Cystic Fibrosis Gene Therapy: Methods for the optimisation of CFTR gene delivery.

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5 Cystic Fibrosis mouse models- *Cftr*^{tm1Unc}-Tg(FABPCFTR)1Jaw/J

5.1 The need for a new cystic fibrosis mouse model

New therapies for the treatment and cure of CF are currently being developed and researched. To aid in pre-clinical development of these therapeutic interventions and to better understand pathophysiology of the disease, several CF mouse models have been developed. In these mice the CFTR gene has either been completely inactivated (null mice) or naturally occurring mutations such as ΔF508 or G551D have been introduced leading to altered chloride transport across epithelial cells [190].

Generally, while CF mice do not mimic human CF lung disease, they do have blocked and inflamed intestines as a direct consequence of the altered ion transport. The lack of human-like lung disease in knockout mice came as a disappointment, but may be due to the expression of alternative calcium-activated chloride channels which act as a substitute for the absent CFTR-mediated chloride channel function [191]. This activation of alternative ion channels occurs in the mice lung but not the human lung. It is interesting to note that it does not occur in the mouse nasal epithelia, meaning the characteristic CF ion transport abnormalities of increased sodium absorption and absent chloride transport are retained in the mouse nasal epithelium [192].

It is well known that altered sodium and chloride transport in CF airway epithelial cells leads to changes in the potential difference (PD) across the apical membrane of these cells. The baseline PD is predominantly a measure of sodium absorption across the apical membrane and the response to the sodium channel inhibitor amiloride is increased in CF. The response to perfusion with a low chloride solution, which induces chloride flux *via* CFTR is reduced or absent. This PD measurement is used widely as a measure the effectiveness of corrective therapy in CF mouse nasal epithelium. Ideally the technique is used to observe treatment over time, with multiple measurements over the course of the treatment or life of the mouse. The best mouse models to assess treatments on are those with very

low or absent residual CFTR function. The UNC-null, G551D or ΔF508 are the most widely used models [169] [193] [194]. The severity of the intestinal disease in these mice varies depending on the strain and genotype and is largely dependent on the level of residual CFTR activity [195]. The intestinal problems impact severely on the lifespan of CF mice. By the age of weaning-21 days- a large proportion of CF pups are dead- approximately 50-95% [195]. In most of these mice the intestinal morbidity and mortality is seen at two distinct time points. Many new born CF mice die within 5 days of birth. The second group of mice die shortly after weaning. The histology of jejunum from CFTR-knockout mice (*cftr*^{Tm1Unc}) shows dilated and distended crypts with mucus, whereas these abnormal jejunum findings are barely visible in crypts of normal mice. Further differences are seen with goblet cell hyperplasia and hypertrophy apparent only in CF jejunum [195]. It has however, been found that manipulation of the diet, with replacement of normal drinking water with an electrolyte solution containing 6% polyethylene glycol before weaning can significantly increase the lifespan of the CF pups to near that of normal mice, as intestinal complications are ameliorated [195, 196]. With this dietary modification a large proportion of *cftr*^{tm1Unc} mice remain alive up to 18 months of age, with this lifespan being similar to that of normal mice.

In summary, CF mice suffer gut disease which causes high pre- and post-weaning mortality, runting and reduced survival after anaesthesia [197]. In addition to these complications, breeding requires heterozygote matings as the CFTR knockout causes infertility, particularly in females. Heterozygote matings result in only ¼ of pups being of CF genotype. These characteristics are obviously not favourable for producing the number of affected animals for large experiments.

To overcome the problems caused by the intestinal obstructions in CF mice, Zhou *et al* developed a gut-corrected CF mouse. This was done by using the rat intestinal fatty acid-binding protein (FABp) gene promoter to direct expression of the wild-type huCFTR complementary DNA to the intestinal epithelial cells of the knockout mice [198].

A chimeric FABp-huCFTR gene construct was microinjected into fertilized oocytes, producing transgenic mice from both heterozygotic CFTR +/- and wildtype FVB/N mice. The FABp-huCFTR transgene was detected by Southern blot analysis in founder mice and their offspring. Human CFTR mRNA was readily detected by reverse transcription PCR in the small intestine of 6 distinct FABp-huCFTR mouse lines. Human CFTR mRNA was not detected in the lungs or nasal epithelium. Human CFTR mRNA was found most abundant in the ileum, jejunum and duodenum and was less abundant in the cecum and colon. Founder lines were bred to CFTR +/- heterozygotes, these heterozygotes were then bred to produce homozygous CFTR -/- mice expressing huCFTR mRNA [198].

Mice that were FABp +/- and CFTR -/- were found to routinely survive weaning and showed prolonged survival. No mice were found to have developed intestinal obstruction at ages ranging from 1.5 to 7.5 months. In contrast, matings of CFTR +/- mice from FVB/N and C57Bl/6 backgrounds without the FABp-huCFTR transgene resulted in survival of less than 5% of the CFTR -/- offspring [198].

Short circuit measurements made from the intestine of CFTR -/- , bitransgenic (huCFTR +/-) / (CFTR -/-) and wildtype mice showed Cl^- secretion to be restored in bitransgenic mice. Correction of the goblet cell hyperplasia was also shown in the ileum *via* histological examination [198].

The establishment of a FABp-huCFTR corrected CF colony appeared advantageous for our therapeutic gene transfer studies. The establishment of this colony would not only be advantageous due to better production of CF animals and simpler husbandry as there would not be a need for additives in the drinking water, these mice would also be immune tolerant to the human CFTR. As we use the human CFTR gene in our gene therapy vectors, using a mouse that already contains the human CFTR gene (driven by the FABp promoter in the intestines) means the possibility of an immune response to the human CFTR gene by the animals treated with our vector can be ruled out. A history of how the WCH colony was developed is described further in this chapter.

5.2 An historical account of the WCH- *Cftr*^{tm1Unc}-Tg(FABPCFTR)1Jaw/J mouse colony

Initially, affected mice bred from the *Cftr*^{tm1Unc} mouse colony were used for the work described in this thesis. However, these mice showed significant morbidity and mortality following repeated anesthesia and PD testing. In addition, breeding of affected animals in the required numbers was laborious due to the inability to breed CFTR -/- males and females together due to the reduced fertility of female CF mice, therefore the breeding colony requires Het +/- female, CFTR -/- male matings, resulting in only 50% of offspring being CFTR knockout mice. Accordingly, it was thought the establishment of a colony of FABp-huCFTR CF mice (genotype) would be advantageous.

Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J mice ordered from Jackson Laboratories arrived in Australia in August 2006. Five females and 7 males were received. Mice were released to Waite animal services following 2 days quarantine. While at the Waite holding facility, 5 breeding pairs were setup. From those breeding pairs, the following offspring were recorded in Table 5-1.

Mice were released to the WCH animal facility in December 2006. As the breeding pairs were breeding well, all pairs except for breeder box 2 were separated.

It was noticed in August 2007 that mice from the Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J colony appeared to be displaying the distinctive yellow and white incisors seen in non-CF animals our Cftr^{tm1Unc} colony. In the latter colony, a check of the teeth can be carried out for mice of post weaning age to determine genotype as CF mice display chalky white incisors and heterozygote mice display distinctly yellow incisors. This can usually only be carried out with certainty post weaning. The appearance of white and yellow incisors was not expected in the Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J colony as it was thought that all

mice ordered and received were homozygous for both the CFTR knockout (white teeth) and the FABp-huCFTR knock-in.

Table 5-1: Original Breeding Pairs (from Waite Campus)

Box number	***Date of litter***	♂***offspring***	♀***offspring***
1	23-11-2006	1	4
	12-12-2006	8	2
	4-01-2007	3	6
2	22-11-2006	3	6
	12-12-2006	3	4
3	23-11-2006	4	4
	17-12-2006	5	5
4	25-11-2006	5	0
5	22-11-2006	6	2
	12-12-2006	7	4

By September 2007, there were no mice left in the Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J colony displaying white teeth which led to more breeding pairs being established to breed CFTR-knockout mice. Also during this time, it was noted that there were deaths in the colony around weaning age. This drew our attention to the FABp-huCFTR status of the mice, with the deaths likely due to intestinal problems arising from the mice lacking the FABp-huCFTR knock-in, with the associated problems not being dealt with as colonlytely was not being added to the drinking water. As with the CFTR-knockout genotyping, these problems had not been considered earlier as it was thought all mice received in August 2006 were homozygous for the FABp-huCFTR knock-in.

As it became obvious mice were not homozygous for FABp-huCFTR, genotyping each mouse became essential to determine its FABp status. This was done to help start establishing a breeding program to

produce mice homozygous for both FABp-huCFTR and the CFTR knockout. This would make genotyping unnecessary with 100% of offspring being CF/FABp, as required for experimentation needs, as opposed to breeding FABp-huCFTR hemizygotes, which limits the productivity of the colony, as 1 in 4 mice will lack FABp-huCFTR and die.

Genotyping protocols were obtained from Jackson Laboratories for determination of CFTR and FABp-huCFTR status.

5.2.1 Genotyping PCRs

The genotyping PCRs were established using gDNA extracted from toe tags, tail snips or ear tags from new born to post-weaning colony mice. Genomic DNA was extracted from the tissue samples using 150 µL of Viagen TM ® tail lysis solution supplemented with 0.4 mg/mL proteinase K, with incubation at 55 °C overnight followed by inactivation of the proteinase K solution at 80 °C for 45 minutes .

Five to twenty nanograms of gDNA was used from each mouse for amplification, with 0.2 mM dNTPs, 1 µM primers, 0.0125 U/µL HotStarTaq polymerase, in a total volume of 20 µL.

The CFTR knockout mice contain a neomycin gene inserted into the CFTR gene. The three primers in this reaction give 3 distinct banding patterns to allow the exact genotype to be determined. These banding patterns are:

Wildtype (+/+) = 526bp (band from the CFTR gene)

Heterozygote (+/-) = 526 + 357bp (bands from the CFTR gene and neomycin gene)

CFTR knockout (-/-) = 357bp (band from the neomycin gene)

