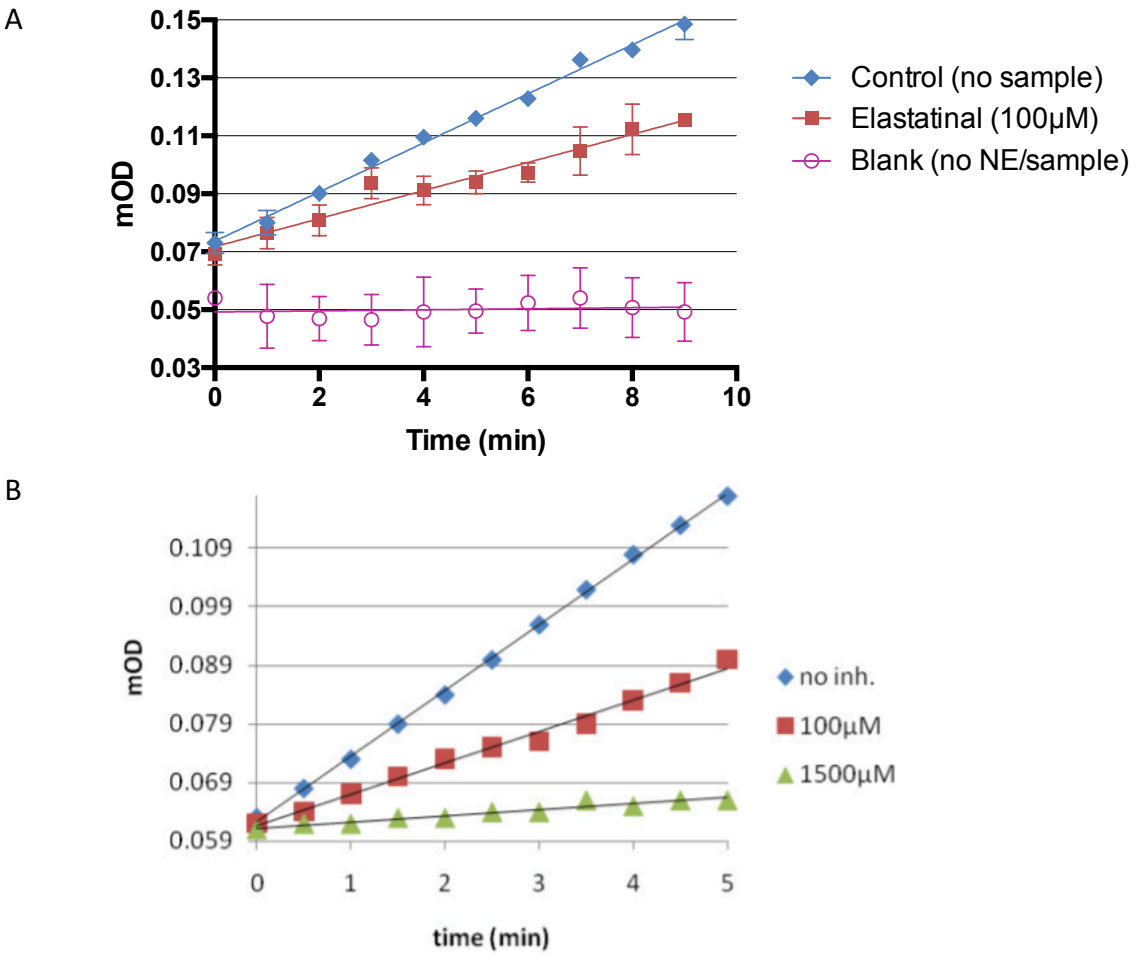


Figure 7-2 Validation of the Enzo colorimetric neutrophil elastase inhibition assay

(A) 20 µl of control, blank and test inhibitor samples supplied with the Enzo neutrophil elastase kit were tested according to the manufacturer's instructions. NE function was measured by absorbance at 405 nm (mOD, mean optical density). Absorbance was measured every minute for ten minutes, and plotted against time to show the reaction velocity. Each data point represents the mean of n=2 samples analysed in duplicate. Error bars represent range. The data shown is representative of three separate validation experiments.

(B) Reaction velocities of control ('no inh') and elastatinal ('100 µM') samples reported by the manufacturer (Enzo Life Sciences Instruction Manual, BML-AK497).

This experiment was performed by Lidia Cammack under my supervision.

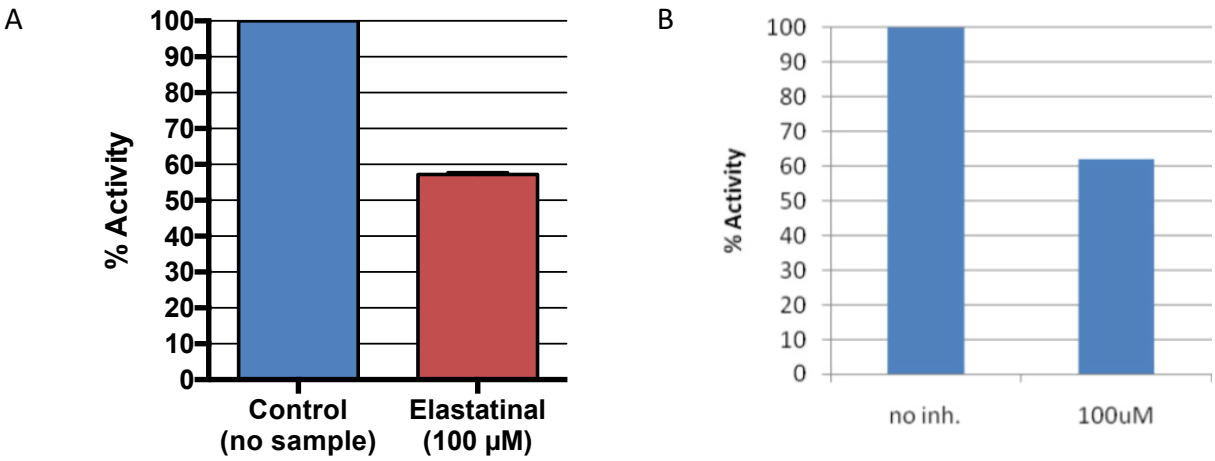


Figure 7-3 Validation of the Enzo colorimetric neutrophil elastase inhibition assay

(A) Percentage neutrophil elastase activity for samples in Figure 7-2 is calculated using the reaction velocity (gradient of line of best fit). Each bar represents mean of n=2 samples analysed in duplicate. Error bars represent range.

(B) Percentage neutrophil elastase activity reported by Enzo Life Sciences (Instruction Manual, BML-AK497). No inh=no inhibitor (i.e. control with no sample). 100uM=100 µM elastatinal (test inhibitor).

This experiment was performed by Lidia Cammack under my supervision.

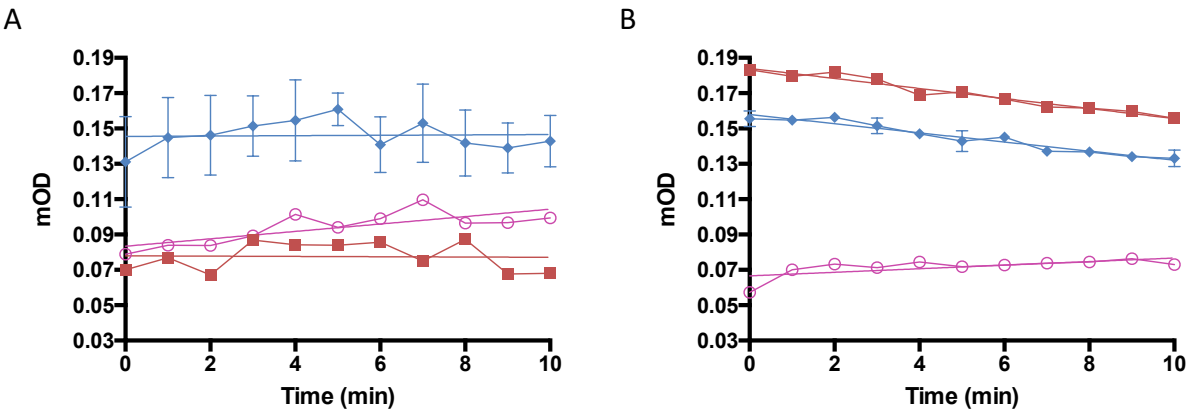


Figure 7-4 Lack of enzyme activity in certain batches of Enzo colorimetric neutrophil elastase assay

20 µl of control (closed diamonds), blank (open circles) and test inhibitor (100 µM elastatinal, closed squares) samples supplied with two batches (a) and (b) of the Enzo neutrophil elastase kit were tested according to the manufacturer's instructions. NE function was measured by absorbance at 405 nm (mOD, mean optical density). Absorbance was measured every minute for ten minutes, and plotted against time to show the reaction velocity.

In some batches of assay kit, no neutrophil elastase enzyme activity could be detected despite proper storage and handling of the kit reagents.

Each data point represents the mean of n=2 samples analysed in duplicate. Error bars represent range. The data shown is representative of two validation experiments per batch.

7.3.2 Assay validation – purified human α_1-antitrypsin completely inhibits neutrophil elastase at high concentrations

In the next step of the validation process, we tested the activity of hAAT purified from pooled human plasma. We performed serial dilutions of purified hAAT from 0.0125 to 20 µM (each sample analysed in duplicate). There was a concentration-dependent inhibition of NE with increasing concentrations of purified hAAT. Because there were only n=2 samples per concentration, it was not appropriate to perform statistical analysis on this data; however, above a concentration of 0.025 µM hAAT, there was noticeable inhibition of neutrophil elastase (Figure 7-5). Above concentrations of 0.1 µM hAAT, there was between 2 to 15% remaining neutrophil elastase activity. This is consistent with what would be expected; each assay reaction contains 0.22 µM neutrophil elastase, and binding of hAAT to NE occurs in an irreversible, 1:1 molar fashion [1].

This data is further proof that the assay works as intended; high concentrations of the potent neutrophil elastase inhibitor, purified human α_1-antitrypsin, inhibited neutrophil elastase in a concentration-dependent manner when diluted in assay buffer.

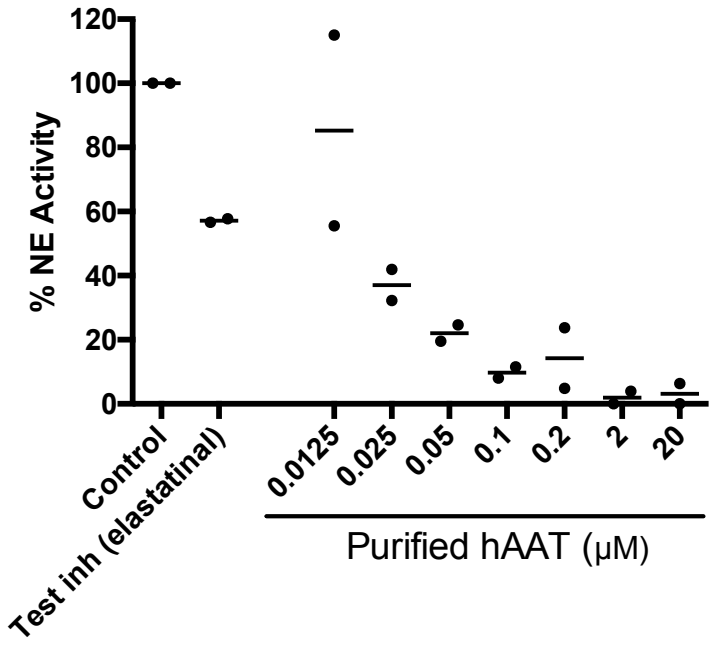


Figure 7-5 Purified human α_1-antitrypsin inhibits neutrophil elastase

Human α1-antitrypsin (hAAT) purified from pooled human plasma was diluted in assay buffer to the concentrations shown, and the percentage neutrophil elastase activity determined. With increasing concentrations of purified hAAT, NE activity decreased.
Each data point represents one sample; horizontal bars represent mean of samples analysed in duplicate. The x-axis shows the concentration of hAAT in μM.

Control=no sample (NE only). Test inh=test inhibitor (elastatinal) at 100 μM. NE=neutrophil elastase.

This experiment was performed by Lidia Cammack under my supervision.

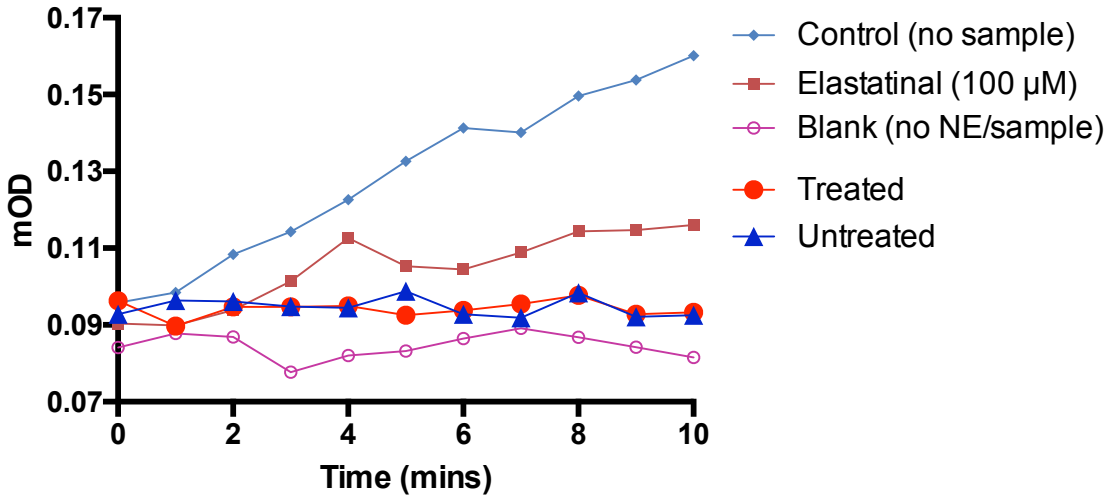
7.3.3 Tissue culture media from A549 cells inhibits neutrophil elastase in the Enzo colorimetric kit

Having demonstrated (a) that the neutrophil elastase assay kit works as expected and (b) that neutrophil elastase is inhibited after treatment with purified hAAT, I next sought to determine if recombinant hAAT made following gene transfer *in vitro* inhibits neutrophil elastase. A transduction experiment in A549 cells was performed, as reported in Figure 4-4B (page 142). In this experiment, the highest doses of rSIV.F/HN (titre unknown; volume given 50 µl or 100 µl virus in 100 µl total volume) produced approximately 10 ng of hAAT per ml of tissue culture supernatant. This is equivalent to approximately 1.92e-4 µM hAAT, which according to the results in Figure 7-5 would not significantly inhibit neutrophil elastase in this assay.

Cell culture supernatant from A549 cells transduced with 50 µl rSIV.F/HN-hCEF-sohAAT in 100 µl total volume was analysed on the neutrophil elastase assay, and compared to cell culture supernatant from untreated cells. The 50 µl dose group was chosen because this group expressed the highest concentration of hAAT. The reaction velocities of the experiment are shown in Figure 7-6A. Both treated and untreated A549 cell supernatant (n=5 samples per group) displayed flat reaction velocities, implying that neutrophil elastase was completely inhibited. When the % neutrophil elastase activity was calculated from the slope of the line of best fit, this finding was confirmed; residual neutrophil elastase activity was 4.3% in the treated group, compared to 0.92% in the untreated group (median values, not significant). This suggests that either (a) there are neutrophil-elastase inhibiting proteases intrinsically expressed in A549 cell media, or (b) that some other property of the cell culture media makes it unsuitable for analysis in this assay. One solution could have been to dilute the cell culture media, however because the hAAT concentration was known to be 1.92e-4 µM, i.e. outside

of the range of sensitivity of the assay, it was decided that this would not be worthwhile. The data from this experiment was therefore inconclusive.

A



B

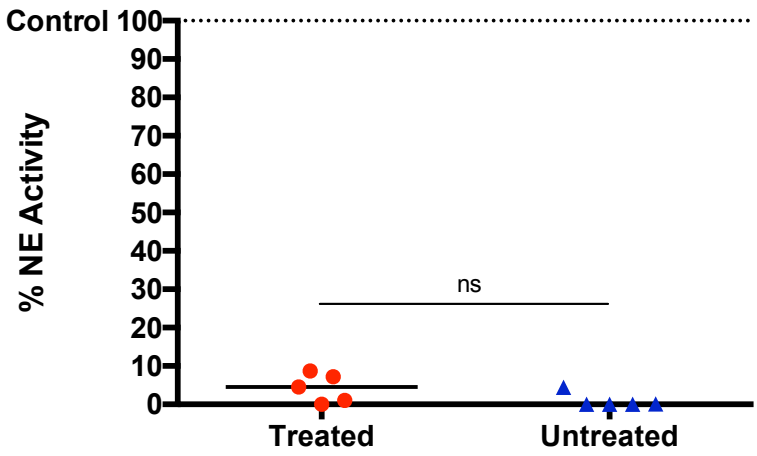


Figure 7-6 Cell culture supernatant from A549 cells inhibits neutrophil elastase

(A) Cell culture supernatant from A549 cells **treated** with rSIV.F/HN-hCEF-sohAAT were analysed for neutrophil elastase inhibitory capability, and compared to cell culture supernatant from **untreated** cells (given Optimem only). The functional titre of the virus batch used could not be determined as it was below the lower limit of detection of the TaqMan transduction assay.

NE function was measured by absorbance at 405 nm (mOD, mean optical density). Absorbance was measured every minute for ten minutes, and plotted against time to show the reaction velocity.

For control, blank and elastatinal, each data point represents the mean of n=2 samples analysed in duplicate. For treated and untreated BALF samples, each data point represents the mean mOD for n=5 samples per group, each analysed in duplicate. Error bars have been omitted for the sake of clarity.

(B) Percentage neutrophil elastase (NE) activity in cells **treated** with rSIV.F/HN-hCEF-sohAAT or left **untreated.** The % NE activity was calculated for n=5 samples per group compared to a control (NE only). Each data point represents an individual well of cells. Horizontal bars represent group medians. The dotted line represents the assay control (neutrophil elastase only), which is anchored to 100% neutrophil elastase activity. ns=not significant (Mann Whitney *U* test).

This experiment was was designed by me and performed by Lidia Cammack under my supervision.

7.3.4 Broncho-alveolar lavage fluid needs to be diluted for accurate interpretation of neutrophil elastase activity

Because it was not possible to explore the ability of hAAT produced by gene therapy *in vitro* to inhibit neutrophil elastase, I next analysed broncho-alveolar lavage fluid (BALF) samples from the *in vivo* experiments. Mice treated with 1e8 to 1.5e8 TTU of rSIV.F/HN-hCEF-sohAAT expressed therapeutic levels of human α_1-antitrypsin (Figure 5-6 and Figure 5-7), and experiments were performed to see if this protein was functional; i.e. if it inhibited human neutrophil elastase.

I chose to analyse BALF rather than lung tissue homogenate because I reasoned that it would contain less cellular debris, which I have already shown can interfere in the neutrophil elastase assay. Please note that in this chapter I will discuss the analysis of *broncho-alveolar lavage fluid* samples; however, in previous chapters, I reported the post-treatment concentration of hAAT in *epithelial lining fluid*, which is derived from the hAAT concentration in BALF by multiplying by the dilution factor. For a more detailed discussion, please refer to Section 3.3.3.

In mice treated with rSIV.F/HN-hCEF-sohAAT in Figure 5-6 and Figure 5-7, the mean concentration of hAAT in broncho-alveolar lavage fluid (BALF) was 2000 ng/ml (0.04 μM), which is predicted to lead to neutrophil elastase inhibition based on the data in Figure 7-5, where an equivalent concentration of hAAT reduced neutrophil elastase assay to approximately 40% compared to the control.

We first analysed undiluted BALF samples from treated and untreated mice. The baseline optical density (OD) readings at time 0 were higher than the baseline readings in the control samples, which prevents accurate interpretation of the data (Figure 7-7A). It would still have been possible to calculate the percentage neutrophil elastase activity (as this is determined by

the slope of the line of best fit, regardless of the initial OD), however we were concerned that the data analysis would not have been reliable. We therefore performed serial dilutions of BALF samples (1:1, 1:10, 1:100 and 1:1000, n=2 samples from treated mice and n =2 samples from untreated mice). Consistent with previous observations, undiluted BALF samples had a higher baseline OD than the control (neutrophil elastase only; Figure 7-7). Diluting BALF samples 1:100 or 1:1000 resulted in a similar reaction velocity to the control, suggesting that all neutrophil elastase inhibitory capability was lost. However, dilution of BALF 1:10 reduced baseline OD readings to a value lower than the control, and did not completely remove neutrophil elastase inhibitory capability of BALF samples. Based on these results, BALF samples in future experiments were diluted 1:10.

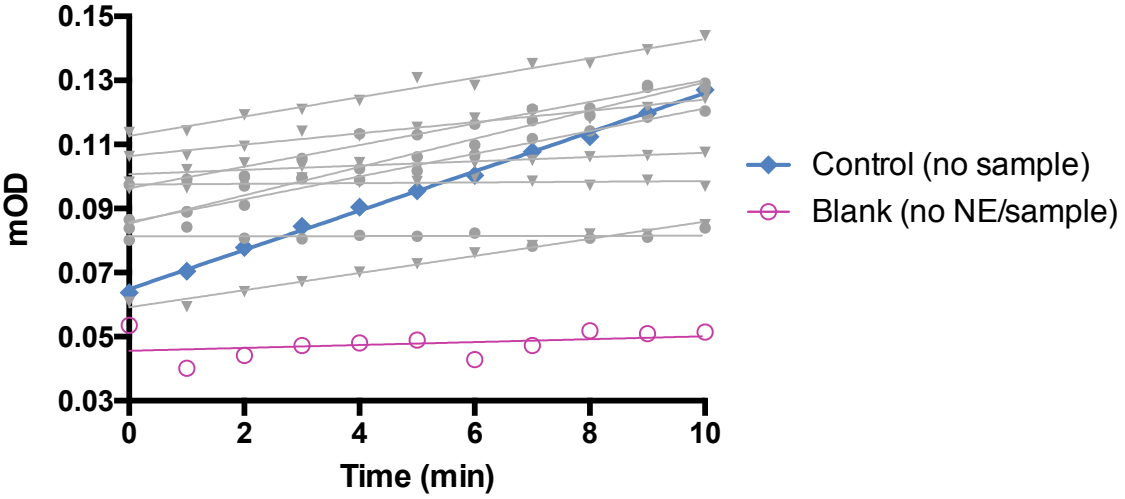


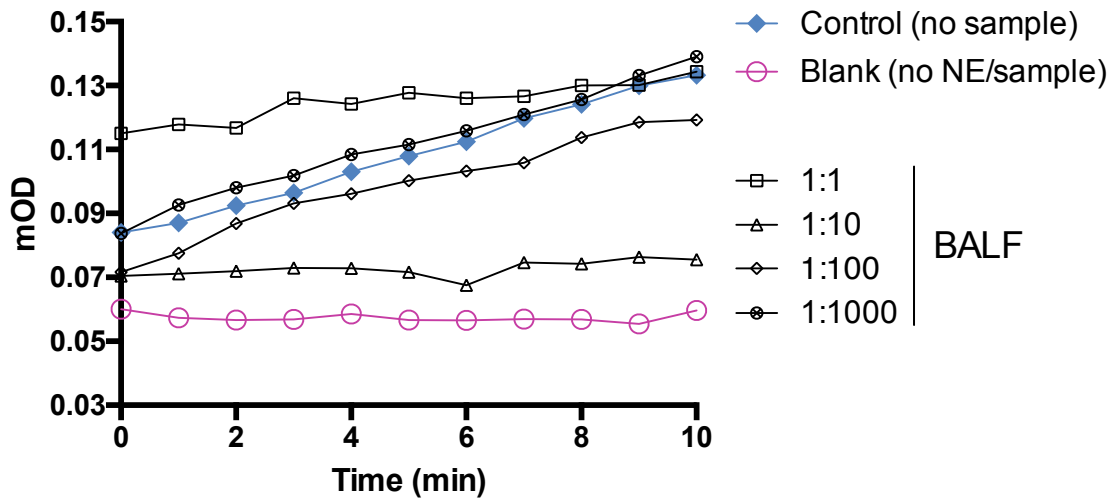
Figure 7-7 Undiluted samples have a high baseline optical density in the neutrophil elastase assay

Broncho-alveolar lavage fluid (BALF) samples from 4 mice treated with rSIV.F/HN-hCEF-sohAAT (grey triangles) and from 4 mice treated with PBS (grey circles) were analysed in undiluted form. Because the baseline mean optical density (mOD) readings at time 0 were higher than control readings, the data cannot be reliably interpreted.

Each data point shows the mean of n=2 samples analysed in duplicate. Error bars are omitted for the sake of clarity.

This experiment was designed by me and performed by Lidia Cammack under my supervision.

A



B

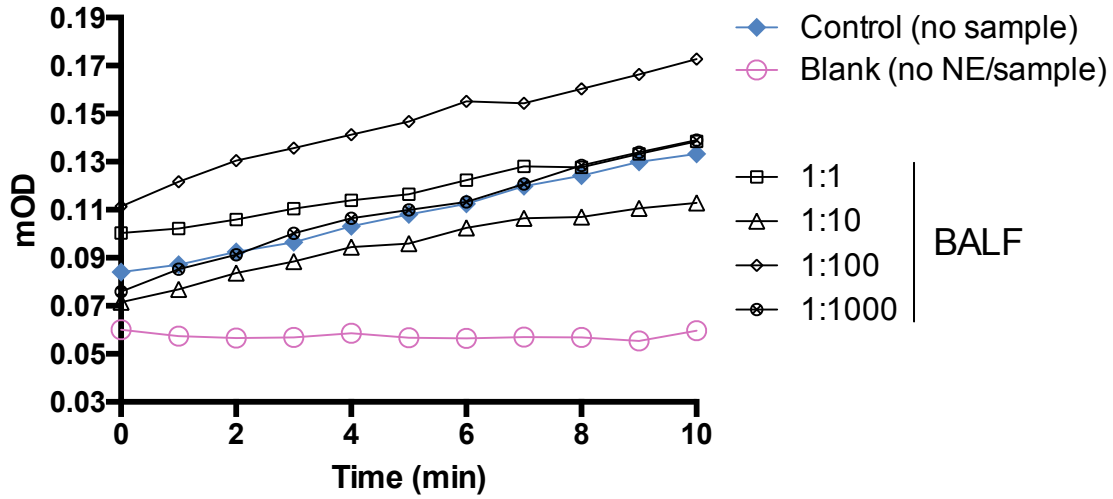


Figure 7-8 Dilution of broncoalveolar lavage fluid samples reduces baseline optical density and allows accurate analysis on the neutrophil elastase assay

Broncho-alveolar lavage fluid (BALF) samples were serially diluted and analysed on the neutrophil elastase assay. Here, one representative set of dilutions from n=2 animals treated with rSIV.F/HN-hCEF-sohAAT (**A**) and one representative set of dilutions from n=2 animals treated with PBS (**B**) are shown.

Consistent with previous observations, undiluted BALF samples (open squares) have a higher baseline mean optical density (mOD) than controls. Diluting samples 1:10 (open triangles) decreases baseline mOD to a level below the control, allowing accurate interpretation of neutrophil elastase inhibition. Dilution to 1:100 (open diamonds) or 1:1000 (crossed circles) removes neutrophil elastase inhibition.

Each data point represents the mean of n=2 samples analysed in duplicate. Error bars are omitted for the sake of clarity.

This experiment was was designed by me and performed by Lidia Cammack under my supervision.

7.3.5 Murine broncho-alveolar lavage fluid does not significantly inhibit human neutrophil elastase

In the final step of assay validation before analysing BALF samples from treated mice, I analysed the ability of BALF from untreated, wild type mice to inhibit human neutrophil elastase in this assay. BALF from untreated mice was compared to control samples, which are anchored to 100% neutrophil elastase activity; I also spiked some untreated BALF samples with purified human α_1-antitrypsin at a concentration of 0.2 μM, as I had previously shown that this concentration of hAAT should completely inhibit NE, and this data therefore will help to determine whether or not the assay has worked.

Spiked and unspiked BALF samples from untreated mice were diluted 1:10 and analysed. In unspiked samples, there was still high neutrophil elastase activity (75±4.2% compared to the control, which is given an arbitrary value of 100% neutrophil elastase activity), whereas in spiked samples there was little neutrophil elastase activity remaining (1.9±0.7%, $p<0.0001$). This data shows that whilst untreated murine BALF does partially inhibit human neutrophil elastase when diluted 1:10, the effect is modest. This supports future analysis of BALF samples from mice treated with rSIV.F/HN-hCEF-sohAAT.

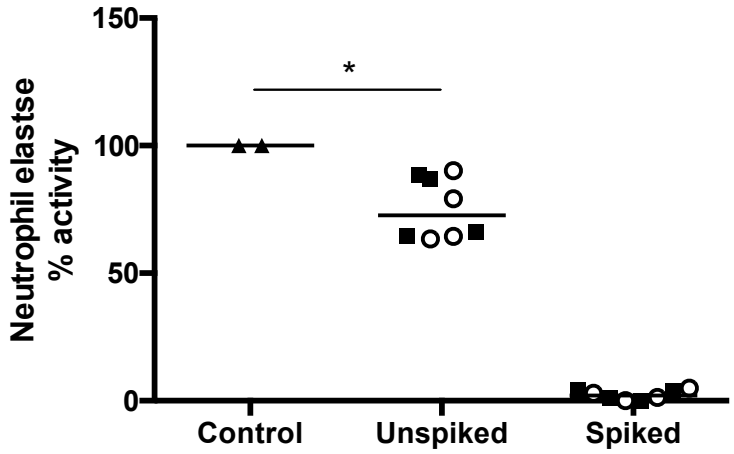


Figure 7-9 Murine broncho-alveolar lavage fluid samples partially, but not wholly, inhibit human neutrophil elastase

Broncho-alveolar lavage fluid (BALF) samples from untreated mice were analysed for their capacity to inhibit human neutrophil elastase (NE). Unspiked samples were compared to samples spiked with 0.2 μM human α_1-antitrypsin, a concentration which has previously been shown to completely inhibit human neutrophil elastase. All BALF samples were diluted 1:10, and the reaction velocities compared to control samples (NE only), which were anchored to 100%.

Data from two separate experiments (represented by closed squares and open circles, respectively) are shown.

Each data point represents the mean neutrophil elastase activity from n=2 samples analysed in duplicate. Horizontal bars represent group medians.

*=$p<0.05$ compared to control (n=2 wells with no sample, i.e. assumed to have 100% neutrophil elastase activity) (Mann Whitney *U* test).

7.3.6 Broncho-alveolar lavage fluid from mice treated with rSIV.F/HN-hCEF-sohAAT inhibits neutrophil elastase

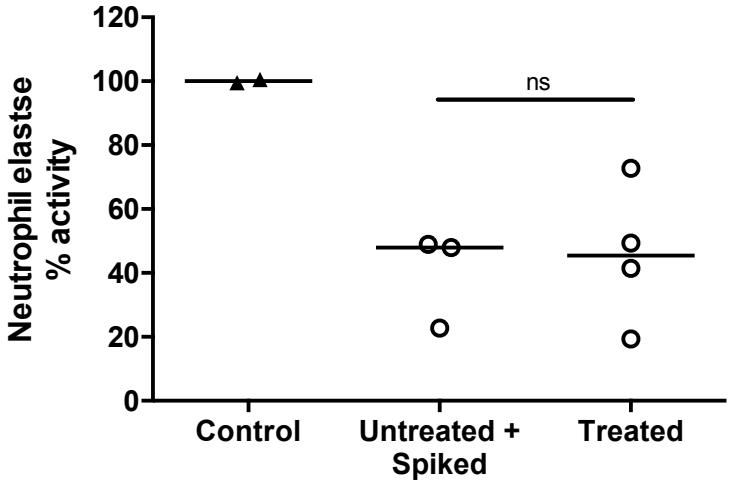
Having shown that (1) murine BALF samples need to be diluted to be analysed on the neutrophil elastase assay, and (2) that BALF from untreated mice does not completely inhibit human neutrophil elastase in the assay, I was able to move on to analyse the effect of treatment with the lentiviral vector rSIV.F/HN-hCEF-sohAAT on the ability of murine BALF to inhibit neutrophil elastase.

BALF from treated mice, with a median hAAT concentration of 2000 ng/ml (0.04 μM) was compared to BALF from untreated mice, which was spiked with an equivalent amount of purified hAAT. Whilst it would have been desirable to analyse treated BALF, untreated BALF *and* spiked BALF on the same assay, this was not possible due to the limited number of samples that can be analysed at one time on the assay kit.

Two separate experiments were performed, which are shown on Figure 7-10. In both experiments, treated and spiked samples inhibited neutrophil elastase; the remaining neutrophil elastase activity was approximately 50%. In the first experiment, with small numbers (n=3-4 per group), there was no significant difference in group medians. In the second experiment (n=6-7 per group), samples from different treated mice inhibited neutrophil elastase slightly more than spiked samples (untreated: median 35% (range 0-57%) NE activity; *spiked:* median 65% (range 39-81%) NE activity; $p<0.05$). As the experiments were performed using the same method, a combined analysis was performed. This analysis showed that there was no significant difference between group medians when comparing BALF from treated mice, and BALF from untreated mice spiked with an equivalent amount of purified hAAT (Figure 7-11, n= 9-11 per group).

Taken together with data from previous experiments, which showed that BALF from untreated mice inhibited neutrophil elastase activity by approximately 25% (Figure 7-9), this data shows that treatment of mice with the lentiviral vector rSIV.F/HN-hCEF-sohAAT, coding for hAAT, produces a functional protein in broncho-alveolar lavage fluid that is able to inhibit human neutrophil elastase.

A



B

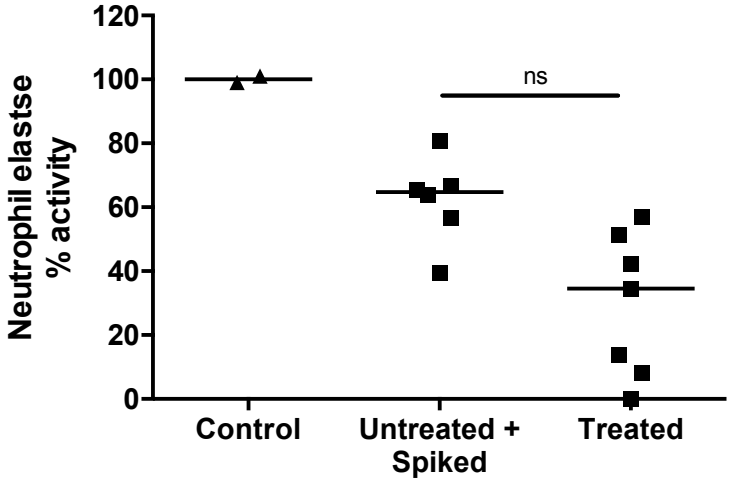


Figure 7-10 Analysis of the effect of treatment with rSIV.F/HN-hCEF-sohAAT on the neutrophil elastase inhibitory capacity of murine broncho-alveolar lavage fluid

Mice were treated with rSIV.F/HN-hCEF-sohAAT (1 to 1.5e8 TTU/mouse) by nasal sniffing, and were shown to have median hAAT levels of ~ 2000 ng/ml. The ability of BALF from these animals to inhibit neutrophil elastase was compared to BALF from untreated animals which had been spiked with equivalent amounts of purified hAAT (2000 ng/ml, 0.04 µM). Two separate experiments were performed with different samples, shown on panels **(A)** and **(B)**. All BALF samples were diluted 1:10.

Each data point represents the mean neutrophil elastase activity from n=2 samples analysed in duplicate. Horizontal bars represent group medians.

*=$p<0.05$, ns=not significant (Mann-Whitney *U* Test).

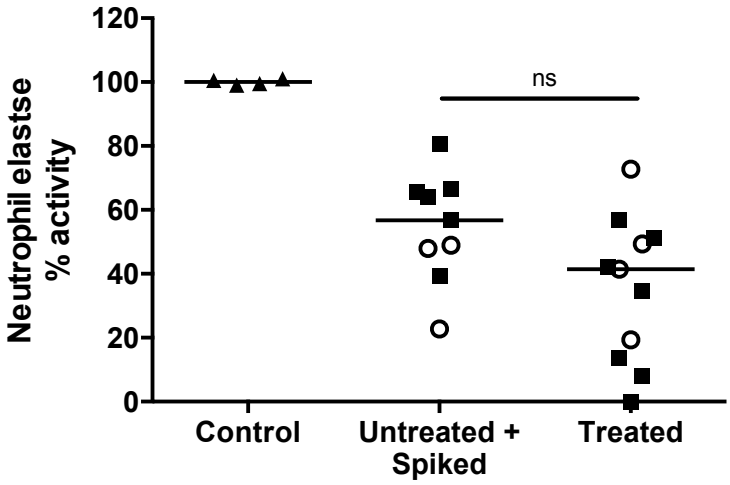


Figure 7-11 A combined analysis of the data from Figure 7-10 shows that there is no difference in the ability of treated and spiked samples to inhibit neutrophil elastase

Here, data from Figure 7-10 is combined to increase group numbers.

Each data point represents the mean neutrophil elastase activity from readings performed in duplicate. Horizontal bars represent group medians. Two separate experiments are represented, shown by closed and open symbols respectively.

ns=not significant (Mann-Whitney *U* Test).

7.4 Discussion

In previous chapters, I have shown that the lentiviral vector rSIV.F/HN-hCEF-sohAAT can produce significant levels of human α_1-antitrypsin in mice and various *ex vivo* human tissue culture models. It is necessary to prove that the recombinant protein is functional; the fact that hAAT is detected on an ELISA does not necessarily imply that it is able to carry out its main physiological function, namely inhibition of neutrophil elastase.

After being transcribed and translated, many proteins undergo post-translational modification, which is necessary for the protein to be functional. These steps may involve protein folding, membrane trafficking, or other chemical modifications. α_1-antitrypsin undergoes post-translational glycosylation at three sites [66], which is relevant for its function. One clinical trial of inhaled α_1-antitrypsin for cystic fibrosis [68] used recombinant hAAT produced in sheep by PPL Therapeutics, Roslin, Scotland [211]. The trial failed to show significance, with no effect of inhaled hAAT on sputum neutrophil elastase activity or the level of sputum hAAT/NE complexes. In a different trial of inhaled hAAT therapy in cystic fibrosis, investigators used hAAT purified from human plasma (Prolastin, manufactured by Bayer, USA). Whilst the trial did not reach its primary endpoint, there was a significant decrease in the levels of neutrophil elastase in sputum [64]. The discrepancy between these two trials is believed to be because of the difference between recombinant hAAT, used by Martin *et al*, which is unglycosylated, to purified hAAT, used by Griese *et al*, which is glycosylated [66]. Whilst there is no reason to believe that α_1-antitrypsin produced following gene therapy in my experiments will not be functional, this example illustrates why it is still necessary to investigate it in detail.

hAAT is a multifunctional protein, with many roles throughout the body [38]. However, the long-held consensus is that the main role of α_1-antitrypsin is the inhibition of serine proteases,

particularly neutrophil elastase. In α_1-antitrypsin deficiency (AATD), there are two major causes of morbidity and mortality; mis-folded protein polymerises in hepatocytes causing liver cirrhosis, whilst a lack of circulating protein causes the early onset of chronic obstructive pulmonary disease due to the unopposed action of proteases in the lung. Whilst liver cirrhosis affects approximately 30% of adults with AATD, the penetrance of lung disease is greater with up to 60% of adults with AATD suffering from COPD [1]. This justifies the decision in this chapter to study the inhibitory effect of α_1-antitrypsin produced by gene therapy on neutrophil elastase.

Many key studies of gene therapy for α_1-antitrypsin in mice fail to report on the functionality of hAAT [22] [88] [92]. Wilson *et al* proved that their gene therapy approach using a VSV-G pseudotyped lentiviral vector ameliorated an animal model of emphysema [94]; this would have been a useful experiment to perform but was not feasible in the time-frame of this project. One study of gene therapy for α_1-antitrypsin in mice did show an inhibitory affect of hAAT in serum, using a neutrophil elastase assay which operated on similar principles to the Enzo assay but using a different substrate [85]. They showed that treatment of muscle with an adeno-associated viral vector lead to significant levels of hAAT in serum; in the *ex vivo* assay, serum from treated mice was shown to reduce elastase activity to approximately 30% compared to uninhibited controls.

Likewise, I have not been able to find any investigation of the functionality of hAAT produced by gene therapy in the human trials that have been conducted to date [81] [84] [95]. However, two trials of inhaled α_1-antitrypsin for the treatment of cystic fibrosis did investigate the levels of free neutrophil elastase after treatment [68] [78], both using a neutrophil elastase assay based on cleavage of the substrate MeO-Suc-Ala-Ala-Pro-Val-pNA, similar to the Enzo neutrophil elastase assay kit used in this chapter.