An example gel is shown in Figure 5-1

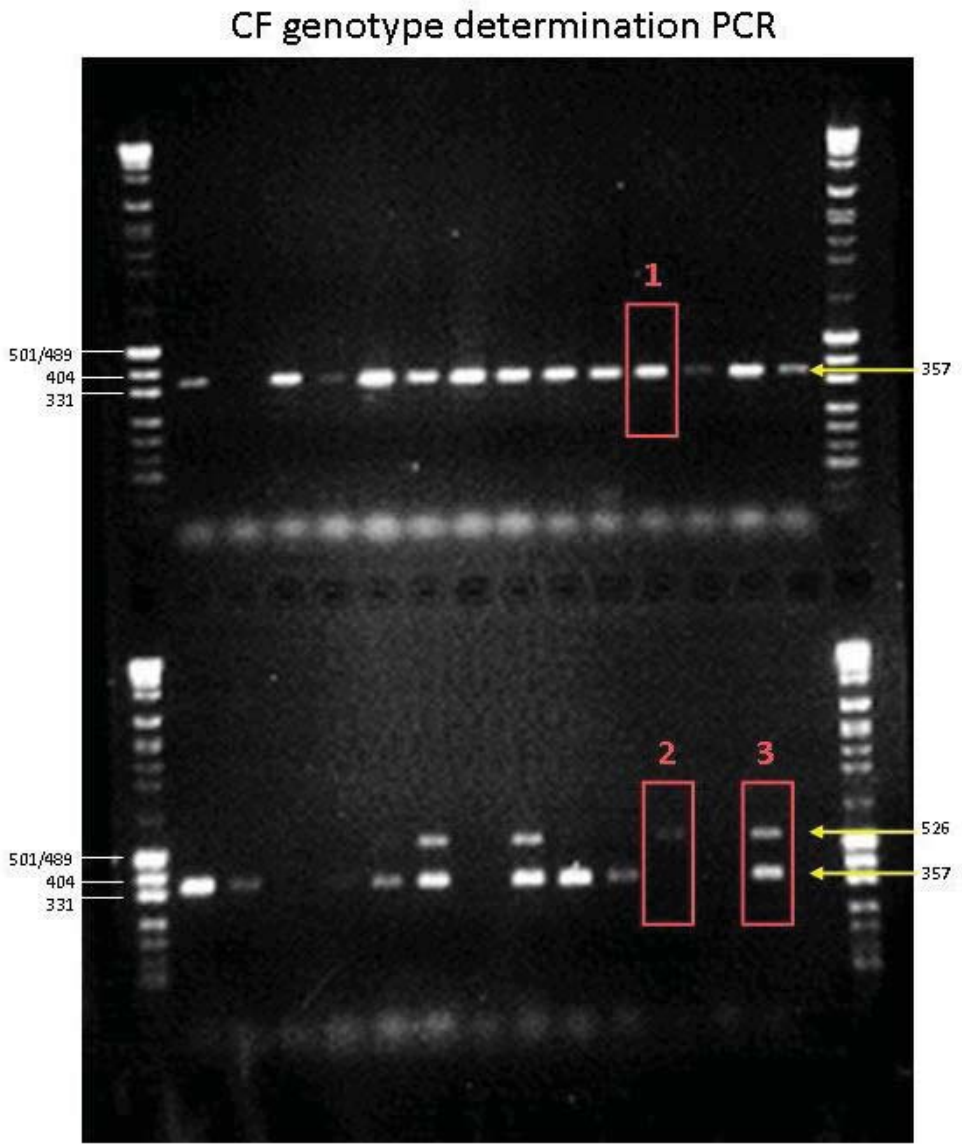


Figure 5-1: Agarose Gel Electrophoresis of PCR products from CF genotyping PCR

Agarose gel shows the banding patterns of the CF genotyping PCR. Markers SppI/EcoRI and pUC19/HpaII shown either end of the gel. 1: band at 357 base pairs indicating CFTR knockout mouse (-/-) 2: band at 526 base pairs indicating homozygous for CFTR knockout (+/+) 3: bands at 536 and 357 base pairs indicating heterozygous for CFTR knockout (+/-).

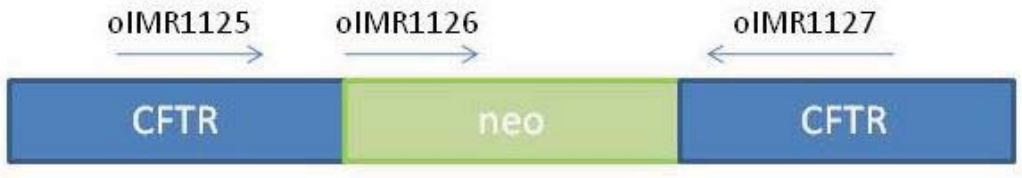
Primers used for the PCR reaction were ordered and chemically synthesized by Invitrogen Life Technologies.

All sequences written 5'→ 3'

oIMR1125: GAG AAC TGG AAG CTT CAG AGG (primer located upstream of the neomycin gene insertion site)

oIMR1126: TCC ATC TTG TTC AAT GGC C (primer located within the neomycin gene)

oIMR1127: TCC ATG TAG TGG TGT GAA CG (primer located downstream of the neomycin gene insertion site)



The PCR cycling conditions are described in section 2.4.3.1.

To determine if mice carry the FABp-huCFTR transgene, a PCR that amplifies a 413bp fragment from the human CFTR transgene was used. As this is a PCR which will not give a result if subjects do not contain the FABp-huCFTR gene, an IL-2 gene fragment was amplified as an internal control to ensure the PCR had worked. It should also be noted that this PCR cannot distinguish mice hemizygous for the transgene from mice that are homozygous for the transgene.

The primers for this PCR are

All primers written 5' → 3'

oIMR0042: CTA GGC CAC AGA ATT GAA AGA TCT (wild-type IL-2 forward primer)

oIMR0043: GTA GGT GGA AAT TCT AGC ATC ATC C (wild-type IL-2 reverse primer)

oIMR1011: AAA CTT CTA ATG GTG ATG ACA G

oIMR1012: AGA AAT TCT TGC TCG TTG AC

An example gel is shown in Figure 5-2

The PCR cycling conditions are described in section 2.4.3.2.

5.3 Results of genotyping

5.3.1 First round genotyping

The breeding pairs that were then being used for breeding, were genotyped for CFTR and FABp-huCFTR status approximately 1 year after arrival at the WCH animal facility. As described previously, the delay in genotyping was due to the incorrect assumption that all mice were homozygous for the CFTR knockout, and for the FABp-huCFTR transgene.

Four main breeding pairs were genotyped, as shown in Table 5-2.