The Enzo neutrophil elastase assay kit is a commercially available, colorimetric drug discovery kit. According to the manufacturer's datasheet, the assay is designed for the screening of potential pharmaceutical inhibitors of neutrophil elastase; unfortunately, we could not find any evidence of this specific kit being used to screen inhibitors of NE (i.e. hAAT) in biological fluids. However, in the four studies we did find where researchers investigated the inhibition of NE in biological fluids, very similar assays were used, with a similar or identical NE substrate (MeO-Suc-Ala-Ala-Pro-Val-pNA) to that used in the Enzo neutrophil elastase assay. As none of the four references stated a manufacturer, I assume that the investigators developed their assays in-house [212] [213] [214] [215]. Developing our own assay would have been feasible in this project, however we reasoned that as the methods used by these investigators were almost identical to the Enzo assay kit, the most appropriate first step would be to analyse our samples using this kit to see if we could demonstrate anti-neutrophil elastase activity.

Before analysing treated samples, we validated the assay kit by showing that (a) the control and blank samples behaved as expected (apart from certain batches of the assay kit, discussed below) and (b) purified human α_1-antitrypsin inhibited neutrophil elastase in a concentration-dependent manner. We then analysed cell culture supernatant from A549 cells treated with rSIV.F/HN-hCEF-sohAAT. hAAT expression in these cells was very modest (10 ng/ml, 1.92e-4 μM), and we did not expect to see significant neutrophil elastase inhibition in the experiment. However, cell culture supernatant from A549 cells (whether treated or untreated) almost completely inhibited neutrophil elastase in this assay. There are two possible explanations for this observation; (1) tissue culture medium from A549 cells, which are derived from human lung adenocarcinoma cells, could constitutively express proteases or other factors that inhibit neutrophil elastase, or (2) the absorbance of tissue culture medium at 405 nm may not be accurately measured by the plate reader due to cellular debris. The

phenomenon of high background absorbance of turbid liquids can result in 'sample scatter'; the plate reader is designed to detect light passing through a liquid in a straight line, however the presence of large particles in the liquid scatters the beam of light, which is falsely recorded as high absorbance [216]. Dilution of tissue culture medium would be expected to reduce this phenomenon, however as the level of hAAT in these samples was already so low it was decided that this would not be worthwhile.

Instead, we looked at the ability of broncho-alveolar lavage fluid (BALF) samples from mice to inhibit human neutrophil elastase. Again, undiluted samples could not be accurately analysed due to a high absorbance at time 0; however, by diluting the lavage fluid 1:10 we were able to obtain usable data. BALF from untreated mice had a small inhibitory effect on human neutrophil elastase, which could be explained in one of two ways. Firstly, murine homologues of neutrophil elastase could break down the assay substrate MeOSuc-Ala-Ala-Pro-Val-pNA, resulting in substrate cleavage, pNA release and increasing optical density readings over time. Activity of murine neutrophil elastase homologues has previously been demonstrated [217]. Secondly, murine homologues of α_1-antitrypsin could inhibit human neutrophil elastase, leading to a positive result. I have been unable to find any reports in the literature regarding the activity of murine α_1-antitrypsin homologues [16] against human neutrophil elastase, but this possibility cannot be ruled out. Finally, BALF diluted 1:10 could still show false neutrophil elastase inhibition due to sample scatter as a result of cellular debris in the solution, as discussed above. Nevertheless, the NE inhibitory capability of untreated murine BALF recorded by this assay was modest; this allowed further studies of the functionality of human α_1-antitrypsin produced in murine BALF following gene therapy.

Importantly, in this chapter I have shown to a reasonable degree of certainty that BALF from mice treated with rSIV.F/HN-hCEF-sohAAT inhibited neutrophil elastase, i.e. the human α_1-antitrypsin protein produced following gene therapy is functional. I have shown this by

comparing the inhibitory capacity of treated BALF with untreated BALF spiked with equivalent amounts of purified hAAT. However, some assay-to-assay variability (as shown in Figure 7-10) makes the data somewhat difficult to interpret. Despite this, a combined analysis of two separate experiments, reported in Figure 7-11, shows that (1) BALF from treated mice inhibited human neutrophil elastase, and (2) the inhibitory capability is roughly equal to untreated murine BALF spiked with equivalent volumes of purified hAAT. Combining the results of two assays like this always requires a great deal of caution, but is unavoidable in this case due to limitations of the Enzo neutrophil elastase assay and our plate reader. The optimal experiment to perform, to prove that hAAT produced by gene therapy is functional, would have involved the following groups:

- BALF samples from untreated mice;
- BALF samples from treated mice;
- BALF samples from untreated mice, spiked with purified hAAT.

However, the assay requires the measurement of optical density every minute, and our Appliskan plate reader can only perform a limited number of readings in this time frame. I determined that, if samples were to be analysed in duplicate (as is preferable), and the necessary controls and blanks were included, a maximum of 13 experimental samples could be analysed per assay. Performing multiple assays sequentially would have been possible but would not have been desirable, as small changes in room temperature or incubation times can affect the assay results [218] and it may have involved repeated freeze-thawing of kit reagents.

We did encounter some problems performing the neutrophil elastase assay. Of the seven kits purchased in total to conduct these experiments, two could not be used because, when validated, unreliable traces of neutrophil elastase activity were obtained. The manufacturer replaced the faulty kits and we carefully validated each new kit, handling all regents with

extreme care (for example, carefully aliquoting the regents to avoid repeated freeze-thaw cycles). I am therefore confident in the data presented here, although we have shown that this assay kit needs to be carefully tested before use.

Unfortunately, time constraints meant that we could not analyse samples from all our experiments – we were not able to investigate the functionality of hAAT after gene therapy in *ex vivo* tissue culture models (air-liquid interfaces, nasal brushings and lung slices). I prioritised analysis of samples from the *in vivo* studies, partly because I felt they were the most relevant and important samples, but also because they were the only samples in which hAAT was concentrated enough to lead to meaningful data in the neutrophil elastase assay. The results of percentage neutrophil elastase activity in serial dilutions of purified hAAT suggest a sensitivity range of the assay of approximately 0.0125 μM to 0.2 μM (equivalent to 625 ng/ml to 10 μg/ml) – the BALF samples from treated mice fall within this concentration range, whilst the samples from lung slices and nasal brushings (median hAAT concentrations of ~ 320 ng/ml and 400 ng/ml respectively) would not have fallen within this range. It was not worthwhile analysing samples from air-liquid interface cultures because earlier work demonstrated that they are not an appropriate model for investigating gene therapy for human α_1-antitrypsin.

The neutrophil elastase assay kit from Enzo is designed for screening pharmaceuticals, and the manufacturer makes no claims about the kit's ability to investigate neutrophil elastase activity in biological fluids. It was the most appropriate kit available at the time of the experiments; however, an in-house assay or an assay kit from an alternative manufacturer may have produced more reliable results in biological tissues.

Despite the limitations of the assay kit, and some concerns over its reliability, I feel that there is enough data to back up the main conclusion of this chapter – that human α_1-antitrypsin produced in BALF following gene therapy in mice is functional. It is important to remember

that the broncho-alveolar lavage fluid samples were diluted 1:10 for this assay; therefore, the true inhibitory effect of hAAT in these experiments is likely to be higher.

8 "The nose as a factory" – using the nasal epithelium for the production of secreted proteins

8.1 Introduction

In Chapter 5, I described *in vivo* experiments of gene delivery to the lung, using it as a factory organ to produce the secreted protein α_1-antitrypsin. An alternative approach for the treatment of some diseases might be to deliver the gene transfer agents to the nasal epithelium, from where recombinant proteins are released into the circulation. It has previously been hypothesised that the nose could be an appropriate factory organ particularly in the context of cystic fibrosis, because in the nose there are no purulent secretions and no pre-existing inflammation, which impair gene delivery attempts to the lung [202]. The nose is also more easily accessible than the lung, and therefore gene delivery to the human nose may be simpler and more cost-effective than treating the lung. Previous work by the Consortium has demonstrated proof-of-concept for using the nose as a factory to produce secreted proteins using Sendai virus [202].

Hypothesis: rSIV.F/HN lentiviral vectors, previously shown to transduce the lung, can also transduce the nasal epithelium and result in the production of proteins.

Aim: use the techniques of nasal perfusion and 'slow nasal sniffing' to selectively transduce the nasal epithelium of mice. Demonstrate protein production using bioluminescent imaging and *ex vivo* assays.

Conclusion: rSIV.F/HN can transduce nasal epithelium, as demonstrated by bioluminescent imaging and *ex vivo* assays. However, it was not possible to selectively transduce the nose, with expression also seen in lung tissue.

8.2 Materials & Methods

C57Bl/6 mice were treated with rSIV.F/HN hCEF-GFPLux. GFPLux is a fusion protein of GFP and Firefly luciferase; both proteins retain their reporter characteristics, although fusing the proteins may quench the signal of both proteins (Uta Griesenbach, personal communication).

Nasal perfusion: animals were treated with 4e7 TTU rSIV.F/HN-hCEF-GFPLux in 100 µl total volume by nasal perfusion at a rate of 6.67 µl/minute, as described in section 2.5.2. Control animals were given 100 µl PBS. The animal was placed with the head tilted backwards at a -30° angle in an effort to avoid liquid dripping into the lung.

At the indicated time-points post-treatment, gene expression was quantified using bioluminescence imaging (BLI) *in vivo*. D-Luciferin (GoldBio, MO, USA) was administered intraperitoneally (150 mg/kg in 200 µl). Mice were anaesthetised and placed in the imaging chamber; the bioluminescence (in photons per second per area per steradian) was measured using an IVIS50 system (Xenogen). A light scale with blue being least intense and red being most intense was used.

Mice were then treated with 100 µl D-luciferin intranasally and culled. The lungs were dissected and placed in the imaging chamber. Subsequently, the lungs were snap frozen and later homogenised, with Firefly luciferase expression quantified as described in Section 2.6.

The nasal epithelium was also dissected and processed as previously described in Section 2.5.3.

Slow nasal sniffing: animals were treated with 7e7 TU rSIV.F/HN-hCEF-GFPLux in 100 µl total volume. Six mice were treated by slow nasal sniffing; the dose was split into 10 µl aliquots, administered to the nasal epithelium at 2 minute intervals. Three control mice were

treated by fast nasal sniffing (100 µl bolus administration). Imaging and tissue harvesting was performed as described above.

Quantification of gene expression: firefly luciferase assays on nasal and lung tissue homogenate were performed as described in Section 2.6. For bioluminescent imaging, photon emission from a pre-defined area (the nose or lung, as appropriate) was measured using LivingImage software, expressed as photons/second/cm^2/steradian (p/s/cm^2/sr).

8.3 Results

8.3.1 A pilot study of gene expression following nasal perfusion

The aim of the pilot experiments in this chapter is to demonstrate that rSIV.F/HN can transduce the nasal epithelium and produce reporter proteins. A lentiviral vector carrying the gene for the GFPLux fusion protein (green fluorescent protein and Firefly luciferase) was used; this non-secreted protein was selected so that it would be possible to determine which tissues had been transduced.

In the first experiment, two mice were treated with 4e7 TTU rSIV.F/HN-hCEF-GFPLux in 100 µl total volume by nasal perfusion. A control animal was given 100 µl PBS. Nasal perfusion is a technique that has been developed by the UK CF Gene Therapy Consortium to deliver gene transfer agents to the nasal epithelium; the aim of this experiment was to validate the technique to see if it was possible to selectively transduce the nose. A fine tip catheter (0.5 mm diameter) is placed 2.5 mm inside one nostril, and the gene therapy vector (or control) slowly perfused by a syringe pump over 15 minutes.

Two days post-treatment, mice were anaesthetised, given 150 mg/kg D-luciferin intraperitoneally and imaged 10 minutes later in a bioluminescent imaging machine (Figure 8-1). There was significant protein expression seen in the nose, validating my nasal perfusion technique and justifying the progression to larger experiments.

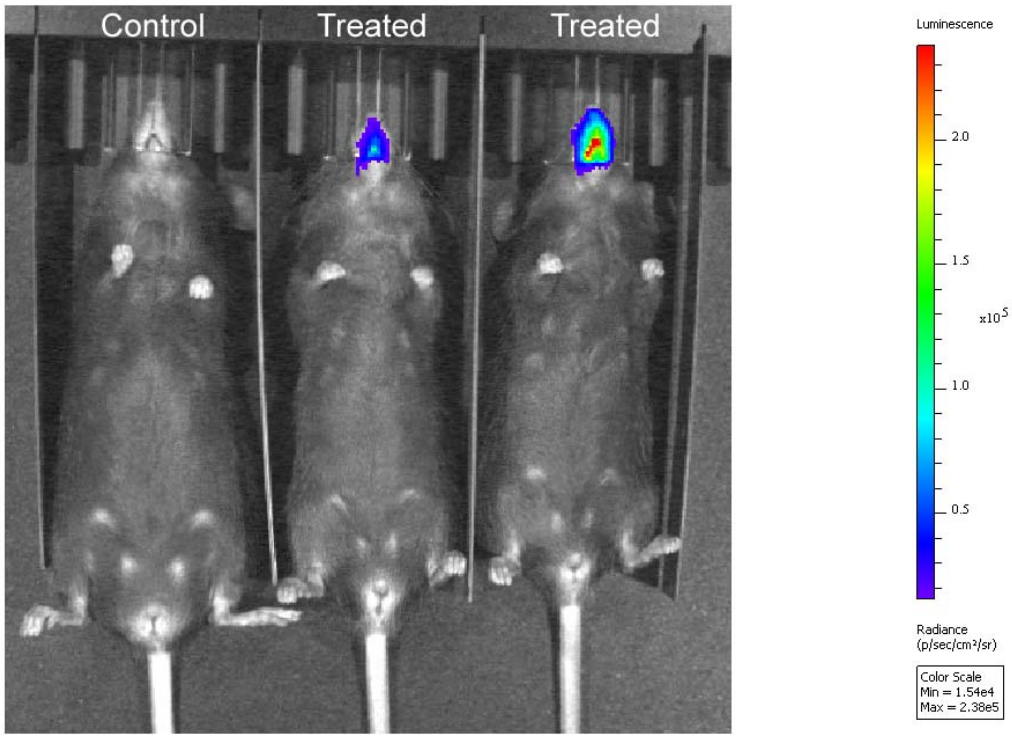


Figure 8-1 A pilot study of gene expression following nasal perfusion with rSIV.F/HN-hCEF-GFPLux

On day 0 of the experiment, two mice were given rSIV.F/HN-hCEF-GFPLux (4e7 TTU in 100 µl total volume) by nasal infusion; one control animal was given 100 µl PBS. On day 2 post treatment, mice were anaesthetised, given 150 mg/kg D-luciferin and imaged after 10 minutes.

8.3.2 Nasal perfusion with rSIV.F/HN hCEF-GFPLux leads to expression of the reporter protein GFPLux in the murine nose

Having demonstrated expression of a reporter protein following nasal perfusion, I next performed a similar experiment in n=5 mice. Mice were given 4e7 TTU of the same batch of rSIV.F/HN hCEF-GFPLux and imaged 8 days post-treatment (the delay was due to a fault with the IVIS imaging software which needed to be repaired). Results of experiments in Chapter 5 showed that gene expression from rSIV.F/HN vectors is stable for many months, so I do not expect this delay to affect interpretation of the data. The results of the bioluminescent imaging are shown in Figure 8-2, showing that gene expression in the noses of treated mice was significantly greater than controls. Based solely on observation of the BLI images produced, it appeared that there was no expression of luciferase from the lungs. However, when photon emission was measured using software, there was slightly increased photon emission in the lungs of some treated mice, although this did not reach statistical significance (Figure 8-3). This data suggests that quantifying photon emission using software is more sensitive than visual observation of images from BLI.

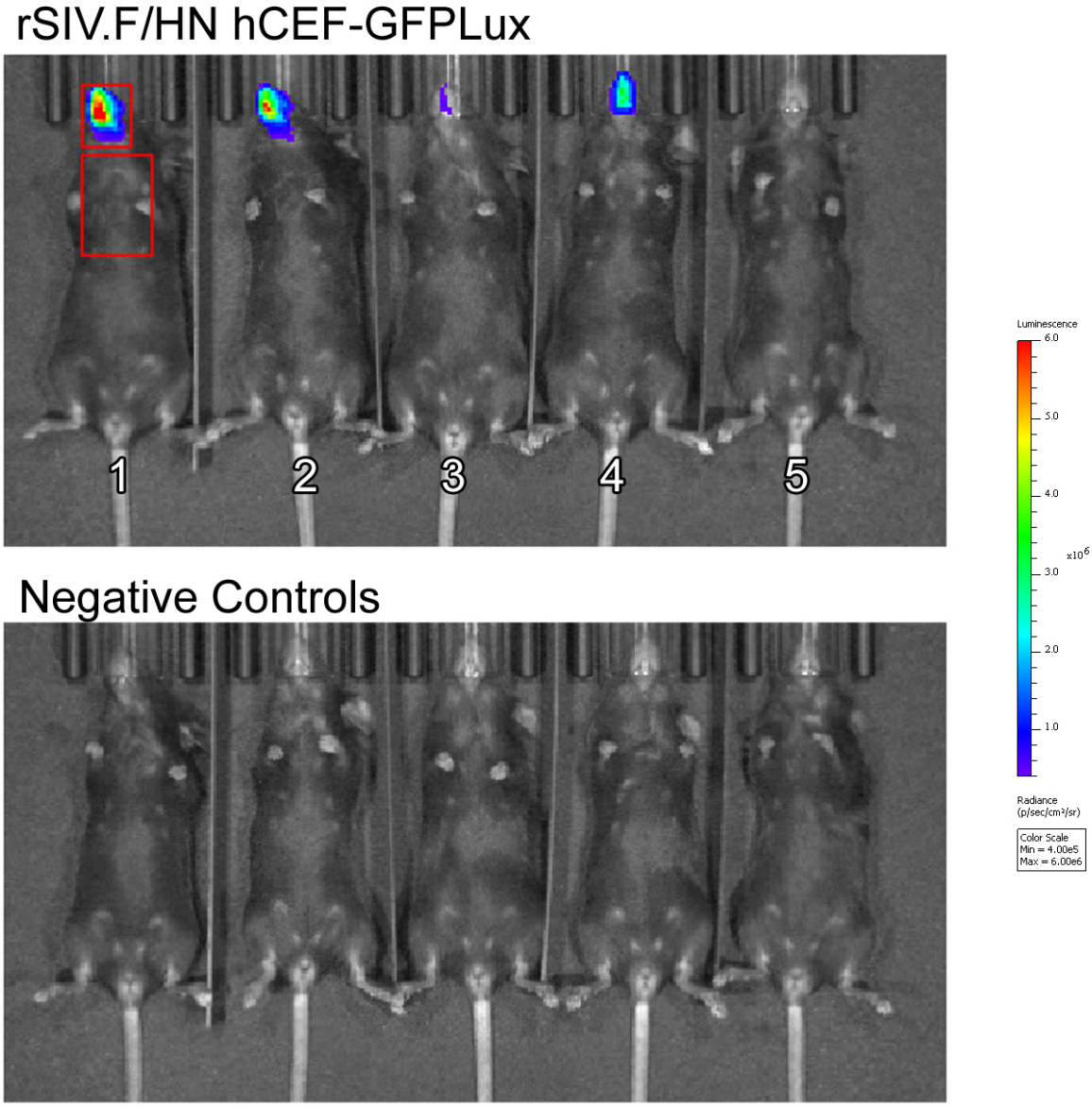
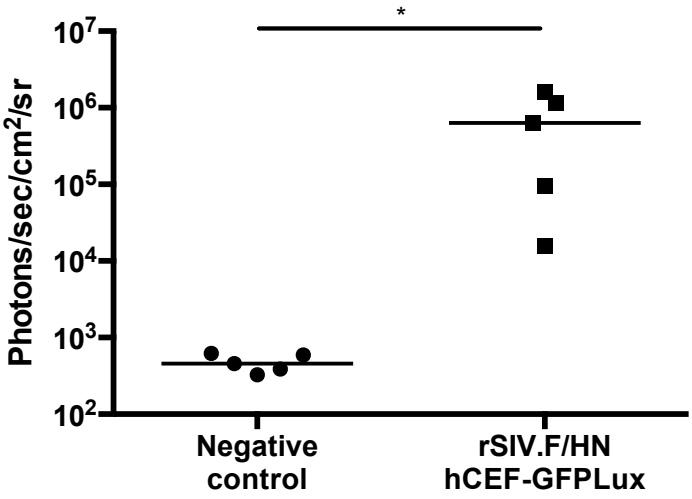


Figure 8-2 Gene expression in the nose following nasal perfusion with rSIV.F/HN-hCEF-GFPLux

On day 0 of the experiment, mice (n=5) were given rSIV.F/HN-hCEF-GFPLux (4e7 TTU) (top panel) or PBS (bottom panel) by nasal perfusion. On day 8, mice were anaesthetised, given 150 mg/kg D-luciferin intraperitoneally and imaged after 10 minutes.

Red squares show the region that was defined as nose (top) and lung (bottom), which was kept consistent for all animals in the experiment.

A.



B.

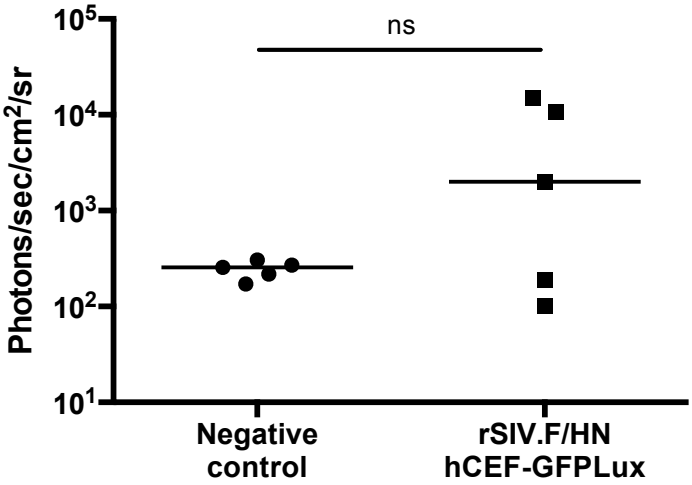


Figure 8-3 Quantification of bioluminescent imaging

On day 0 of the experiment, mice (n=5) were given rSIV.F/HN-hCEF-GFPLux (4e7 TTU) or PBS by nasal perfusion. On day 8, mice were anaesthetised, given 150 mg/kg D-luciferin intraperitoneally and imaged after 10 minutes. Photon emission was quantified in **(A)** nose and **(B)** lung and expressed as photons per second per cm^2 per steradian (photons/sec/cm^2/sr).

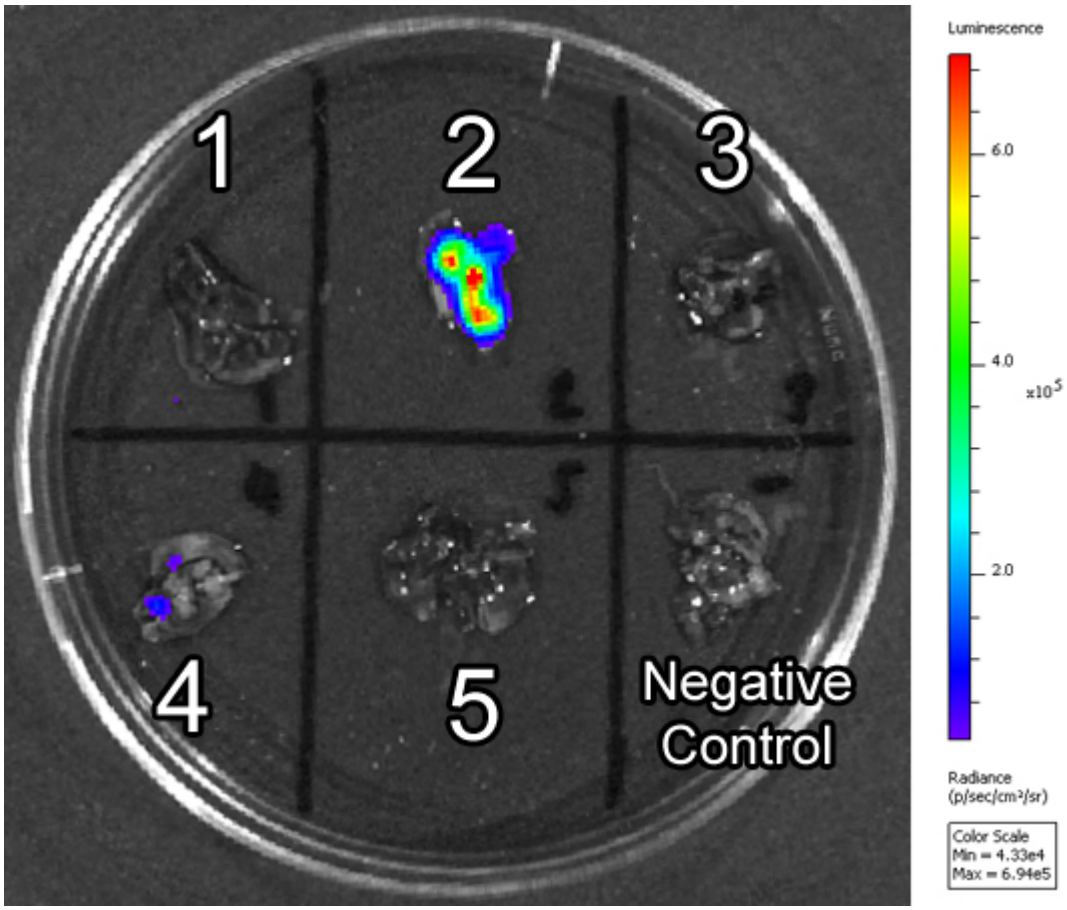
**=$p<0.01$, ns=not significant (Mann Whitney *U* test).

8.3.3 Ex vivo *imaging is a more sensitive method of quantifying gene expression in the lung than* in vivo *imaging*

The results in Figure 8-2 and Figure 8-3 suggest that following nasal perfusion, there is no gene expression in the lung. However, to ensure that this conclusion was correct I next wanted to perform *ex vivo* imaging. Mice were administered 100 µl D-luciferin to the lung by nasal instillation, and culled. The lungs were dissected, placed on a petri dish, and imaged. Mindful of the need to be compliant with the 3Rs, only one control animal was culled, allowing the other untreated mice to be used in other experiments.

The results of the *ex vivo* imaging are shown in Figure 8-4. Unlike the *in vivo* images, high levels of gene expression were seen in two out of the five treated mice, suggesting that imaging the lungs *ex vivo* is more sensitive than *in vivo*. Because only one negative control animal was imaged, it was not appropriate to perform statistical analysis in this experiment, however when photon emission was quantified three lungs had above-background levels of photon emission (Figure 8-4B). This data suggests that *ex vivo* imaging of lungs is more sensitive than *in vivo* imaging.

A.



B.

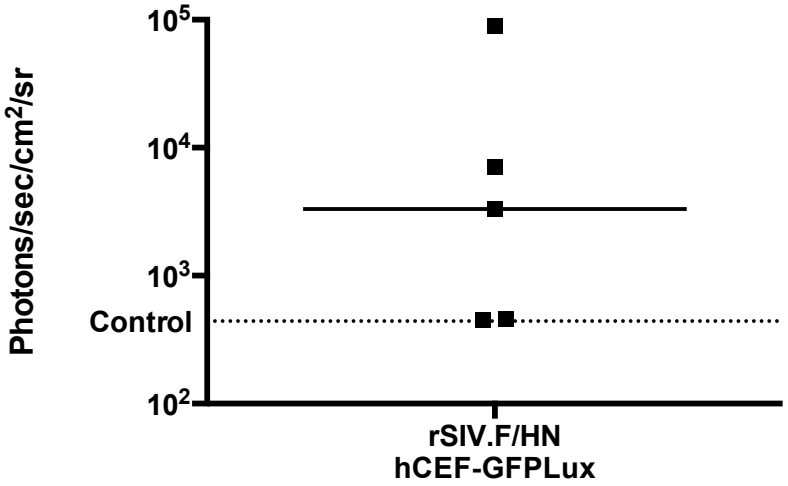


Figure 8-4 *Ex vivo* imaging of the lungs of mice treated with rSIV.F/HN-hCEF-GFPLux following nasal perfusion

On day 0 of the experiment, mice (n=5) were given rSIV.F/HN-hCEF-GFPLux (4e7 TTU) or PBS by nasal perfusion. On day 8, mice were given 100 µl D-luciferin intranasally and culled. The lungs were dissected and imaged **(A)**, and photon emission quantified in photons/sec/cm²/sr **(B)**. Each data point represents one lung, horizontal bars represent group medians. Dotted line represents radiance in the negative control lung. No statistical analysis was performed due to the low *n* number in the negative control group.

8.3.4 Luciferase assays on tissue homogenates are more sensitive at detecting gene expression than in vivo *or* ex vivo *imaging*

Having shown that several mice produced reporter protein in the lungs, I next analysed reporter gene expression in tissue homogenates. When the lung tissue from animals treated with rSIV.F/HN-hCEF-GFPLux by nasal sniffing were analysed, I observed significant levels of luciferase expression compared to archived negative control samples from C57Bl/6 mice (Figure 8-5A). Even the treated animal with the lowest levels of luciferase expression in the lung showed two log orders higher expression than negative controls, suggesting that bioluminescent imaging of luciferase expression is far less sensitive than *ex vivo* tissue homogenate assays. In nasal tissue homogenates, levels of luciferase expression were also significantly greater than negative control samples (Figure 8-5B).

The median luciferase expression in nasal tissue homogenate was 1.4e6 (range 2.7e5-1.2e7) relative light units (RLU) per mg protein, compared to 4.5e4 (range 89-9e5) RLU per mg protein in lung tissue homogenates, suggesting that the nasal perfusion technique leads to two log orders higher expression in the nose than in the lung.

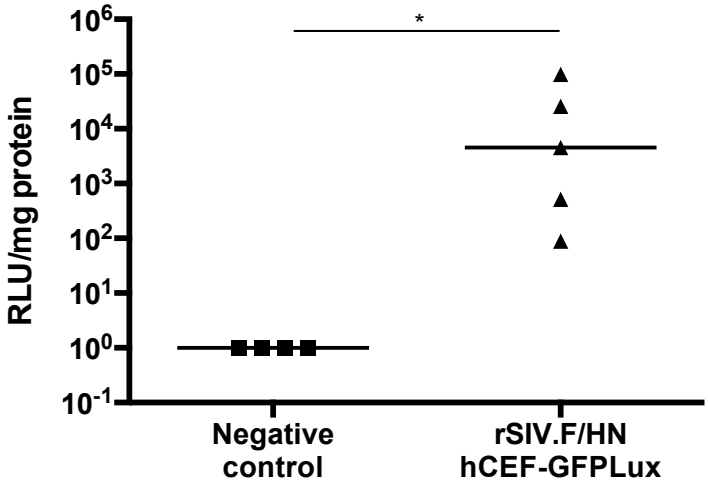
I next examined the correlation between the two methods of luciferase expression quantification (bioluminescent imaging and *ex vivo* luciferase assay on tissue homogenates). When the two methods of quantification of luciferase expression were compared, there was a strong positive correlation in both lung and nose tissue (Figure 8-6):

- Lung: Spearman rank 0.91, $p<0.01$
- Nose: Spearman rank 0.73, $p<0.05$

Taken together, the results of this experiment suggest that in my hands, nasal perfusion as a gene delivery technique (1) leads to log orders higher expression in the nose than in the lung,

but (2) still leads to significant gene expression in the lung, meaning that if a 'nose-only' gene delivery technique is desired an alternative or improved method will need to be found. Furthermore, (3) there is an order of sensitivity of detecting luciferase expression, with *in vivo* bioluminescent imaging being least sensitive, followed by *ex vivo* imaging and *ex vivo* luciferase assays in tissue homogenates being progressively more sensitive. The relative sensitivity of the detection methods has to be taken into account when interpreting the different luciferase detection assays.

A.



B.

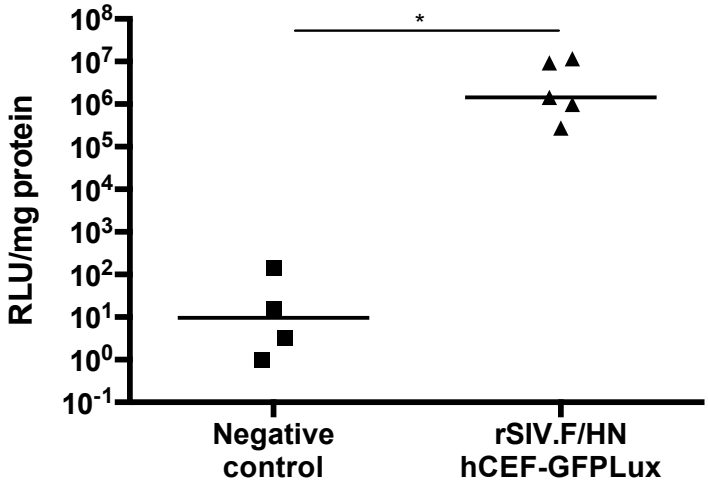


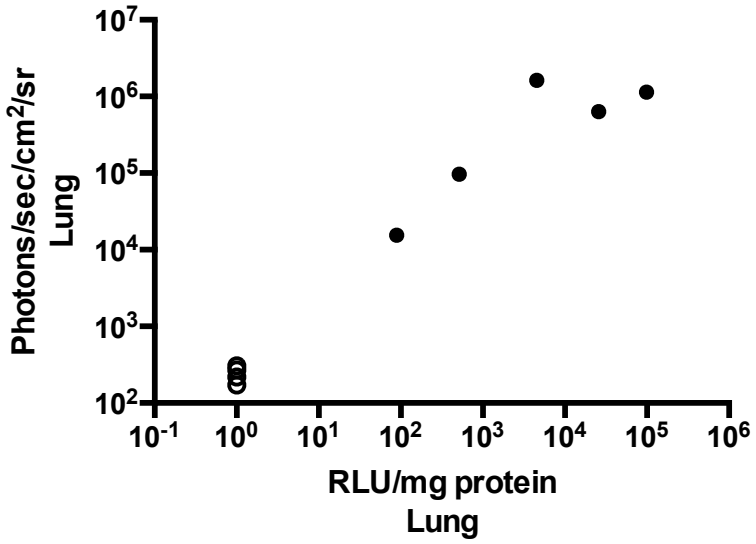
Figure 8-5 Quantification of luciferase in lung and nasal tissue homogenates from animals given rSIV.F/HN-hCEF-GFPLux by nasal sniffing

On day 0 of the experiment, mice (n=5) were given rSIV.F/HN-hCEF-GFPLux (4e7 TU) or PBS by nasal perfusion. On day 8, mice were culled and luciferase expression quantified in (**A**) lung and (**B**) nasal tissue homogenates, compared to archived negative control samples from C57Bl/6 mice, and corrected for total protein in each sample.

Each data point represents one animal. Horizontal bars represent group medians. RLU=relative light units.

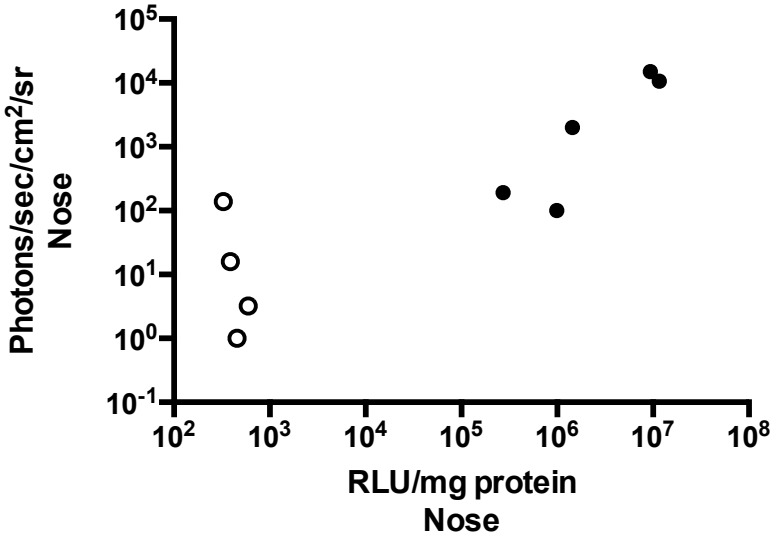
*=$p<0.05$ (Mann Whitney *U* test).

A.



Spearman rank 0.91
p<0.01

B.



Spearman rank 0.73
P<0.05

Figure 8-6 Correlation between quantification of luciferase expression by bioluminescent imaging and *ex vivo* luciferase assay on tissue homogenate

Mice (n=5) were given rSIV.F/HN-hCEF-GFPLux (4e7T TU, closed symbols) or PBS (open symbols) and the luciferase expression in **(A)** lung and **(B)** nose was quantified by bioluminescent imaging and *ex vivo* luciferase assays on tissue homogenates. Here, the two measures of luciferase expression are compared.

8.3.5 Slow nasal sniffing is an alternative delivery technique but still leads to transduction of lung tissue

Because I was unable to demonstrate that nasal perfusion does not solely transduce lung tissue, I explored an alternative gene delivery technique of "slow nasal sniffing". This is similar to the nasal sniffing delivery method used in previous chapters, with the exception being that the 100 µl of gene transfer agent is delivered in ten boluses of 10 µl each at two minute intervals, rather than the whole volume at once. I hypothesised that prolonged contact time with the nasal epithelium would lead to higher levels of gene transfer with slow nasal sniffing, with no dripping into the lung.

Mice were treated with 7e6 TTU rSIV.F/HN-hCEF-GFPLux either by (A) slow nasal sniffing (n=6) or (B) fast (i.e. regular) nasal sniffing (n=3). The animals were imaged at day 28; the delay was due to a fault which closed the imaging suite for a number of weeks, but I do not expect this to affect interpretation of the data as I have already shown that treatment with rSIV.F/HN vectors leads to stable gene expression for many months (Chapter 5). A smaller dose of virus was used than in previous experiments to allow more mice to be treated.

In vivo bioluminescent imaging was then performed (Figure 8-7), which showed that all mice expressed luciferase in the nose, whether the delivery method was fast or slow nasal sniffing. Similar to the experiments described above, on the basis of imaging alone it appeared that there was no luciferase expression in the lung. When photon emission was calculated using software, there was significantly more luciferase expression in both the nose and lung of animals treated with slow nasal sniffing compared to fast nasal sniffing (Figure 8-8). There was also a strong positive correlation between photon emission when animals were imaged supine (face up) and prone (face down) (Pearson rank 0.99, $p<0.0001$, Figure 8-8C).

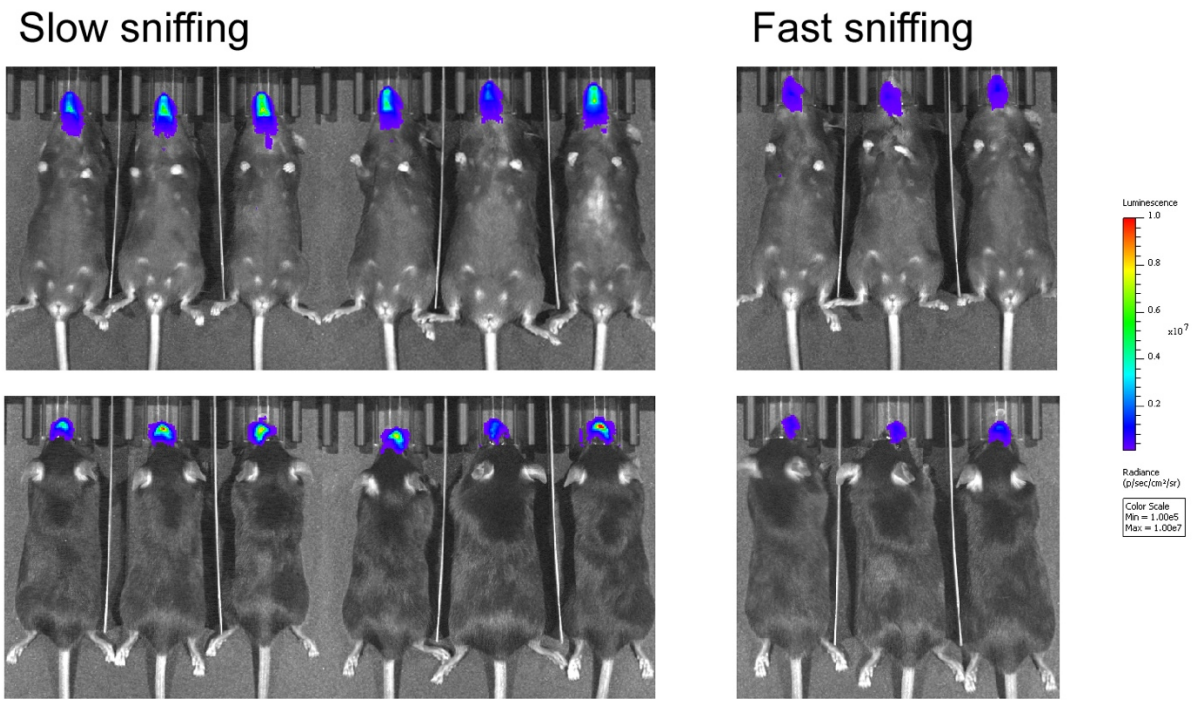
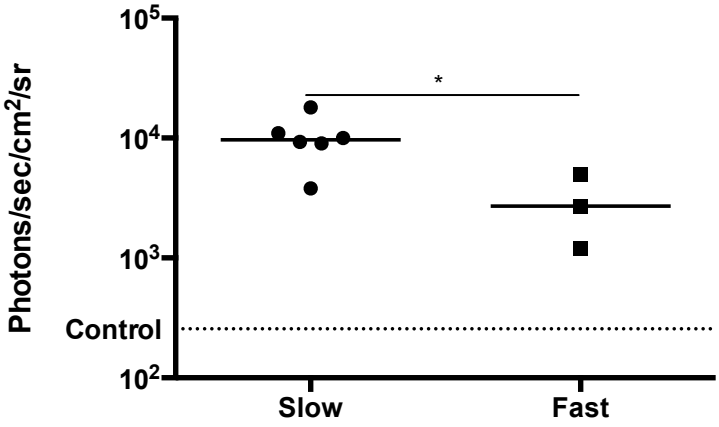


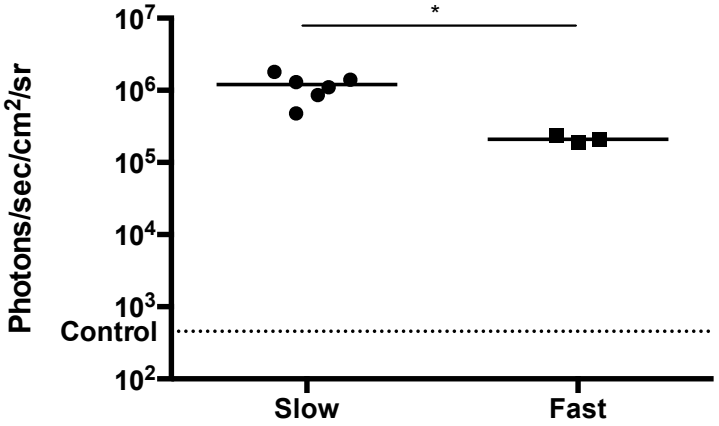
Figure 8-7 Expression of luciferase in the nose following fast and slow nasal sniffing

On day 0 of the experiment, mice were given 7e6 TTU rSIV.F/HN-hCEF-GFPLux either by slow nasal sniffing (n=6) or fast nasal sniffing (n=3). On day 28, animals were given 150 mg/kg D-luciferin intraperitoneally and imaged at 10 minutes. The animals were imaged supine (top panels) and prone (bottom panels).