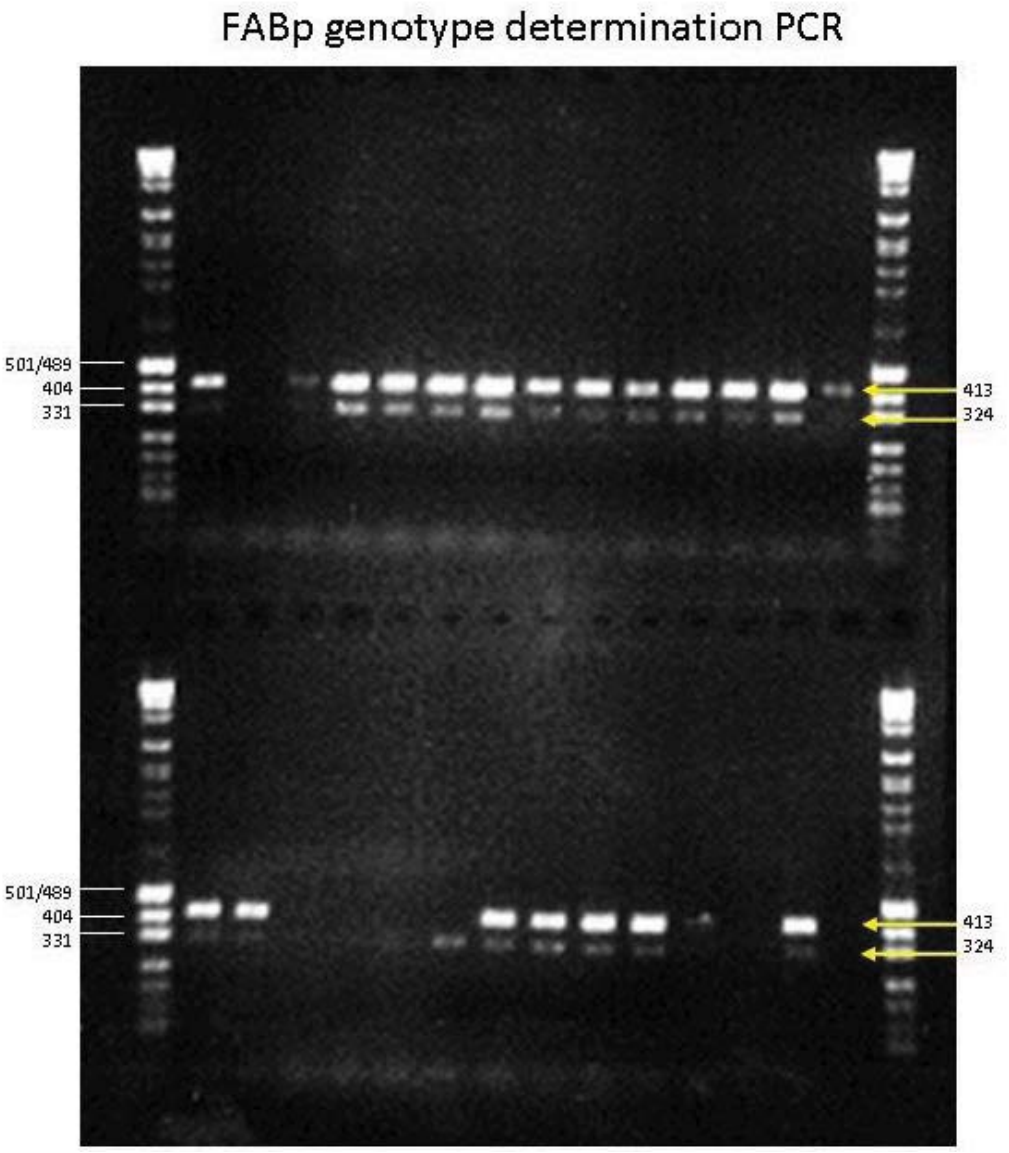


Figure 5-2: Agarose Gel Electrophoresis showing PCR products of FABp genotyping PCR

Agarose gel with SppI/EcoRI and pUC19/HpaII markers. Bands shown at 413 base pairs indicating the presence of the FABp transgene, bands at 324 show the IL-2 internal control to ensure PCR integrity

Table 5-2: Genotyping of first generation Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J mice

Breeder identification	Sex	Theoretical CFTR genotype from incisor colour	PCR confirmed CFTR genotype	PCR confirmed FABP-huCFTR status*
8C	♀	Heterozygous	Heterozygous	+ve
	♂	Heterozygous	Heterozygous	+ve
3B	♀	CFTR-knockout	CFTR-knockout	+ve
	♂	CFTR-knockout	CFTR-knockout	+ve
8B	♀	Heterozygous	Heterozygous	-ve
	♂	te><Author>WelCFTR-	CFTR-knockout	+ve
9B	♀	Heterozygous	Wild type	+ve
	♂	CFTR-knockout	CFTR-knockout	-ve

* This analysis cannot distinguish hemi/homozygous animals.

5.3.1.1 Breeder pair 8C

The family tree for breeder pair 8C is shown in Figure 5-3. The mice used in breeder pair 8C were 3rd generation mice, with the female being progeny of breeder box 6 and the male from breeder box 8. Both breeder box 6 and 8 were setup from the progeny of original mice received in August 2006. This breeder pair, although confirmed as a het +/- by het +/- pairing was continued as the genotyping results showed a positive FABp-huCFTR status for both animals, which is advantageous for further breeding.

5.3.1.2 Breeder pair 3B

Breeder pair 3B was setup using brother/sister matings of a CF male and a het female. Again, this pairing is of 3rd generation mice, breed from the original Box 1 mice received in 2006. The FABp-huCFTR status of Box 3B was also found to be positive, with this breeding pair continued for further expansion of the colony. (See Figure 5-4)

5.3.1.3 Breeder pair 8B

Breeder pair 8B was made up of a 2nd generation heterozygous female from the original breeder box 4, and a 3rd generation CF male from the same lineage as the heterozygous male described in breeder box 8C (see Figure 5-5). After genotyping, it was found that the female heterozygous mouse was negative for the FABp-huCFTR gene, indicating that this mouse does not express the huCFTR in the gastrointestinal organs. The consequence of this is likely premature death of this mouse, but also the introduction of an FABp negative breeding line into the colony. As the FABp-huCFTR PCR cannot distinguish between mice that are hemizygous or homozygous for the transgene, breeding an FABp negative animal increases the chance of breeding more FABp negative mice. This is not advantageous for our breeding program, as it will not overcome the limitation we have with the Unc CF colony and the intestinal problems associated with them. Therefore this breeding pair was discontinued.

5.3.1.4 Breeder pair 9B

The mice used to establish breeder pair 9B were a 2nd generation female from original box 4 and a 3rd generation CF male originally derived from box 5 (see Figure 5-6). The genotyping results from this pair were interesting as it was also discovered that inscisor colour does not distinguish between mice that are heterozygous for the CFTR knockout and mice that are wild type. The female mouse used in this breeding pair presented with yellow inscisors, however, after genotyping it was found not to be heterozygous, but rather wild type as it only gave a PCR result of a band at 526 bp, indicating a +/+ genotype. The male used in this breeding pair was found after genotyping to be FABp-huCFTR negative. As this breeding pair presented many problems for continued breeding, the pair was separated.

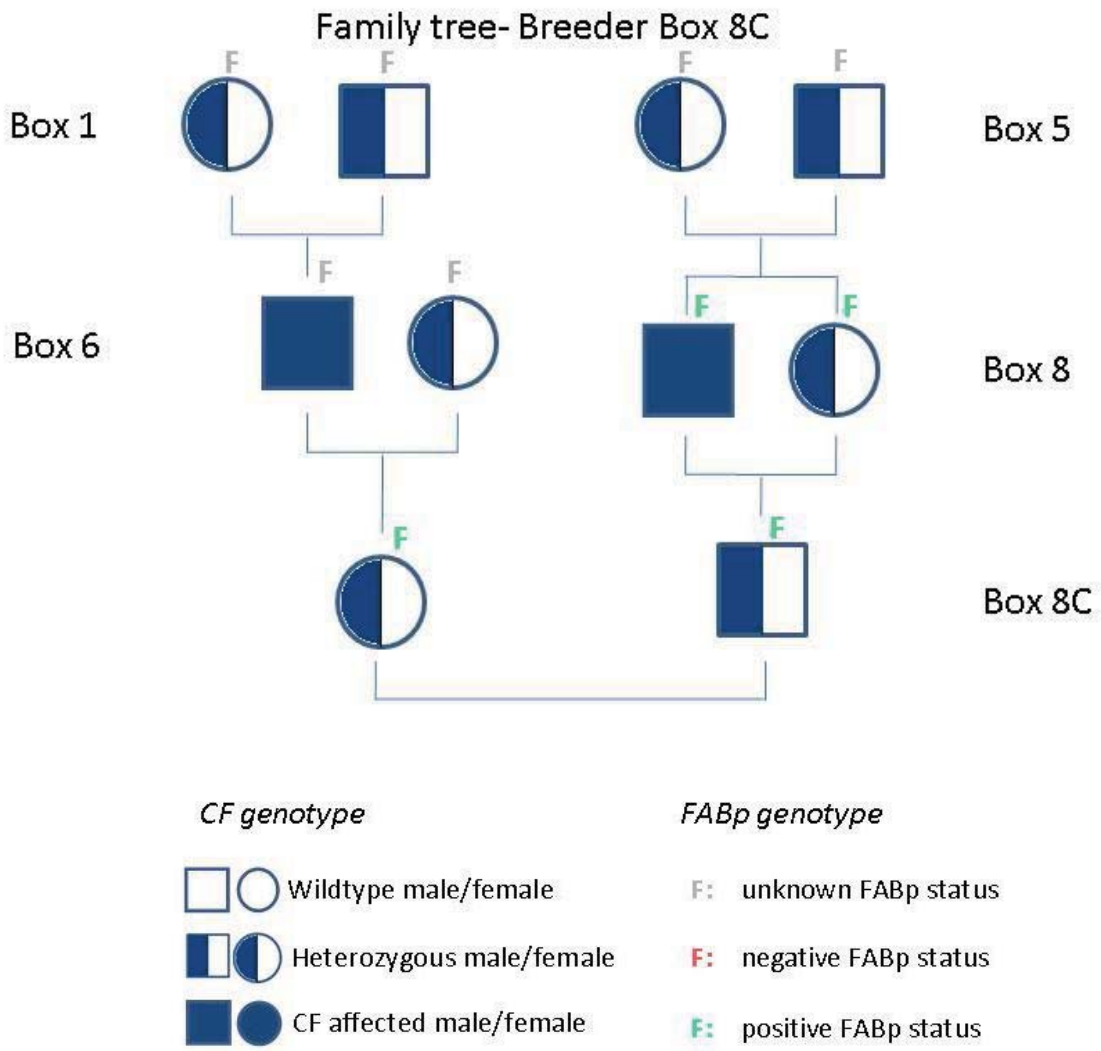


Figure 5-3: Family tree- Breeder Box 8C

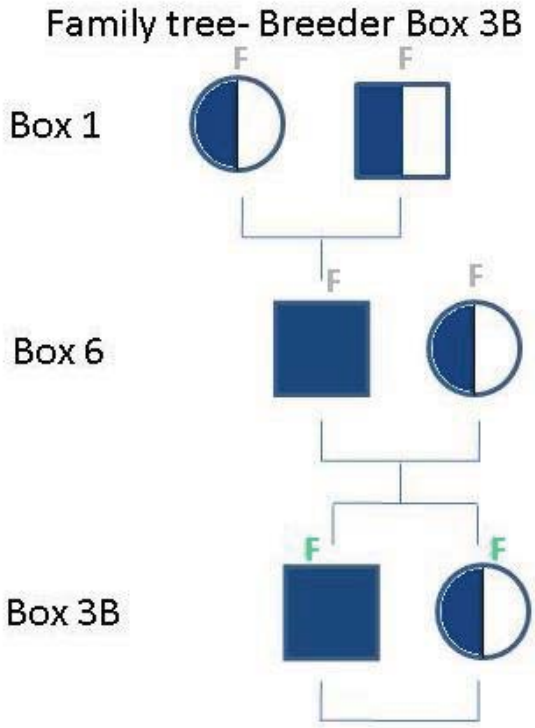


Figure 5-4: Family tree- Breeder Box 3B

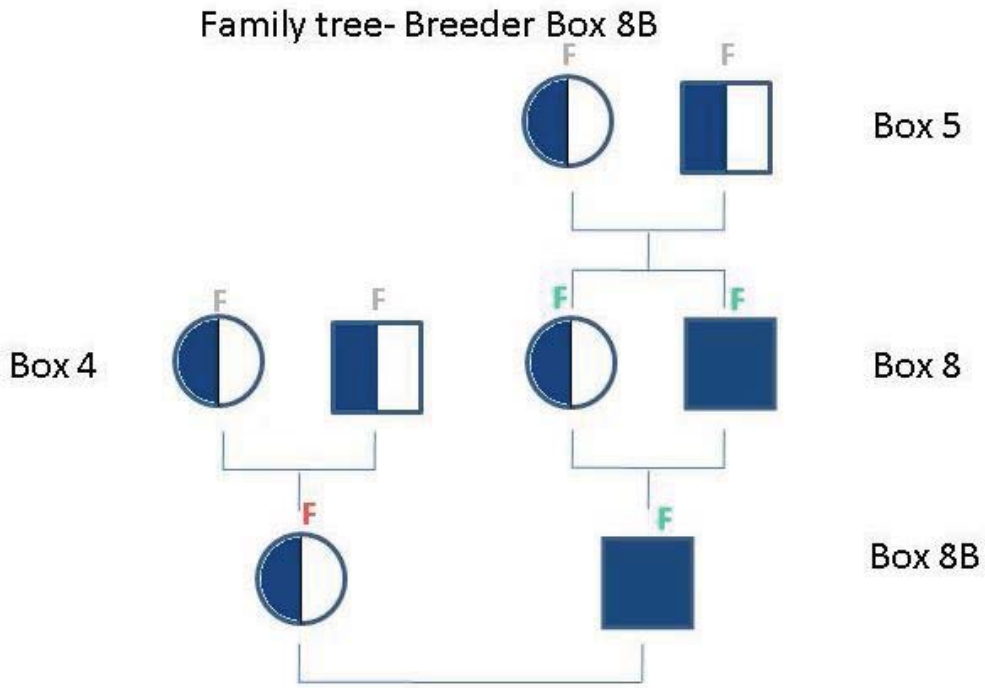


Figure 5-5: Family tree- Breeder Box 8B

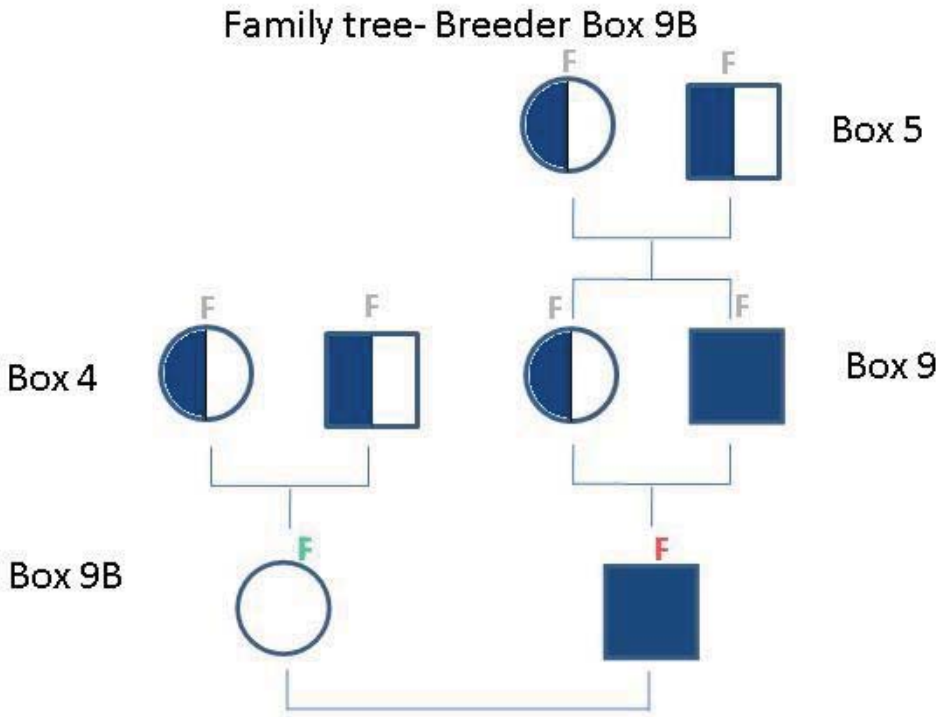


Figure 5-6: Family tree- Breeder Box 9B

5.3.2 Second round genotyping

Available progeny from breeding pairs genotyped in 5.3.1, as well as other available mice of the same generation, were genotyped for both transgenes. See Table 5-3.

As Table 5-3 shows, all progeny from genotyped breeding pairs gave a positive PCR product for the FABp-huCFTR transgene.

As these mice were still young, they were used to determine their true FABp-huCFTR status (heterozygous or homozygous for FABp-huCFTR) by breeding with normal C57Bl/6 mice.

5.3.3 Determination of FABP-huCFTR status

As the FABp genotyping PCR cannot detect if the tested mouse carries one or two copies of the FABp-huCFTR transgene (i.e. hemizygous versus homozygous mice), a different breeding program was required to accurately determine FABp status.

Genotyped pups that had a positive FABp-huCFTR status were bred with normal wild type C57Bl/6 mice. Once pups were born, they were immediately sacrificed and genotyped for their FABp status.

If all the pups give a positive FABp PCR result, this indicates that the CFTR^{tm1Unc}-Tg(FABPCFTR)1Jaw/J mouse bred with the wild type normal is in all probability homozygous for the FABp-huCFTR transgene. However, if any pups are found that are negative for the FABP transgene, then it can be concluded with absolute confidence that the CFTR^{tm1Unc} mouse bred with the wild type normal is heterozygous for the FABp-huCFTR transgene.