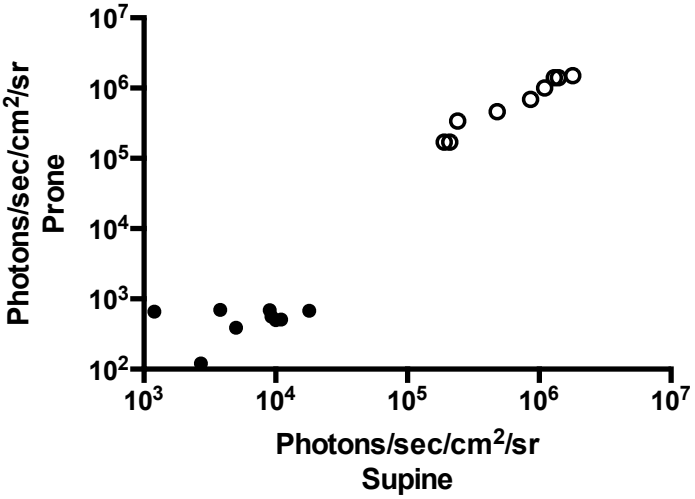
A.



B.



C.



Pearson rank 0.99, p<0.0001

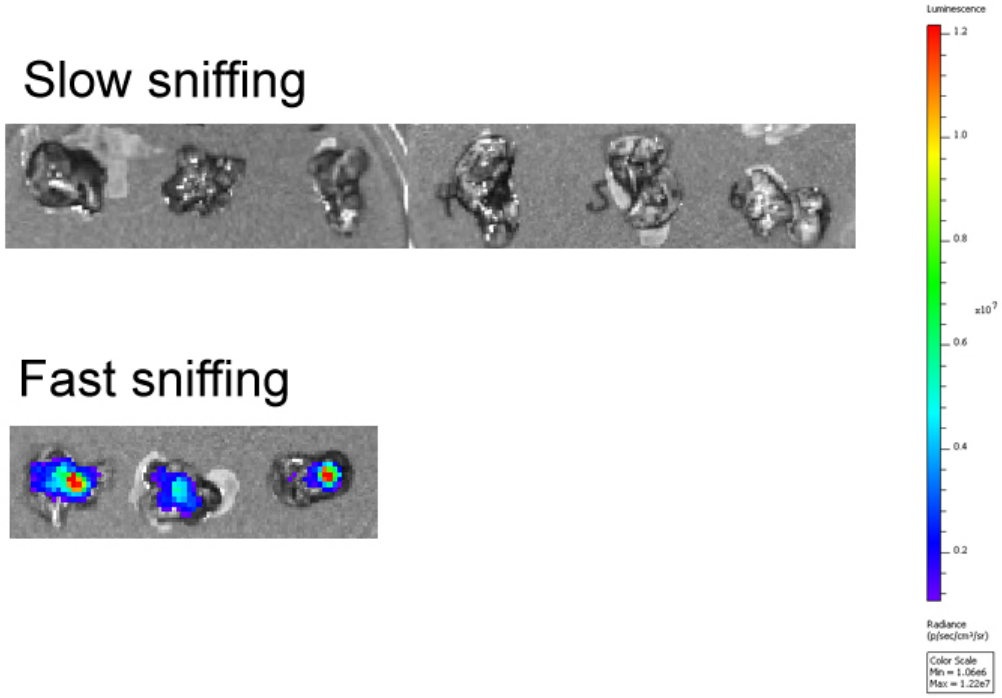
Figure 8-8 Quantification of luciferase expression in the nose and lung of animals treated with slow and fast nasal sniffing.

Mice were treated with 7e6 TTU rSIV.F/HN-hCEF-GFPLux either by slow nasal sniffing (n=6) or fast nasal sniffing (n=3). Photon emission was quantified in supine animals at day 28 in **(A)** lung and **(B)** nose. Dotted lines represent control values of photon emission in lung and nose from untreated animals in previous experiments (Figure 8-3).

Animals were also imaged prone, and the correlation between the prone and supine readings for nose (open symbols) and lung (closed symbols) compared **(C)**. Each data point represents one animal, horizontal bars represent group medians. *=p<0.05 (Mann Whitney *U* test).

The mice were then given intranasal D-luciferin, culled, and the dissected lungs imaged *ex vivo* (Figure 8-9). There was no luciferase expression in the lungs of animals treated by slow nasal sniffing. However, in the lungs of animals treated by fast nasal sniffing, there was significant ($p<0.05$) gene expression, implying that slow nasal sniffing may selectively transduce the nasal epithelium.

A.



B.

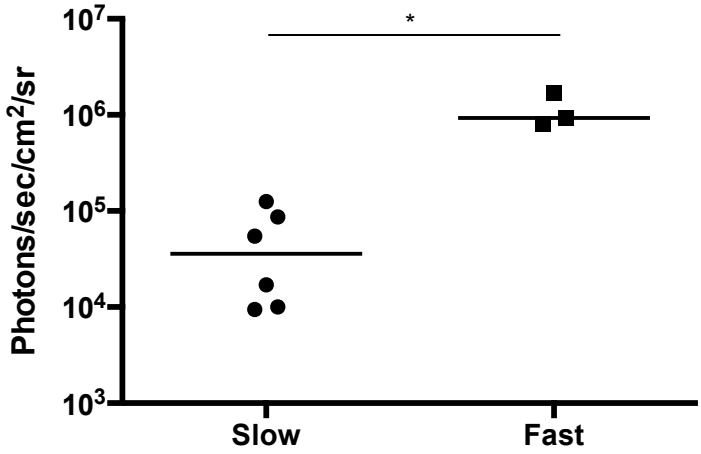


Figure 8-9 Expression of luciferase in the lungs following fast and slow nasal sniffing

(**A**) On day 0 of the experiment, mice were given 7e6 TTU rSIV.F/HN-hCEF-GFPLux either by slow nasal sniffing (n=6) or fast nasal sniffing (n=3). On day 28, animals were given 100 µl D-luciferin intranasally, culled, and the dissected lungs imaged at 10 minutes.
(**B**) Quantification of luciferase expression. Each data point represents one animal, horizontal bars represent group medians. *=$p<0.05$ (Mann Whitney *U* test).

8.3.6 Slow nasal sniffing leads to increased gene expression in the nasal epithelium, but does not prevent transduction of lung tissue

As previous experiments showed that *ex vivo* assays in tissue homogenates are far more sensitive than bioluminescent imaging, I then homogenised nasal and lung tissue and determined the luciferase expression (Figure 8-10). In nasal tissue homogenate, luciferase expression in animals treated by slow nasal sniffing was significantly higher than archived negative control samples; there was no statistically significant gene expression in animals treated with fast nasal sniffing compared to negative controls. This suggests that a prolonged contact time between the gene therapy vector and nasal epithelium can boost gene expression.

In lung tissue homogenate, the level of gene expression in the lungs of animals treated with slow and fast sniffing was very similar, despite the fact that *ex vivo* imaging (Figure 8-9) showed virtually no gene expression in the lungs of animals treated by slow nasal sniffing:

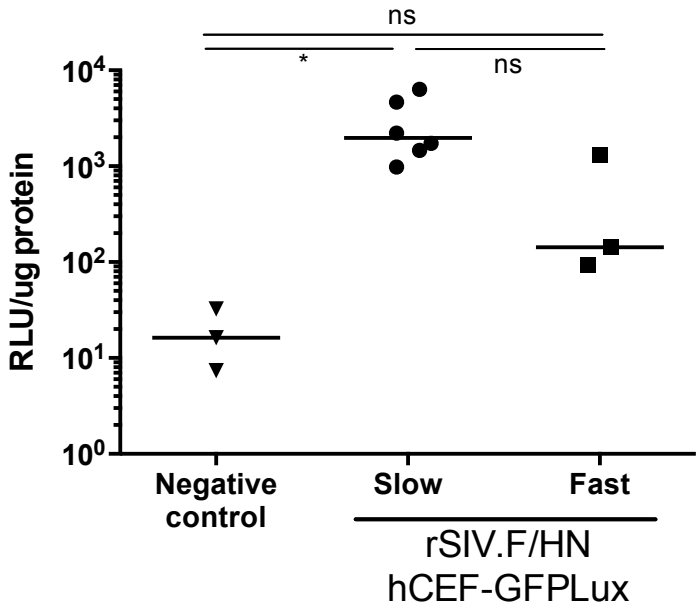
- *Slow sniffing*: median 11.8 (range 6.2-29.4) RLU/mg protein
- *Fast sniffing*: median 28.2 (range 21.1-43.5) RLU/mg protein
- *Controls*: median 0.39 (range 0.1-0.47) RLU/mg protein

It should be noted that the levels of luciferase expression in this experiment were slightly lower than previous experiments (i.e. Figure 8-5), possibly due to the lower viral titre used in this experiment.

Taken together, the data in this experiment shows that slow nasal sniffing does lead to increased expression of a reporter protein in the nasal epithelium compared to treatment by fast nasal sniffing; however, like nasal perfusion this technique is not able to selectively transduce the nasal tissue and leave the lung untransduced.

In summary, I have been able to demonstrate that rSIV.F/HN transduces nasal epithelium, however in my hands I have been unable to find a technique that selectively transduces nasal epithelium and not the lung.

A.



B.

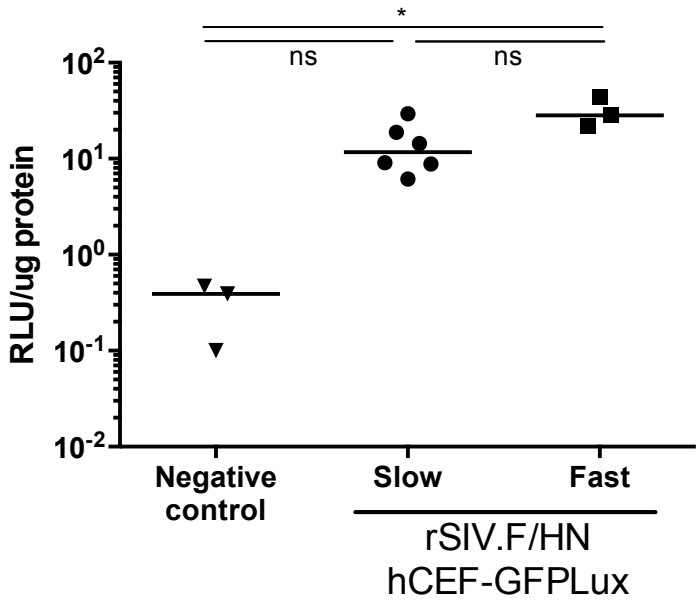


Figure 8-10 Quantification of luciferase expression in the nasal and lung tissue homogenates following fast and slow nasal sniffing

On day 0 of the experiment, mice were given 7e6 TTU rSIV.F/HN-hCEF-GFPLux either by slow nasal sniffing (n=6) or fast nasal sniffing (n=3). On day 28, animals were culled and luciferase expression quantified in (**A**) nasal and (**B**) lung tissue homogenates, compared to archived negative control samples from C57Bl/6 mice, and corrected for total protein in each sample.

Each data point represents one animal. Horizontal bars represent group medians. RLU=relative light units.

*=$p<0.05$, ns=not significant (Mann Whitney *U* test).

8.4 Discussion

In this chapter, I have described pilot experiments that assess two different delivery technique for the delivery of gene transfer agents (GTAs) to the nasal epithelium. There are several situations in which this delivery method may be appropriate in humans. Firstly, in the treatment of lung diseases such as cystic fibrosis, the target organ is diseased, which may make gene transfer less efficient; for example, in the case of cystic fibrosis, purulent secretions and inflammation in the lung impair gene delivery efforts [202]; it is also conceivable that in late-stage α_1-antitrypsin deficiency, the destruction of lung tissue could reduce the surface area available for gene transfer. Secondly, in the context of gene therapy for secreted proteins for the treatment of diseases such as haemophilia, diabetes and others, it might be desirable to use the nose as a "factory" organ, for example if gene transfer to the nose was either more efficient, more efficacious, or less invasive than gene transfer to other more commonly used factory organs such as the liver or skeletal muscle.

The nose is often used in clinical trials as a surrogate for lung delivery, because it has a similar cellular composition and is assumed to be safer for first-in-man trials [101]. The nose has been used as the target organ in initial trials of CF gene therapy using viral [219] and non-viral [167] vectors. The nose has also recently been used for the gene transfer of antibodies against the influenza virus [220]. Interestingly, the nose was the target organ of choice in the first clinical trial of α_1-antitrypsin gene therapy [81]. The authors treated one nostril with a liposome complexed to a plasmid carrying hAAT cDNA, using the contralateral nostril as the negative control. Unfortunately, spillover occurred from one nostril to the other, making conclusions difficult to draw from this study. Despite being used in clinical research, I have been able to find few references describing gene transfer to the nose of mice, making it difficult to compare my data to previously published work.

Previously the UK CF Gene Therapy Consortium has investigated the nasal epithelium as a factory for systemic protein delivery, using Sendai viral vectors to transduce the nasal epithelium [202]. The results of this study showed that Sendai viral vectors could be used to produce significant levels of the secreted protein Interleukin-10 in serum. However, other research has shown that Sendai viral vectors cannot be repeatedly administered [116], which is not the case with rSIV.F/HN [134]. I therefore investigated delivery of rSIV.F/HN to the nasal epithelium, performing pilot experiments to optimise delivery techniques and demonstrate the production of reporter proteins.

In these pilot experiments, I have not used secreted proteins (such as *Gaussia* luciferase or α_1-antitrypsin) as doing so would make it impossible to determine which organ has been transduced. Instead, the non-secreted firefly luciferase was used (in a chimeric construct with Green Fluorescent Protein); ultimately, future work would use secreted proteins.

Bioluminescent imaging (BLI) is a powerful tool that allows gene expression in one animal to be monitored over time, reducing the number of animals that need to be used in scientific experiments. The Consortium has previously shown that when mice are given rSIV.F/HN-FLux by nasal sniffing, gene expression persists at significant levels for the lifetime of the mouse (24 months) [134]. I therefore used BLI to image mice which had been treated with a similar lentiviral vector, coding for the chimeric GFPLux protein, by nasal perfusion. With nasal sniffing, the gene transfer agent is given in a 100 µl bolus, whereas with nasal perfusion the same volume of gene transfer agent is slowly administered by a fine catheter placed in the nostril, delivered by a syringe driver. It has previously been shown that this delivery method can selectively transduce nasal epithelium, but not the lung, using a Sendai virus vector carrying luciferase cDNA [202] which was not the case in my hands using rSIV.F/HN. Importantly, the technician who performed the Sendai virus experiments between 2000 and

2002 has since left the laboratory; this loss of expertise and minor changes in experimental technique may explain the discrepancy between these results.

When I treated animals with rSIV.F/HN carrying luciferase cDNA, BLI suggested that the nasal epithelium had been selectively transduced; there was high signal intensity in the nose, and no detectable signal in the lung. However, when the lungs were excised it was clearly demonstrated that gene transfer to the lung had occurred. This became even more apparent when a luciferase assay on post-mortem tissue homogenates was performed. The sensitivity of the different gene expression detection methods suggests that there is a degree of *in vivo* photon quenching through tissue and skin, and shows that it is important not to rely on bioluminescent imaging alone when looking for gene expression, but instead to regard it as a useful tool e.g. to study gene expression over time.

As my aim was to determine a method of selectively transducing the nasal epithelium, I experimented with a new technique I developed, called slow nasal sniffing. This is similar to the traditional nasal sniffing delivery method I have used elsewhere in the thesis, with the exception that the volume of gene transfer agent is delivered in ten aliquots of 10 µl over the course of 20 minutes. Interestingly, slow sniffing was shown to be approximately one log order more efficient at delivering gene transfer agents to the nasal epithelium compared to fast sniffing; therefore, I hypothesise that longer contact times with the nasal epithelium leads to more selective nasal transduction. It would be interesting to explore this further, i.e. by stretching the administration time of nasal perfusion or slow nasal sniffing to an hour or more, to see if there is any effect on protein expression. It is also interesting to note that even slow sniffing led to high levels of gene expression in the lung, supporting previous data that rSIV.F/HN only requires a very short contact time to enter cells [134]. Unfortunately, it was still not possible to selectively transduce the nasal epithelium and not the lung with this new

delivery method. It will therefore be necessary to further improve and optimise these two techniques in the future.

In two of the three experiments in this chapter, there was a delay between gene transfer and bioluminescent imaging and tissue harvesting due to faults with the imaging machine. I cannot rule out the possibility that this would affect the conclusions drawn from the data, however previous experiments described in this thesis (Chapter 5) and elsewhere [134] have shown that SIV leads to stable, long-lasting gene expression.

In summary, the pilot experiments presented in this chapter provide proof-of-concept that two delivery techniques can preferentially transduce nasal epithelium instead of the lung. However, the two techniques will need to be further investigated and optimised if the aim of selectively transducing only the nasal epithelium is to be achieved. Future work will also include experiments using a secreted protein such as *Gaussia* luciferase, to investigate whether or not nasal perfusion or slow nasal sniffing leads to secretion of proteins into the serum. This approach could open up opportunities for using the nose as a factory to produce secreted proteins for the treatment of various systemic diseases.

9 Analysis of sputum biomarkers in cystic fibrosis patients

9.1 Introduction

One of the appeals of gene therapy with α_1-antitrypsin is that it has a number of potential clinical applications. The most straightforward application of α_1-antitrypsin gene therapy is for the treatment of α_1-antitrypsin deficiency, a disease which needs better treatment options; whilst protein replacement therapy is licensed in several countries, it has not been approved in the UK and the clinical efficacy remains unproven at the current time [52]. α_1-antitrypsin has also been proposed as a treatment for many diseases with an inflammatory component, including multiple sclerosis, rheumatoid arthritis and diabetes [38] and gene therapy may be a suitable approach for treatment of these diseases.

An intriguing question is whether α_1-antitrypsin gene or protein replacement therapy could be useful in the treatment of cystic fibrosis. In the cystic fibrosis lung, there is a protease:antiprotease imbalance, which leads to progressive destruction of the lung tissue architecture [221] [222]. It is therefore reasonable to assume that treatment with the antiprotease α_1-antitrypsin would be of clinical benefit; this hypothesis has been tested in a number of trials (discussed in section 1.1.5.3), and whilst no clinical efficacy has been proven the trials performed to date have generally been small, short-term studies or otherwise have been inadequately controlled, as discussed above.