Table 5-3: Genotyping of first generation Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J mice

Identification	CF Genotype	FABP-huCFTR status
4-1	CF -/-	+ve
4-2	CF -/-	+ve
3A-1	CF -/-	+ve
3A-2	CF -/-	+ve
8C-1	CF -/-	+ve
8C-2	CF -/-	+ve
8C-3	CF -/-	+ve
8C-4	CF -/-	+ve
8C-5	CF -/-	+ve
8C-6	CF -/-	+ve
8C-7	CF -/-	+ve
8C-8	CF -/-	+ve
8C-10	CF -/-	+ve
8C-11	CF -/-	+ve
8C-12	CF -/-	+ve
3B-1	CF -/-	+ve

Both male and female Cftr^{tm1Unc}-Tg(FABP-huCFTR) mice were bred with their corresponding wild type mouse.

From the Cftr^{tm1Unc}-Tg(FABP-huCFTR) male 8C-8 bred with a normal female, from 6 pups born, 3 were FABp positive and 3 FABp negative, therefore indicating that this male is heterozygous for FABp-huCFTR. Therefore this mouse was not used for further breeding.

The female Cftr^{tm1Unc}(FABp-huCFTR) mice setup to mate with a wild type male proved more difficult to breed from. Three female Cftr^{tm1Unc}-Tg(FABp-huCFTR) mice, 8C-10, 8C-11 and 8C-12, were housed for breeding with one C57Bl/6 wild type male. It was noted on many occasions that mice were pregnant by the animal house staff, and females were removed from the male C57Bl/6 to a separate cage to allow identification of mother and pups. This occurred with all females, and in all cases the females were found not to be pregnant. These phantom pregnancies were noted 3-4 times per mouse. Following the phantom pregnancies, which led to re-caging every 2-3 weeks, the age of the females were thought to be too old for breeding- particularly if they were to be returned to the colony for breeding. The decision was made not to breed female CF mice with the normal males, as this was taking a significant amount of breeding time that could be used to grow the colony, rather than further explore the FABp-huCFTR status of the mice.