In preparation for the Phase IIb trial of non-viral gene therapy for cystic fibrosis, the UKCFGTC performed two studies of biomarkers in CF patients [101]:

1. An longitudinal *tracking study* [223] in CF patients undergoing exacerbations, which aimed to identify key biomarkers of inflammation (patients attended at

the beginning of an exacerbation and at the end of a two-week course of antibiotics);

2. A longitudinal *run-in study* (unpublished) in stable CF patients, which aimed to identify suitable primary and secondary endpoints for the upcoming gene therapy trial and also characterise the most suitable patient population for this trial [101]. Patients attended up to four clinic appointments over the course of 12 months, where various clinical markers of lung function were recorded including forced expiratory volume in one second (FEV_1) % predicted and lung clearance index (LCI). At these visits, patients also gave serum and sputum samples, which were analysed for various biomarkers including neutrophil elastase.

As sputum samples from the run-in study were available to me, I took the opportunity to analyse them for α_1-antitrypsin. To the best of my knowledge, there are no existing reports in the literature of measurement of α_1-antitrypsin in sputum from cystic fibrosis patients. I hoped that analysing these samples would provide useful information about correlations between levels of hAAT in sputum from cystic fibrosis patients and other biomarkers and measures of disease severity.

Hypothesis: analysis of sputum samples from stable cystic fibrosis patients will offer new insights into the disease, and could support the use of α_1-antitrypsin as a therapeutic molecule in these patients.

Aim: validate an α_1-antitrypsin ELISA kit in sputum from cystic fibrosis patients, and subsequently analyse levels of α_1-antitrypsin in patients involved in the UK CF Gene Therapy Consortium Run-In study.

Conclusion: α_1-antitrypsin can be detected in the sputum of stable cystic fibrosis patients, with a median concentration of 7.3 µg/ml.

9.2 Materials & Methods

9.2.1 Collection of sputum samples

In the UK CF Gene Therapy Consortium Run-in study, stable patients with cystic fibrosis attended clinic at The Royal Brompton, London or Western General Hospital, Edinburgh, four times over the course of a year. At each clinic visit, measures of lung function (FEV_1 % predicted and LCI) were recorded. Where possible, spontaneous or induced sputum samples were collected using a previously described method [224] by the study nurses and processed according to standard operating procedures. Briefly, four volumes of 0.1% dithiothreitol (DTT) were added to each sample, vortexed and then placed on a roller mixer in a cold room for 15 minutes. A further 4 volumes of PBS was added and the samples labelled and stored at -80°C pending further analysis.

Measurement of neutrophil elastase in sputum was performed by Jackie Donovan and reported in her thesis [225].

Analysis of α_1-antitrypsin in sputum was performed by myself as described below, occasionally with the assistance of Loren Cameron, an undergraduate student whom I supervised during a three-month laboratory placement. In total, 213 sputum samples from 77 patients were analysed for human α_1-antitrypsin.

9.2.2 Competitive ELISA for human α_1-antitrypsin

A competitive ELISA from Abcam (catalogue number ab108798) was performed using the reagents supplied in the kit, according to the manufacturer's instructions. It should be noted that this hAAT ELISA kit is different from the hAAT ELISA kit used elsewhere in this thesis, for reasons discussed below. Briefly, the α_1-antitrypsin standard included with the kit was

diluted in assay buffer to 40 µg/ml, and then further diluted to make a standard curve (sensitivity range 0.039 to 40 µg/ml). 25 µl of standard or sample was placed a 96-well plate pre-coated with an anti-α_1-antitrypsin antibody, and immediately 25 µl of biotinylated human α_1-antitrypsin added to each well. After a two hour incubation, wells were washed five times in wash buffer and 50 µl streptavidin-peroxidase complex added for a further 30 minutes. The wells were washed again, and 50 µl chromogen substrate added per well. After 20 minutes, the reaction was stopped by the addition of 50 µl stop solution to each well and the absorbance read at 450 nm in a microplate reader (Appliskan). All standards and samples were analysed in duplicate.

9.2.2.1 Assay validation: effect of dithiothreitol

To investigate the effect of dithiothreitol (DTT) on the ELISA, two separate standard curves (0.039 to 40 µg/ml hAAT) were prepared. One set of standards was diluted in 0.9% NaCl; the other set of standards was diluted in 0.9% NaCl and spiked with DTT to a final concentration of 0.1%, to mimic sputum preparation. The ELISA was performed as described above.

9.2.2.2 Assay validation: spiked sputum samples

To validate the Abcam ELISA in processed sputum samples, two sputum samples from different patients were divided into four aliquots and either analysed unspiked, or spiked with known amounts of purified human α_1-antitrypsin (Sigma, UK) to a final concentration of 0.5, 5 or 20 µg/ml. The assay was performed according to the manufacturer's instructions. The concentration of hAAT in the two unspiked samples was subtracted from the spiked samples to calculate the assay recovery of hAAT in µg/ml.

9.2.3 Statistical analysis

Data were analysed using GraphPad Prism (version 6). Normality was assessed using the D'Agostino-Pearson omnibus normality test, which in every case showed that the data set was non-parametrically distributed. Correlations were therefore assessed using Spearman rank correlation.

9.3 Results

9.3.1 Selection and validation of an appropriate human α_1-antitrypsin ELISA kit to analyse sputum samples

A literature search was performed to investigate the expected concentration of human α_1-antitrypsin in human sputum. I identified seven publications in which human α_1-antitrypsin was measured in sputum (Table 9-1); the vast majority of papers measured hAAT in patients with α_1-antitrypsin deficiency, chronic obstructive pulmonary disease (COPD), bronchiectasis or asthma. One study [226] reported levels of hAAT in sputum induced from healthy controls. Surprisingly, I was unable to find any reports that measured hAAT in sputum from cystic fibrosis patients.

In the papers identified, there was a large variation in α_1-antitrypsin concentrations, which is probably a result of it being an acute phase protein [36]. In healthy subjects, hAAT in sputum ranged from 0 to 5 µg/ml; in patients with asthma, COPD and bronchiectasis, hAAT in sputum ranged from 5 to 75 µg/ml. Most publications used an in-house ELISA to measure hAAT; one used an ELISA kit from Immunadiagnostic, and one publication used an alternative detection method.

The ELISA kit used thus far in this thesis was a sandwich ELISA from Abcam (Cambridge, UK), with a sensitivity of 0.39 to 100 ng/ml. Based on my literature search, sputum samples would have needed to be diluted 50- to 500-fold when using this ELISA kit, which could have introduced an unacceptable degree of error. I therefore investigated alternative ELISA kits and decided to test a different ELISA kit from the same manufacturer, which was a competitive ELISA with a sensitivity of 0.039 to 40 µg/ml.

Table 9-1 Concentration of human α_1-antitrypsin in sputum samples

Publication	hAAT detection method	Disease group		n	$[hAAT]_{sputum}$ (μM)	$[hAAT]_{sputum}$ (μg/ml)
Vignola, Bonanno, *et al*, 1998[1] [226]	Nephelometric assay	Control		16		0 – 5
		Healthy smokers		10		0 – 5
		Asthma		19		4 – 52
		Bronchitis *or* COPD		10		5 – 75
Hill, Campbell, *et al*, 1999 [227]	ELISA (in-house)	COPD at start of exacerbation		11	0.91	47
		AATD (Pi*ZZ) baseline		11	0.07	3.6
		AATD (Pi*ZZ) at start of exacerbation		11	0.25	13
Stockley, Bayley, *et al*, 2001[2] [228]	ELISA (in-house)	Chronic obstructive bronchitis		7	0.37	19
		Bronchiectasis		14	1.06	55
Stockley, Bayley, *et al* 2002 [229]	ELISA (in-house)	AATD (Pi*ZZ)		11	0.17	8.8
Woolhouse *et al*, 2002 [230]	ELISA (in-house)	Bronchiectasis/chronic bronchitis	(-) DTT	32	0.03	1.6
			(+) DTT	32	0.05	2.6
Ropcke, Holz, *et al*, 2012 [231]	ELISA (Immunadiagnostik)	Healthy smokers		23		0.808
		COPD		24		0.554
Sinden & Stockley, 2013 [232]	ELISA (in-house)	AATD (Pi*ZZ)		28	0.05	2.6
		COPD		22	0.405	21

A summary of published data of measurements of human α_1-antitrypsin in humans. In publications where hAAT concentration was reported in μM, I have also converted values to μg/ml as described in Section 7.2.1. hAAT=human α_1-antitrypsin. AATD=α_1-antitrypsin deficiency. COPD=chronic obstructive pulmonary disease. DTT=dithiothreitol (+/- denotes samples treated or untreated with DTT).

[1] Interpretation of data from this paper is complicated because the authors do not report absolute hAAT values, simply presenting their data on a graph. I have therefore quoted the approximate range of hAAT concentrations by reading the graph.

[2] In this paper, hAAT was measured in sputum at four separate clinic visits. I have calculated the mean concentration of hAAT across the four visits for each patient group.

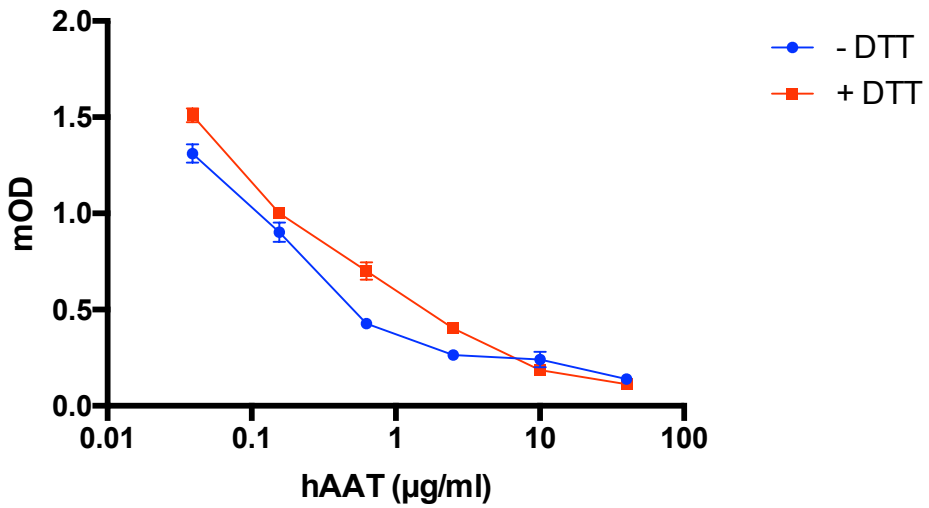
9.3.2 Effect of dithiothreitol on human α_1-antitrypsin ELISA kit

The first step in the validation of this competitive ELISA kit was to see if dithiothreitol (DTT), a mucolytic agent used to prepare soluble (sol) phase sputum, interfered with the assay. Sputum samples are often solubilised with DTT as it leads to improved recovery of cells [233]; however, it has previously been reported that treatment with DTT leads to the over-estimation of human α_1-antitrypsin in sputum by both ELISA [230] and a Multi-Analyte Profiling kit [234]. The reasons for this are unclear, but it has been hypothesised that DTT may interfere with three-dimensional protein structure and therefore affect epitope presentation, or it may directly interfere with antibody-based assays [230].

I was using samples from a study in which the priority was other assays, and was not involved in the study design, and therefore had no choice but to use DTT-treated samples; I therefore tested whether or not DTT interfered with the Abcam ELISA kit by preparing standard curves of hAAT with and without DTT. I intended this method to be similar to a previous publication [230].

When the two standard curves were compared, the lower and higher standards were shown to have similar mean optical density readings (Figure 9-1A). The two middle standards (0.625 and 2.5 µg/ml) were found to have slightly higher mean optical densities (mOD) in DTT-treated samples. The mOD values in DTT-treated standards were then expressed as a percentage of the untreated standards, which were anchored to 100%. This showed that treatment with DTT lead to over-estimation of hAAT concentration by around 50 to 65%. This is a similar order of magnitude to previous reports [230]. The potential over-estimation of hAAT in DTT-treated sputum samples must be borne in mind when considering the rest of the data presented in this chapter.

A.



B.

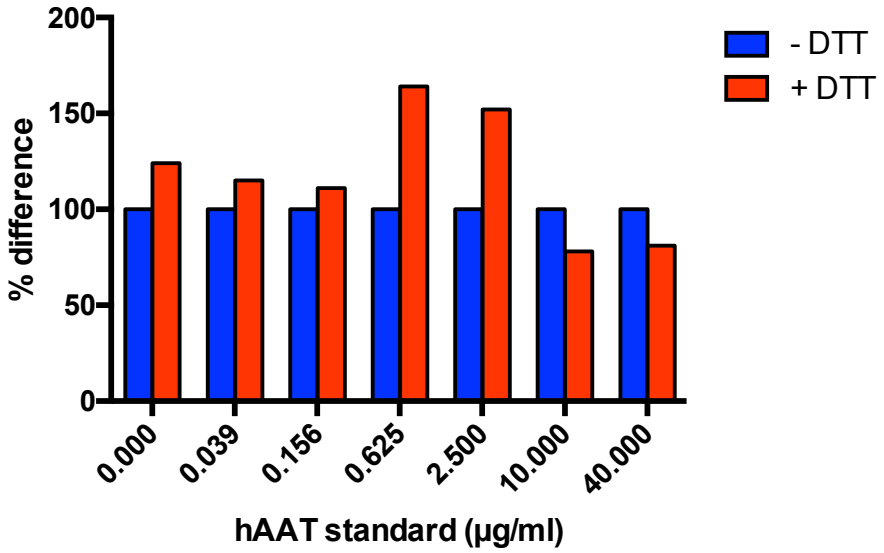


Figure 9-1 Dithiothreitol (DTT) may lead to overestimation of human α_1-antitrypsin concentration in sputum samples

A. Human α_1-antitrypsin standards (0.039 – 40 µg/ml) were prepared in NaCl and spiked with DTT to a final concentration similar to that in processed sputum samples. Dots represent the mean optical density (mOD) reading for each standard analysed in duplicate. Error bars represent the range.

B. Here, the mean optical density of DTT-treated hAAT standards, as reported in (A), is expressed as a percentage of non DTT-treated hAAT standards which are anchored to 100%.

9.3.3 Recovery of human α_1-antitrypsin in spiked samples

In the second stage of assay validation, sputum samples from two cystic fibrosis patients were spiked with known amounts of human α_1-antitrypsin standard, to assess whether the assay kit accurately measures hAAT in processed sputum samples. The sputum samples were spiked to a final concentration of 0.5, 5 or 20 µg/ml of purified hAAT. The hAAT concentration above baseline (unspiked samples) in shown on Figure 9-2.

The data showed that most spiked hAAT was recovered, and there was a dose-response relationship. However, the absolute value of hAAT detected above baseline was not always equivalent to what was expected. For example, in samples spiked to a final hAAT concentration of 20 µg/ml, recovery of hAAT was 12.6 and 7.8 µg/ml in patients 1 and 2 respectively. However, this was deemed to be within an acceptable margin of error.

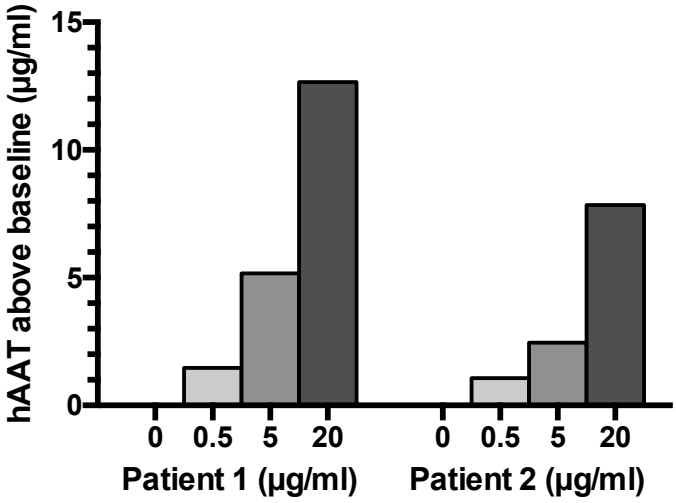


Figure 9-2 Recovery of known amounts of human α_1-antitrypsin in processed sputum samples

Sputum samples from two patients were spiked with purified human α_1-antitrypsin (hAAT), to a final concentration shown on the x-axis in µg/ml. An ELISA was then performed to quantify hAAT in all spiked and unspiked samples. The hAAT concentration in the unspiked sample was subtracted from the spiked samples, and data expressed as hAAT recovery in µg/ml.

9.3.4 Analysis of sputum samples from stable cystic fibrosis patients for human α_1-antitrypsin

Having shown that DTT does not interfere with the ELISA, and that the ELISA detects spiked human α_1-antitrypsin, I then analysed 217 sputum samples from 77 different patients for hAAT. The concentration of hAAT in these samples is shown in Figure 9-3 and Table 9-2. The median concentration of human α_1-antitrypsin was 7.3 µg/ml, with an interquartile range of 4.3-10.1 µg/ml. These values are similar to the concentration of hAAT reported in earlier publications (Table 9-1), and this will be discussed further at the end of the chapter.

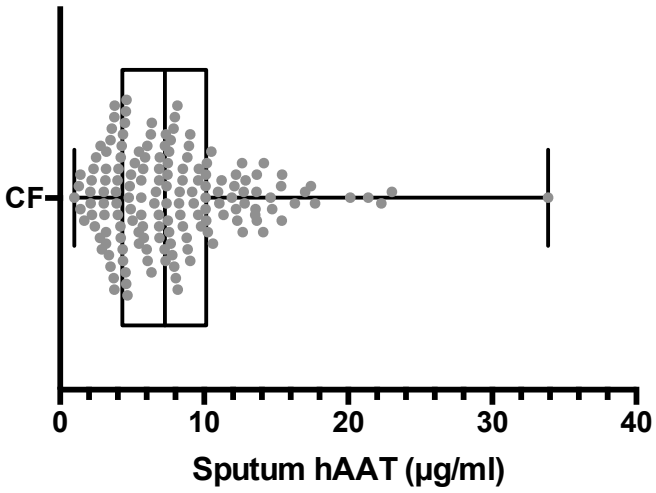


Figure 9-3 Concentration of human α_1-antitrypsin in sputum samples from stable CF patients

217 sputum samples from 77 patients with stable cystic fibrosis were collected over a one-year period. The concentration of α_1-antitrypsin was determined by ELISA. Each data point represents one patient; vertical lines from left to right on the box and whisker plot represent the range (lower), 25th percentile, median, 75th percentile and range (upper).

Table 9-2 Concentration of human α_1-antitrypsin in sputum samples from stable CF patients

	$[hAAT]_{sputum}$ µg/ml
Range (lower)	0.979
25th percentile	4.312
Median	7.283
50th percentile	10.14
Range (upper)	33.88

217 sputum samples from 77 stable cystic fibrosis patients were analysed for α_1-antitrypsin using a commercially available ELISA kit.

9.3.5 Stability of human α_1-antitrypsin over a twelve-month period in stable cystic fibrosis patients

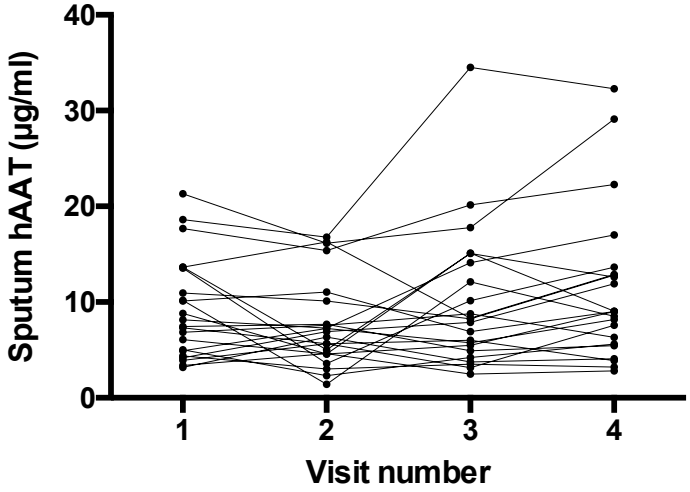
Having determined the concentration of α_1-antitrypsin in sputum samples, I next performed further data analysis to investigate some questions of interest. The first question I addressed was how stable were the levels of α_1-antitrypsin in the sputum of stable cystic fibrosis patients throughout the study.

Patients were selected for the run-in study on the basis of having stable cystic fibrosis. They attended clinic four times over the one-year study period, giving a sputum sample at each visit if possible. In total, there were 22 patients who (a) attended clinic four times, (b) were able to give a sputum sample at each visit, and (c) still had stored sputum samples from each visit by the time of my research.