5.3.4 The Current WCH Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J colony

As it stands, the current colony is now producing CF mice in numbers that can be used for experimentation. Breeding pairs are setup from progeny of mice that have been genotyped and have given a positive result for the FABp-huCFTR transgene. Currently, there are only CF mice being bred, with litter sizes being approximately 4-5 mice per litter. As there have not been deaths post weaning, this would indicate that the mice we are breeding contain the FABp-huCFTR transgene. The current

colony has been maintained by setting up breeding pairs using the progeny of breeding lines 8A and 3B, which had positive results for FABp-huCFTR genotyping and breed efficiently to produce CF mice. Maintenance of the colony has been continued by Ms P Cmielewski, and FABp genotyping has been discontinued at this point.

5.3.5 Lessons learned for Cftr^{tm1Unc}FABP-huCFTR breeding

To maintain the colony it was found that unlike the Cftr^{tm1Unc} colony, breeding pairs must be setup at a young age- approximately 4 weeks of age, just post weaning. From this experience it has also been noted that mice stop breeding at approximately 4 months of age, which differs somewhat from our experience with the Unc colony, with these mice starting breeding at 6-8 weeks of age, and continuing to breed for as long as a year, with regular pregnancies.

It has been found by others that the Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J mice stop breeding after 4-5 litters (unpublished observation, personal communication, A van Heeckeren), therefore careful maintenance of the colony is required to ensure young mice are available for breeding.

It was further noted that an advantage of this colony over the Unc colony is that CFTR-knockout females are fertile, which allows for CF/CF matings, with all progeny being a CFTR-knockout. It was seen however, that even in young CF/CF matings the number of progeny per litter was very small; on average, 2.7 pups were born. Although it is an advantage to be able to breed both affected females and males to produce a colony entirely of affected mice, the small litter sizes makes maintenance of a colony difficult- as these progeny then have to be set up for breeding. Testing of female fertility in CF mouse models has shown decreased female fertility, evidenced by fewer litters and fewer pups per litter when compared to non-CF mice. Sperm transport is impaired in the female CF reproductive tract, leading to decreased oocyte fertillisation. There has also been an observed decrease in the size of

reproductive organs, abnormal estrous cycling and aberrant ovulation patterns indicating that there may be additional abnormalities contributing to the observed decreased fertility [199]. When CF female mice were exposed to exogenous hormones, a correction of organ size and ovulation were observed, leading to somewhat corrected fertility [199]. Therefore, the creation of a FABp colony does not address all the reproductive issues associated with a CFTR-knockout colony. It may be useful to use transgenic technology to correct CFTR expression in other non-respiratory tissues, such as the reproductive tract. There are advantages of the Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J colony for our research as we are using gene therapy vectors to deliver the human CFTR gene. As the Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J colony has the huCFTR correction in the gastrointestinal organs, compared to gene delivery in the original Cftr^{tm1Unc} colony, it is unlikely that an immune response will be mounted from the Cftr^{tm1Unc}-Tg(FABPCFTR)1Jaw/J colony to the huCFTR, as these mice have already been exposed to it. For the research described in this thesis, the FABp colony is advantageous as the immune response issues can be ruled out as a reason for low level or no CFTR gene correction.

Breeding pairs utilizing female mice heterozygous for the CFTR-knockout and male homozygous CFTR-knockout mice where both male and female also had a positive FABp-huCFTR PCR result continued to be setup to allow for maintenance of a strong colony to produce enough mice for both breeding and experimental work.

As approximately 3-4 generations of genotyping has been carried out, with all mice returning a positive FABp result, it would appear that we have now established a breeding colony homozygous for the FABp-huCFTR transgene. Work continues to expand the colony, to allow sufficient numbers of mice for both breeding and mice to be used for further experiments.

6 Development of DNA Aptamers to human CFTR

6.1 Aim

Experiments described in this chapter outline the attempted development of DNA aptamers to human CFTR, to create a new tool for the assessment of CFTR transgene expression.

6.2 Aptamers

Aptamers are single stranded nucleic acid molecules that offer an alternative to traditional monoclonal antibody technology. A random library of short single strand DNA sequences is screened for molecules that bind to the desired target by multiple rounds of affinity purification, utilising PCR to amplify the molecules purified from each round of selection [200]. The extremely high diversity of the original library (10^{15} sequences) and the nature of the interaction of the aptamer and target means that aptamers with extremely high affinity (sub nM to pM) and specificity can be isolated.

Aptamer technology offers an attractive alternative approach to antibodies in that it is rapid, technically straight forward and negates the need for animals.

A gene therapy approach to a cure for cystic fibrosis is a complex and challenging area. Initial studies are conducted in animal models to prove “proof of concept” safety and efficacy. For analysis of these experiments highly specific reagents are required for analysis of the CFTR expression from gene vectors.

Monoclonal antibodies to CFTR are available (such as those described in chapter 4) however, until recently antibodies specific to human CFTR and suitable for immunohistochemistry of nasal tissue sections have been difficult to use for detection of CFTR due to problems with availability and problems with processing tissues which require decalcification, a process which destroys the epitope recognized by the antibody. Recently there have been 4 antibodies to CFTR that have been shown to

have higher affinity and specificity compared to those previously described (Cystic Fibrosis Foundation Therapeutics, personal communication). Despite this, the development of a new tool, such as aptamers, offers a way to rapidly isolate high affinity reagents to CFTR that would expand the range of tools available for analysis of CFTR expression.

Development of monoclonal antibodies was a milestone for the recognition of biological molecules (specifically proteins) with high specificity and affinity. There are however some limitations to monoclonal antibody technology. The technology is only well established in the mouse, making the development of reagents for murine proteins difficult. Molecules that are conserved across species can be difficult to raise antibodies against. Other proteins are simply not immunogenic.