This presents an opportunity to investigate the change in human α_1-antitrypsin concentration in sputum over time, shown in Figure 9-4A. Sputum hAAT appeared stable across the four clinic visits, with no statistical difference in group medians. There was some visit-to-visit variability in sputum α_1-antitrypsin, probably reflecting the fact that α_1-antitrypsin is an acute phase protein; the median coefficient of variation in these 22 patients was 30.4% (range 14.5-57.8%). 19 of the 22 patients studied had four readings of FEV_1 % predicted, shown in Figure 9-4B; the FEV_1 % predicted readings were very similar throughout the study period, with a median coefficient of variation of 4.7% (range 1.2-18.5%).

This data shows that levels of α_1-antitrypsin in the sputum of cystic fibrosis patients were relatively stable, but there was more visit-to-visit variability than in measures of lung function (FEV_1 % predicted).

A.



B.

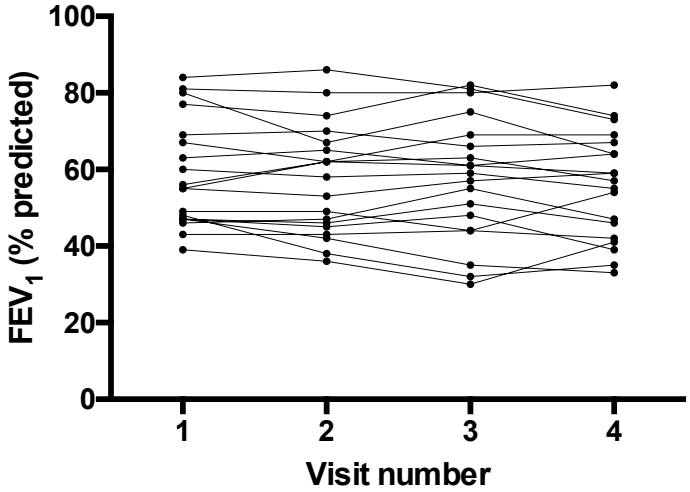


Figure 9-4 Stability of α_1-antitrypsin in sputum samples over one year

22 patients with stable cystic fibrosis visited clinic four times over a one-year period, giving a sputum sample on each occasion.

A: Concentration of hAAT in sputum samples. Each data point represents one sputum sample; samples for each patient are joined by solid lines. There was no statistically significant difference between group medians (Kruskal-Wallis test).

B: For 19 out of the 22 patients in panel A, FEV_1 % predicted data was available for each clinic visit. Each data point represents one measurement of FEV_1 % predicted; readings for each patient are joined by solid lines. There was no statistically significant difference between group medians (Kruskal-Wallis test).

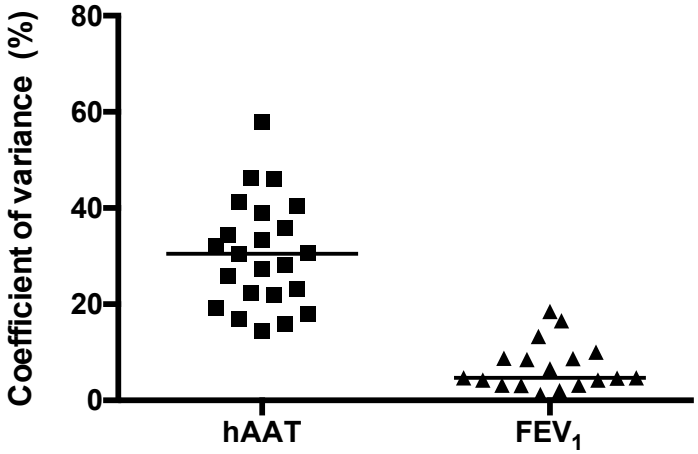


Figure 9-5 Coefficient of variance in sputum human α_1-antitrypsin and markers of pulmonary function in cystic fibrosis patients

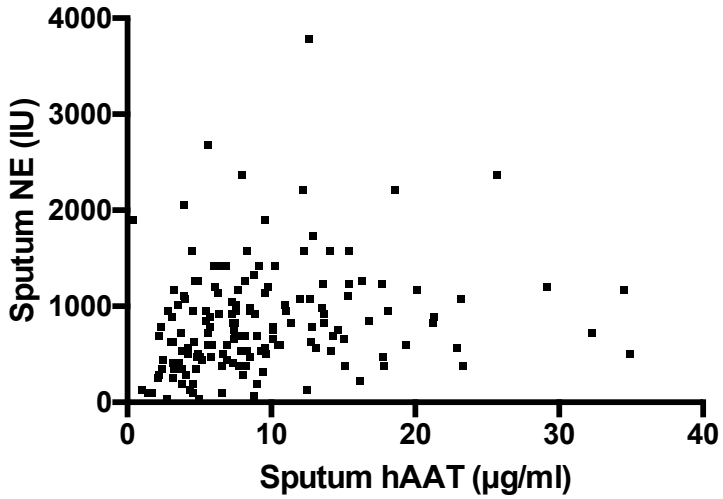
Coefficient of variance in sputum human α_1-antitrypsin (hAAT) and forced expiratory volume in one second % predicted (FEV_1), in stable cystic fibrosis patients who had hAAT measured in four sputum samples over the course of one year.

Each data point represents the coefficient of variance in one patient, based on four readings of hAAT or FEV_1. Horizontal lines represent group medians.

9.3.6 There is a significant but modest positive correlation between sputum α_1-antitrypsin and neutrophil elastase in stable cystic fibrosis patients

I next wanted to see if there was any correlation between sputum α_1-antitrypsin and sputum neutrophil elastase. It has previously been shown that in the epithelial lining fluid of CF patients, levels of NE, hAAT and NE:hAAT complexes were all increased compared to healthy controls [235]; it would be interesting to see if this pattern is retained in sputum (although it should be noted that I was not able to measure NE:hAAT complexes, as discussed later).

The neutrophil elastase concentration in the run-in study sputum samples was determined by Jackie Donovan as part of her thesis [225]. In total, there were 157 sputum samples for which it was possible to determine concentrations of both α_1-antitrypsin and neutrophil elastase. There was a significant ($p<0.0001$) yet modest correlation (Spearman rank 0.304, 95% confidence interval 0.150-0.443) between sputum hAAT and neutrophil elastase.



Spearman rank 0.304 (95% confidence interval 0.150-0.443)
$p<0.0001$

Figure 9-6 There is a significant but modest positive correlation between sputum α_1-antitrypsin and sputum neutrophil elastase

The concentration of human α_1-antitrypsin (hAAT) and neutrophil elastase (NE) was determined in 157 sputum samples from stable cystic fibrosis patients. There was a moderate but significant positive correlation between sputum hAAT and NE.

IU=international units.

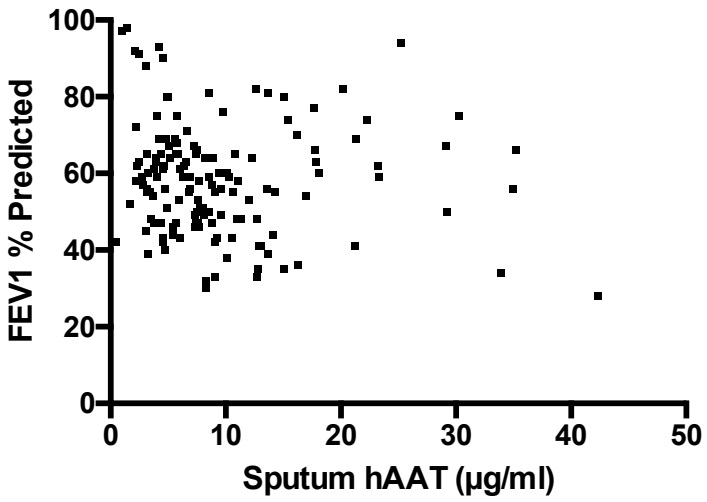
9.3.7 There is no significant correlation between sputum α_1-antitrypsin and physiological markers of lung function in cystic fibrosis patients

The next question I wanted to address was whether or not there is any correlation between sputum markers (neutrophil elastase and α_1-antitrypsin) and physiological markers of lung function (FEV_1 % predicted and lung clearance index, LCI). FEV_1 % predicted is the most commonly reported measure of lung function in cystic fibrosis patients, and is the primary outcome measure of choice in most clinical trials including the recent Phase IIb trial of non-viral gene therapy in cystic fibrosis [105]. A higher FEV_1 % predicted reflects better lung function.

However, it has been suggested that FEV_1 % predicted may not be the best measure of small airways disease, and lung clearance index (LCI) may be more sensitive, particularly in younger patients [236]. LCI is derived from multiple breath washout tests, and is a measure of how many breaths it takes to remove an inert tracer gas; therefore, unlike FEV_1 % predicted, a lower LCI value (i.e. fewer breaths to remove the tracer gas) represents better lung function [223].

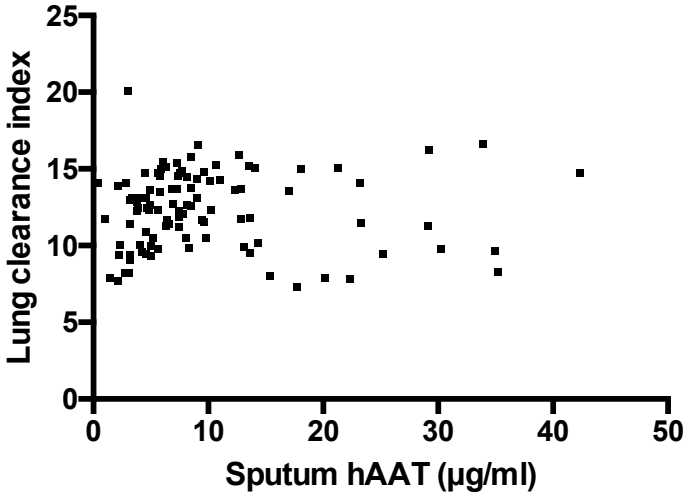
There was no statistically significant correlations between sputum α_1-antitrypsin and either physiological marker of lung function (FEV_1 % predicted or LCI, Figure 9-7). It should be noted that it was not always possible to measure FEV_1 % predicted and lung clearance index at each clinic visit. In total, there were 142 matched readings for sputum hAAT and FEV_1 % predicted, and 100 matched readings for sputum hAAT and LCI.

A.



Spearman rank -0.130 (95% confidence interval -0.293-0.041)
Not significant

B.



Spearman rank 0.126 (95% confidence interval -0.079-0.320)
Not significant

Figure 9-7 There is no significant correlation between sputum α_1-antitrypsin and physiological markers of lung function in cystic fibrosis patients

A. The concentration of α_1-antitrypsin (hAAT) was compared to the FEV_1 % predicted at the time of each clinic visit for 142 samples. Each data point represents one sample from a patient with stable cystic fibrosis.
B. The concentration of α_1-antitrypsin (hAAT) was compared to the lung clearance index at the time of each clinic visit for 100 samples. Each data point represents one sample from a patient with stable cystic fibrosis.

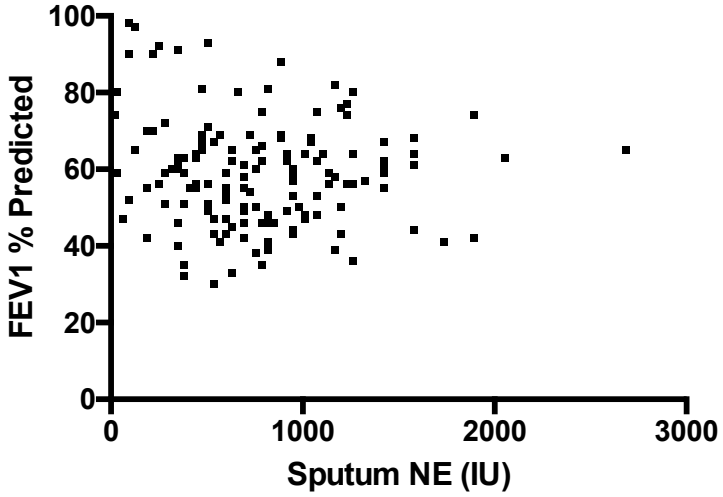
9.3.8 There is a significant but modest positive correlation between neutrophil elastase and lung clearance index, but not FEV_1 % predicted

I next performed a similar analysis to see if there was any correlation between sputum neutrophil elastase and physiological markers of lung function. There was no correlation between sputum NE and FEV_1 % predicted, although there was a modest but positive correlation between sputum NE and LCI (Spearman rank 0.339, 95% confidence interval 0.153-0.503, $p<0.001$, Figure 9-8).

I also calculated the ratio between NE and hAAT for each sputum sample, but did not observe a correlation between NE:AAT ratio and markers of lung function (Table 9-3). It is important to note that the ratio of NE:AAT is not the same as a measurement of NE:AAT complexes, as described in the discussion section of this chapter.

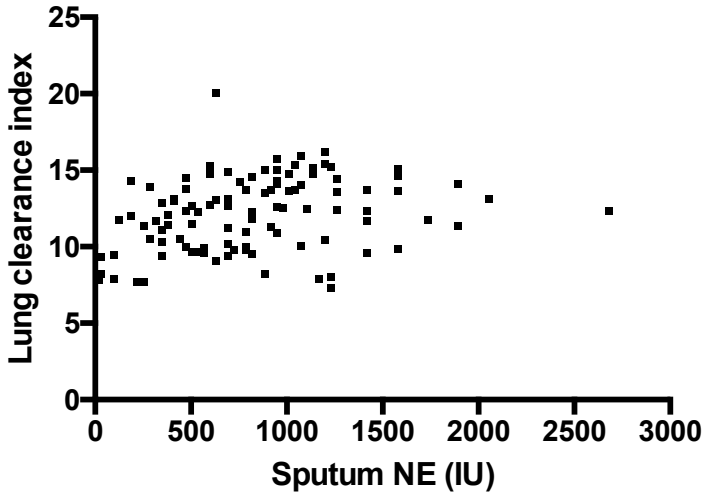
Taken together, this data suggests that there is no clinically meaningful correlation between sputum α_1-antitrypsin, neutrophil elastase and two common markers of lung function, FEV_1 % predicted and lung clearance index.

A.



Spearman rank -0.076 (95% confidence interval -0.239 – 0.091)
Not significant

B.



Spearman rank 0.339 (95% confidence interval 0.153 – 0.503)
p<0.001

Figure 9-8 There is a significant albeit modest correlation between sputum neutrophil elastase and lung clearance index but not FEV_1 % predicted

A. The concentration of neutrophil elastase (NE) was compared to the FEV_1 % predicted at the time of each clinic visit for 148 sputum samples. Each data point represents one sample from a patient with stable cystic fibrosis.
B. The concentration of neutrophil elastase was compared to the lung clearance index at the time of each clinic visit for 105 samples. Each data point represents one sample from a patient with stable cystic fibrosis.

IU=international units.

Table 9-3 Correlation between sputum α_1-antitrypsin, neutrophil elastase, NE:AAT ratio and markers of lung function in stable cystic fibrosis patients

	FEV_1 % predicted			Lung clearance index		
	Spearman rank	95% CI	p	Spearman rank	95% CI	p
hAAT	-0.139	-0.293 to 0.041	ns	0.126	-0.079 to 0.329	ns
NE	-0.076	-0.239 to 0.091	ns	0.339	0.153 to 0.503	<0.001
NE:AAT ratio	0.115	-0.065 to 0.290	ns	0.102	-0.114 to 0.310	ns

Concentration of α_1-antitrypsin (hAAT) and neutrophil elastase (NE) was determined in sputum samples from patients with stable cystic fibrosis. The ratio between NE and AAT was also determined for each sputum sample. These values were compared to the measurements of FEV_1 % predicted and lung clearance index at the time of each clinic visit, and the Spearman rank determined.

9.3.9 Correlation of human α_1-antitrypsin and other sputum biomarkers in stable cystic fibrosis patients

Finally, I wanted to see if there was any correlation between sputum α_1-antitrypsin or neutrophil elastase and other inflammatory markers which had been analysed as part of the run-in study. The following markers were measured by other investigators as part of the study:

- Sputum:
    - Total DNA
    - Extracellular DNA
    - Calprotectin
    - IL-8
    - Myeloperoxidase
    - Neutrophils
    - Total cell count
    - Viable cell count
- Serum:
    - Calprotectin
    - IL-8
    - Neutrophils

The correlations between these markers and sputum α_1-antitrypsin and neutrophil elastase are shown on Table 9-4. Generally there was no significant correlations between α_1-antitrypsin and the measured inflammatory markers; however, there was a significant, albeit weak, correlation between sputum hAAT and extracellular DNA, sputum calprotectin and serum calprotectin (Table 9-4).

There was a highly significant correlation between sputum neutrophil elastase and various inflammatory markers, shown on Table 9-4.

Table 9-4 Correlation between sputum α_1-antitrypsin, sputum neutrophil elastase and other sputum and serum inflammatory markers in stable cystic fibrosis patients

		α_1-antitrypsin			Neutrophil elastase		
		Spearman rank	95% confidence interval	p	Spearman rank	95 % confidence interval	p
Sputum	Total DNA	0.064	-0.183 to 0.302	ns	0.515	0.277 to 0.694	****
	Extracellular DNA	0.255	0.008 to 0.472	*	0.787	0.649 to 0.874	****
	Calprotectin	0.327	0.017 to 0.580	*	0.236	-0.086 to 0.514	ns
	IL-8	0.220	-0.095 to 0.495	ns	0.556	0.295 to 0.740	***
	Myeloperoxidase	0.250	-0.056 to 0.513	ns	0.749	0.575 to 0.858	****
	Neutrophils	0.073	-0.206 to 0.342	ns	0.058	-0.226 to 0.334	ns
	Total cell count	-0.078	-0.334 to 0.189	ns	0.347	0.081 to 0.566	**
	Viable cell count	-0.069	-0.331 to 0.202	ns	0.332	0.062 to 0.556	*
Serum	Calprotectin	0.294	-0.002 to 0.542	*	0.248	-0.054 to 0.509	ns
	Neutrophils	0.163	-0.110 to 0.413	ns	0.382	0.119 to 0.594	**
	Serum IL-8	0.099	-0.199 to 0.380	ns	0.120	-0.182 to 0.401	ns

The indicated inflammatory markers were determined in sputum or serum of stable cystic fibrosis patients and compared to sputum α_1-antitrypsin and neutrophil elastase. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

9.4 Discussion

In this chapter, I have presented an analysis of the concentration of α_1-antitrypsin in sputum samples taken as part of a longitudinal clinical study (currently unpublished). This was an opportunistic study that I performed for two main reasons: (1) there are, to my knowledge, no reports on the levels of α_1-antitrypsin in sputum specifically from cystic fibrosis patients (some publications report sputum hAAT in patients with bronchiectasis [230] [228], but it is not clear whether this includes cystic fibrosis patients or not); and (2) α_1-antitrypsin gene and protein replacement therapy has been proposed as a treatment for cystic fibrosis, and an analysis of endogenous α_1-antitrypsin in sputum from these patients is therefore of interest.

Firstly, it is necessary to consider the reliability of the Abcam ELISA kit used in this study. I validated the assay kit in two stages: (1) by showing that DTT did not interfere with the detection of hAAT by the kit, and (2) by showing that spiked hAAT is detected. I felt that the validation data presented in this chapter justified the use of this assay kit, however there was a suggestion that DTT may lead to over-representation of α_1-antitrypsin, which is consistent with two previous publications [230] [234]. Crucially, all the previous reports of α_1-antitrypsin in sputum use DTT-treated samples, and most used an ELISA to detect hAAT; so it reasonable to assume that there is a similar margin of error in all the studies. It is also important to note that, to my knowledge, no-one has solved this problem apart from using non-DTT-treated samples (which was not an option in this study). The effect of DTT on protein detection assays highlights just one limitation when comparing similar studies; and such comparisons, described below, must be treated with a degree of caution. It is reasonable to assume that there may be a margin of error as high as 50 to 65% in the data presented in this chapter.

When I spiked sputum samples with purified human α_1-antitrypsin, I found that the assay kit detected slightly less hAAT than expected, particularly in highly concentrated samples. In one experiment using a different ELISA kit, I found that a purified human α_1-antitrypsin preparation had a different activity from the hAAT standard supplied with the kit and I feel that the this is the most likely explanation for my observations here. I would have liked to perform two experiments to investigate this further: (1) by spiking samples with the same purified α_1-antitrypsin provided in the kit (which was not possible, due to limited regents supplied with the kit); and (2) analysing different purified α_1-antitrypsin preparations from different manufacturers on the same ELISA kit, to investigate the variable activity of different preparations; this experiment could not be performed for time and cost reasons.

One further limitation of the data presented in this chapter is that I have no control samples from either healthy patients, healthy smokers, or other disease groups. As discussed above, this was an opportunistic study and I was not able to design it in the way that would have been most useful for my purposes (it is also important to note that inducing sputum production from healthy volunteers is not a trivial matter and requires a high degree of patient co-operation; therefore, most other studies also do not have healthy control groups). Therefore, I am unable to conclusively determine if α_1-antitrypsin is up-regulated in the sputum of cystic fibrosis patients; instead, the discussion presented below draws data from various publications to try and reach some form of conclusion of hAAT in cystic fibrosis patients. Despite these three limitations, I still feel that useful information can be determined from the data in this chapter and feel it is worthwhile discussing it further.

In this chapter, I have determined that the mean concentration of hAAT in 217 sputum samples from 77 patients with stable cystic fibrosis is 7.3 µg/ml. Of the seven previous studies of hAAT concentration in sputum (summarised in Table 9-1), I find the report by Vignola and colleagues [226] to be of the most value because they reported sputum hAAT

concentration in four different patient groups: healthy non-smokers, healthy smokers, asthma, and COPD. When compared side-by-side (Figure 9-9), it is firstly apparent that the concentration of hAAT I have detected in these samples is of the same order of magnitude as previously reported studies. The median level of hAAT I detected in CF patients is slightly higher than Vignola and colleagues presented in healthy non-smokers and healthy smokers; and similar to patients with asthma and chronic bronchitis (COPD). In other reports of hAAT in sputum of patients with a pulmonary disease *apart* from cystic fibrosis (summarised on Table 9-1), the median hAAT concentration ranges from 1.5 µg/ml to 55 µg/ml; a similar amount to what I have seen in this study.

It has previously been shown that hAAT is up-regulated in the epithelial lining fluid of CF patients compared to healthy controls [235] and, although I do not have a control group for comparison, my data suggests that this pattern may also be the same in sputum from CF patients. The CF lung is in a state of persistent neutrophilic inflammation [237], and it is this pro-inflammatory state that leads to the increased production of hAAT both in the liver [37] and local inflammatory cells [238]. hAAT helps to mediate the inflammatory process by the inhibition of the neutrophil chemo-attractants IL-8 and LTB-4 [64] [229], both of which are produced by bronchial epithelial cells in response to neutrophil elastase in a paracrine positive feedback loop [212] [239].

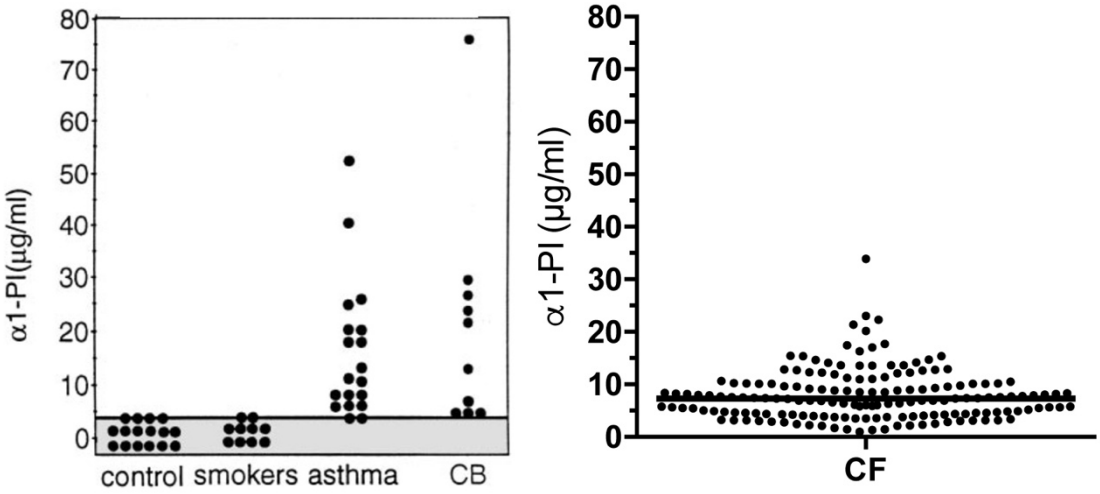


Figure 9-9 A comparison between my data and previously published reports of α_1-antitrypsin in sputum samples

In this figure, I attempt to compare my measurements of α_1-antitrypsin in sputum with a previously published study [226] of sputum hAAT in other groups.

On the left panel is the data presented by Vignola and colleagues of hAAT (α1-PI) concentration in sputum of controls, healthy smokers, asthmatics and patients with CB (chronic bronchitis/chronic obstructive pulmonary disease). The authors do not provide an explanation of the shaded area and horizontal line; I presume that this line denotes the lower limit of detection of their assay.

On the right panel is the data I have presented in this chapter from 77 patients with stable cystic fibrosis (CF) (up to four samples per patient).

α1-PI is α_1-protease inhibitor, another name for human α_1-antitrypsin.

Despite being able to analyse sputum samples from 77 patients, I only had a complete set of four samples from 22 patients (either because a patient missed a clinic visit, was not able to express sputum at one visit, or the sputum sample had been used up by the time of my study). In this small subset, I observed some visit-to-visit variability in the concentration of α_1-antitrypsin with a coefficient of variation of 30.4%; with a larger sample size, it is possible that there would have been less variability. In contrast, FEV_1 % predicted was remarkably stable in these patients, with a coefficient of variation of 4.7%. This is most likely because α_1-antitrypsin is an acute phase protein [240] which, like C-reactive protein (CRP), is up-regulated in response to inflammation. Levels of hAAT in the circulation can increase more than four-fold in response to inflammation, whereas FEV_1 % predicted tends to be a relatively stable marker [241].

Sputum protein measurements are also known to be more variable than serum measurements [242], which is another reason that sputum hAAT may have been more variable in the current study; it would have been useful to compare sputum hAAT in my studies to serum hAAT and C-reactive protein to investigate a link to any acute phrase response, but this was not possible.

I then compared the levels of hAAT in sputum with other biomarkers. There was a statistically significant correlation between neutrophil elastase and α_1-antitrypsin in sputum, which is to be expected for reasons discussed above. However, measuring hAAT and NE alone does not present a complete picture, because NE:AAT complexes (consisting of one molecule of each protein, irreversibly bound and therefore inactivated) are also present in the CF lung [235]. Some groups have developed ELISAs for the NE:AAT complex [243] and and it would have been very interesting, although unfortunately not feasible, to investigate the levels of NE:AAT complexes in sputum. It is not clear whether the Abcam ELISA kit I used in this study would detect NE:AAT complexes as well as free hAAT; the antibodies used in

the assay are directed at multiple epitopes of the α_1-antitrypsin molecule, so it is possible that NE:AAT complexes are included in the concentration of α_1-antitrypsin I have reported.

I was not able to demonstrate a statistically significant correlation between sputum α_1-antitrypsin and physiological markers of lung function (FEV_1 % predicted and LCI) and most other sputum and serum biomarkers, although I cannot rule out the possibility that a larger study with more samples may identify a link. This is a significant finding, because on the one hand it directly implies that treating CF with α_1-antitrypsin (either by a protein replacement or gene therapy approach) may not be of clinical benefit, at least when determined using these two outcome measures. Despite this, it should be noted that in inflammatory lung diseases (such as CF) the inflammatory burden is in the airways and interstitium, and sputum is a 'surrogate' marker; therefore, trying to address the protease:antiprotease balance in the lung could still be a justifiable strategy.

The UK CF Gene Therapy Consortium conducted a second longitudinal study, the Tracking study [244], where CF patients undergoing an exacerbation were investigated before and after a two-week course of antibiotics. It would have been very interesting to look at α_1-antitrpyisn expression in these patients, to see if the expected pattern (high levels of α_1-antitrypsin in the acute exacerbation phase, returning to baseline levels with clinical resolution) is observed. However, there were no samples remaining for me to analyse in this project.

Finally, α_1-antitrypsin has recently been detected in exhaled breath condensate (EBC) in healthy control and COPD patients at concentrations of around 250 pg/ml [245]. EBC is essentially a highly diluted (2000 to 10,000-fold), vaporised form of the airway surface lining fluid [246] and is easily and cheaply obtained (of note, it is significantly easier to obtain EBC from a healthy control than an induced sputum sample). It would be very interesting to look

at levels of α_1-antitrypsin in EBC from different patient groups, including healthy controls, CF patients and those with α_1-antitrypsin deficiency.

In summary, the data in this chapter suggest that α_1-antitrypsin is present in the sputum of cystic fibrosis patients in greater amounts than in healthy non-smokers and smokers, and at around the same level as would be observed in patients with asthma, COPD or bronchiectasis. There were no correlations of note between sputum hAAT and other physiological, sputum and serum biomarkers, although it is not possible to rule out α_1-antitrypsin gene or protein replacement therapy as a treatment for cystic fibrosis purely on the basis of the data presented here.

10 Summary and future directions

In this thesis, I have investigated strategies for gene therapy for the treatment of α_1-antitrypsin deficiency. I have used two vectors, the "gold standards" for viral and non-viral airway gene transfer, to deliver hAAT cDNA to the murine lung; the lipid formulation GL67A and a lentiviral vector based on SIV, pseudotyped with the F and HN surface proteins from Sendai virus.

Key findings presented in this thesis include:

1. Treatment with the non-viral gene transfer agent GL67A resulted in significant levels of hAAT in the murine lung, however this was not followed up further as the levels of hAAT were sub-therapeutic;
2. Treatment with rSIV.F/HN vectors carrying hAAT cDNA resulted in therapeutic levels of hAAT in the murine lung with doses of 1e8 TTU or higher;
3. Expression of hAAT following lentiviral transduction was sustained for at least 19 months, the lifetime of a mouse;
4. rSIV.F/HN vectors were delivered in 10 "split" doses over two weeks with no loss of efficacy, which may be necessary to deliver the viral titre required in man;
5. hAAT secreted into epithelial lining fluid was functional and inhibited neutrophil elastase in an *ex vivo* assay;
6. rSIV.F/HN vectors lead to expression of hAAT in precision-cut human lung slices, and primary human nasal epithelial cells;
7. rSIV.F/HN transduced murine nasal tissue as well and lung tissue, although it was not possible to selectively transduce nose.

In addition, I separately showed that hAAT was raised in the sputum of stable cystic fibrosis patients, suggesting that the body up-regulates production in response to production of neutrophil elastase.

The ultimate aim of my work, and the work of the UK CF Gene Therapy Consortium as a whole, is to produce gene transfer agents that can be translated to the clinic. This thesis adds to the large body of evidence supporting rSIV.F/HN vectors, and a first-in-man clinical trial for CF is planned for 2017 pending the results of in-house toxicology studies, which are currently being performed.

The results of these toxicology studies and the Phase I trial will inform the future development of rSIV.F/HN vectors coding for hAAT. The vector selected for the lentiviral CF trial is rSIV.F/HN-hCEF-soCFTR; the only difference between this vector and the rSIV.F/HN-hCEF-sohAAT vector used in most of my experiments is the transgene. Therefore, if rSIV.F/HN-hCEF-soCFTR passes regulatory-compliant toxicology studies, it is likely that rSIV.F/HN-hCEF-sohAAT could be rapidly translated into the clinic.

Producing large quantities of lentiviral vector in a GMP-compliant manner is one significant hurdle in translation to the clinic; recent progress using animal-free suspension cultures has been encouraging, and should allow for the scale-up of lentiviral vector production prior to a clinical trial.

Pending the results of toxicological studies, I feel that the data presented in this thesis provides sufficient pre-clinical evidence for the effectiveness of rSIV.F/HN-hCEF-sohAAT in animal models and *ex vivo* human tissue culture models. Nevertheless, there are a number of unanswered questions that should be addressed prior to clinical translation, which would provide further support for clinical translation.

1. *Is the best lentiviral vector for hAAT gene therapy being used?*

In this thesis, I was not able to compare different lentiviral viral vector constructs *in vivo*. It is therefore possible that modifications to the rSIV.F/HN-hCEF-sohAAT vector, such as an alternative promoter, could lead to higher expression of hAAT. The hCEF promoter was selected for gene transfer to the airway, however an alternative promoter could be more efficient in the alveoli and interstitium, the target tissue for AAT gene therapy.

2. *Is rSIV.F/HN-hCEF-sohAAT effective in large animals?*

   In this thesis, I performed a proof-of-concept study in n=3 sheep, but was not able to demonstrate production of hAAT or the reporter protein *Gaussia* luciferase using the vector titre available at the time of the experiment. It would be useful to investigate this further, probably by delivering multiple doses.

   It has also been hypothesised that the hCEF promoter used is not active in sheep (Ian Pringle, personal communication) so an alternative promoter may need to be used; alternatively, studies in another large animal model (e.g. pig) could be useful. Importantly, hCEF was the promoter used in the successful clinical trial of non-viral gene therapy for cystic fibrosis, implying that it is active in humans [105].

3. *Does the expression profile remain after aerosolisation of rSIV.F/HN?*

   In the clinic, the most likely route of lentiviral vector administration would be aerosolisation, the method used in the recent non-viral cystic fibrosis trial [105]. The Consortium has shown that aerosolised rSIV.F/HN vectors retain their function (unpublished), however efficacy and duration of gene expression following aerosolisation of these vectors is currently unknown, and should be addressed before the commencement of a clinical trial.

4. *Can rSIV.F/HN-hCEF-sohAAT be repeatedly administered?*

   The Consortium has previously shown that rSIV.F/HN vectors coding for reporter genes can be repeatedly administered at monthly intervals with no loss of efficacy

[134], however I was not able to perform an experiment using rSIV.F/HN-hCEF-sohAAT due to financial constraints. Whilst I do not expect a different result for a virus coding for hAAT compared to a reporter gene, this study would still be informative.

5. *Does gene therapy with rSIV.F/HN-hCEF-sohAAT improve the phenotype in animal models of α_1-antitrypsin deficiency or emphysema?*

   I have also been unable to investigate gene therapy in animal models of α_1-antitrypsin deficiency. It has historically proven difficult to produce an animal model of AATD, as deletion of the SerpinaA1 gene (a murine AAT homologue) results in embryonic lethality [247]. Recently, a new animal model of AATD has been developed using CRISPR/Cas9 to knock down all five murine AAT homologues [248]; the data has yet to be published in a peer-reviewed journal, but in future this model may aid studies of gene therapy for α_1-antitrypsin deficiency. There are, however, several animal models of emphysema [249] and studies in these models may be useful.

   I do not feel that the lack of an animal model in this thesis should prevent translation of hAAT gene therapy to the clinic; animal models are useful but not essential in the development of new therapeutics, and the lack of an effective animal model for cystic fibrosis [250] has not prevented clinical translation of CF gene therapy [105].

6. *Does hAAT produced by gene therapy retain other functions of the molecule?*

   In this thesis, I have demonstrated that hAAT produced by gene therapy is functional and inhibits neutrophil elastase; however, this represents just one function of α_1-antitrypsin, and the conclusion would be strengthened if it could be shown that hAAT produced following gene therapy performs other known functions, i.e. the inhibition of other proteases (including proteinase-3, myeloperoxidase, cathepsin G and others [1]) and any of the other anti-inflammatory, anti-bacterial and cell protective effects of

α_1-antitrypsin. Such studies could be done *in vitro* or *in vivo*, for example using an LPS challenge model.

7. *Could hAAT gene therapy be useful in other diseases?*

   Gene therapy with hAAT is not only limited to the treatment of α_1-antitrypsin deficiency, but could also be beneficial for a number of other patients, such as those with non-AATD associated COPD/emphysema, in which there is also a protease:antiprotease imbalance [94]. Furthermore, thanks to its anti-proteolytic and anti-inflammatory effects, hAAT has been proposed as a treatment in cystic fibrosis (discussed in Chapter 9) and a whole host other of diseases. A search of the US Clinical Trials database in April 2016 identified recently completed or ongoing studies using α_1-antitrypsin for the treatment of graft-versus-host disease/transplant rejection, Type 1 Diabetes, and HIV infection; α_1-antitrypsin has also been proposed as a treatment for Type 2 Diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease [38]) and systemic lupus erythematosus [251]. It would be interesting to study hAAT gene therapy using rSIV.F/HN vectors in animal models of these diseases, to see if there is any potential therapeutic benefit.

8. *Can the nose be used as a factory to produce secreted proteins?*

   In this thesis I present pilot studies of gene transfer to the nose of mice, but was not able to selectively transduce nasal tissue. Further improvements in the technique, or different delivery methods, could lead to selective nasal transduction which could be more appropriate than delivery to the lung for the treatment of some diseases.

It is important to consider some of the limitations of the research presented in this thesis. Firstly, financial constraints due to the high costs of vector production meant that the design of some experiments was sub-optimal. One batch of lentiviral vector, sufficient to treat 20 mice with 100 µl of vector/mouse, costs between £3000 and £15,000 depending on the

production method; this means that in many experiments the viral titre used, and/or the number of animals treated, was limited. As the bench fees for my studentship were £5000 per annum, external funding was necessary to perform most of the experiments. Personnel and financial constraints also meant that the viral titre was not determined in several experiments, and whilst I do not feel that this affected the interpretation of the data, it would certainly be preferable for the viral titre to be known for all experiments.

Secondly, some of the *in vitro* data presented in the thesis is inconclusive; for example in Chapter 4 I was unable to control for the effects of pseudotransduction. However, I feel it is important to stress that rSIV.F/HN lead to expression of human α_1-antitrypsin for almost two years in mice, strongly implying that pulmonary cells were successfully transduced and that the vector was functional; I therefore decided that further *in vitro* experiments were not essential.

The most advanced gene therapy vector for hAAT in clinical development is an rAAV vector that has been studied in three clinical trials [82] [84] [87]. This vector was given intramuscularly, with the aim of producing a secretory 'factory'; currently, only sub-therapeutic levels of hAAT production have been demonstrated. I feel that the lung is a more appropriate target organ for AATD. Lung disease accounts for most of the mortality due to α_1-antitrypsin deficiency [1]; a therapy that can be directly administered to the lung may reduce the titre needed, thereby reducing costs. Furthermore, the long-term expression seen following gene transfer with rSIV.F/HN, a vector that can be repeatedly administered, is also advantageous over the rAAV vectors used in the previous hAAT human gene therapy trials.

The studies presented in this thesis concentrate on techniques that could be used to treat AATD-associated lung disease, whilst treatment of AATD-associated liver disease is largely unexplored. AATD can lead to liver cirrhosis due to polymerisation and accumulation of mutant Z protein, a gain-of-function mutation that would not be affected by protein

augmentation (whether or not protein levels were augmented by intravenous administration, inhalation of purified hAAT, or gene therapy). However, it is possible that other genetic techniques could target the Z-hAAT gene for the treatment of AATD-associated liver disease. For example, knock-down of the gene in hepatocytes by Zinc-finger nucleases [139] or siRNA [135] [136] could prevent accumulation of Z-hAAT; and it is not implausible that a dual-function lentiviral vector, carrying the correct hAAT gene and sequences that knock-down the mutant gene, could be developed and delivered to the liver. An rAAV vector with similar properties is currently in pre-clinical development [142]; researchers have coupled an artificial miRNA targeting hAAT with the cDNA of normal ('M') AAT, delivering it to the murine liver via tail vein injection, demonstrating not only that Z-hAAT was down-regulated but also that M-hAAT was up-regulated for at least three months post-treatment. Furthermore, recent advances in CRISPR/Cas9 genome editing technology raises the intriguing prospect that the hAAT gene could be corrected [140], again possibly using a dual-function vector that also delivers a normal hAAT gene to the liver.

There are also many other potential applications of the lentiviral vector platform developed by the UK CF Gene Therapy Consortium. A number of research projects studying the use of this vector platform to produce recombinant proteins (such as Factor VIII for the treatment of haemophilia A) or monoclonal antibodies (such as infliximab for the treatment of Crohn's Disease and other autoimmune diseases, or antibodies to the influenza virus) have been initiated. It may also be useful to study alternative delivery methods – giving the vector intramuscularly or by hydrodynamic tail vein injection may be more appropriate for the treatment of non-pulmonary disease. Sendai virus has a broad tissue tropism (Uta Griesenbach, personal communication), suggesting that SIV vectors pseudotyped with F and HN proteins could also work in other tissues.

α_1-antitrypsin deficiency represents a disease with no clinically proven treatment options; IV augmentation therapy [52] and gene therapy with intramuscular rAAV vectors [87] have not been shown to lead to improvements in lung function. New treatment strategies for this disease are desperately sought, and gene therapy with lentiviral vectors may improve lung function, morbidity and mortality in AATD patients. The work presented in this thesis provides proof-of-concept that a lentiviral vector can deliver α_1-antitrypsin cDNA to the lung, resulting in the production of a functional, secreted protein at therapeutically relevant levels, thereby placing gene therapy for α_1-antitrypsin deficiency with a lentiviral vector on a translational pathway.

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