Aptamers are single stranded nucleic acids of approximately 50-100 base pairs in length. They can be either DNA or RNA and can bind to their target with both high affinity and specificity. This is possible due to a three-dimensional shape adopted by the aptamer based on intrastrand base pairing that allows interaction over many residues with the target. This differs from antibodies where the number of interacting residues is relatively small [200]. Aptamers with pM binding affinities have been isolated and binding affinities in the low nM range are common [201].

Target molecules for aptamers are diverse and range from simple chemicals such as ethanolamine to diverse proteins [201]. Aptamers can also be used for detection, separation and purification of molecules and cells. The small size of aptamers allows them to access epitopes that are sterically inaccessible to antibodies.

SELEX technology (**S**ystematic **E**volution of **L**igands by **Ex**ponential enrichment) is used to isolate aptamers. In this process, aptamers recognizing the target of interest are enriched through multiple cycles,- 10-15, of binding and elution, with amplification of binding aptamers by PCR after each step,

followed by cloning of individual molecular species. Cloning allows characterization of aptamers by sequencing and their individual testing [200] [202]. Large quantities of aptamer can then be generated by PCR or chemical synthesis.

The aptamer system appears technically uncomplicated and doesn't require the use of animals or cell culture, which means aptamers can be isolated to toxic or non-immunogenic molecules.

Several aptamers have been FDA approved for pre-clinical and clinical trials. One such FDA approved aptamer is an anti-vascular endothelial growth factor aptamer for the treatment of ocular vascular disease [200].

6.3 Development of aptamers to the CFTR gene

As the basic technology for cystic fibrosis gene therapy has been developed by a number of groups, highly specific reagents are required for analysis of CFTR gene transfer. Monoclonal antibodies to CFTR are available, however these have often been of poor quality with low affinity and specificity, as evidenced by the relatively small number of CF gene therapy papers which make use of immunohistochemistry as a form of analysis. The few "good" antibodies to CFTR can be hard to access, therefore an attempt to select aptamers to the human CFTR protein for the quantification of CFTR gene transfer in our gene therapy studies in the murine nasal airway system was deemed to be a worthwhile enterprise.

A random 50mer library was made by chemical synthesis with primer binding sites at both 5' and 3' ends to allow PCR amplification. The primer sequences also contained restriction enzyme binding sites (SacI and BamHI) to facilitate directional cloning.

5’ 3’

AGCTCAGAATAACGCTCAA -- **N**50 -- TTCGACATGAGGCCGGATC

Primers were made to both the 5’ and 3’ binding sites of the library to allow for aptamer amplification

Apt-For (forward primer)

5’ AGCTCAGAATAACGCTCAA 3’

Apt-Rev (reverse primer)

5’ GATCCGGCCTCATGTCGAA 3’

Primers were purchased from Invitrogen on a 40 nM scale, the library was purchased on a 10 µM scale. Modifications to the primers were also made: Apt-For was prepared with FITC-fluorescent (for visualization) or biotin (for binding of streptavidin labeled secondary reagents) labels and Apt-Rev was prepared with a HEGL-A_{20} modification. The HEGL-A20 modification acts as a hydrophilic spacer, which blocks Taq polymerase extension, and the A20 sequence allows for strand separation by size with the addition of an extra 20 adenosine residues on the antisense strand of the amplified aptamers.

6.3.1 Initial screening of aptamer library

Five micrograms of the aptamer library in 500 µL of aptamer binding buffer was denatured at 90 °C for 10 minutes and cooled to 4°C and incubated for 15 minutes before being warmed to room temperature.

The library was then incubated with untransduced CHOK-1 cells ($1x10^6$ cells) to remove aptamer sequences recognizing cellular molecules.

For detailed method describing the initial screening of the aptamer library, see section 2.2.16.

Briefly, the aptamer library/cell mixture was incubated at room temperature with gentle agitation for 30 minutes. Cells were removed *via* centrifugation. Unbound aptamer was then incubated with CHOK-1 cells transduced with lentivirus containing the CFTR gene and incubated with gentle agitation for 30 minutes at room temperature.

Cells were then washed in binding buffer *via* repeated centrifugation / resuspension, followed by cell lysis by resuspension in 100 µL 1% Triton X-100 at 0°C for 5 minutes. Lysed cells were centrifuged at low speed to remove nuclei from the supernatant.

One hundred microlitres of 2 x elution buffer was added to the supernatant from the lysis step to elute aptamer sequences.

Aptamer sequences were precipitated with 0.6 M sodium chloride and 5 volumes of ethanol overnight, before being recovered *via* centrifugation for one hour and the aptamer pellet was then resuspended in 20 µL of TE buffer.

One microlitre of recovered aptamer sequences were amplified in a PCR reaction containing 100 µM of forward-FITC labeled primer and reverse primers-HEGL-A20, 10 mM dNTP's with the appropriate 10 x buffer, and made to 100 µL with H_2O. The PCR reaction is described in section 2.2.16. This PCR was carried out in duplicate to increase the amount of aptamer recovered.

The PCR reactions were precipitated with 0.3M sodium acetate and 3 volumes of ethanol. Samples were incubated at room temperature for 30 minutes followed by centrifugation to pellet amplified aptamers for 60 minutes. Aptamers were resuspended in 10 µL of Urea denaturing gel loading buffer.

Prior to running aptamer samples on a 10% w/v denaturing polyacrylamide gel, samples were denatured at 95 °C for 3-4 minutes and then quenched on ice.

As the forward primer contains a FITC label, once samples were run to the end of the gel, the fluorescent band was cut from the gel and aptamers eluted from the gel with 0.3M sodium acetate and 2 mM EDTA at 80 °C for 45 minutes with occasional mixing. Separation was achieved by the increased size of reverse strand due to the HEGL-A20 sequence.

Aptamers were precipitated with 3 volumes of absolute ethanol, and spun down for use in the subsequent screening for CFTR aptamers.

6.3.2 Subsequent screening of aptamers

Aptamers generated from the initial screening, were used in the subsequent screening process. The protocol adapted for the subsequent screen is essentially the same as the initial screening process, however, the previous round aptamers, rather than the aptamer library, were used as the aptamer source. The heat denatured aptamers were then incubated with CHOK-1 cells expressing the CFTR gene to bind CFTR recognizing sequences. The protocol is then the same as that described in section 2.2.17.

The recovered aptamers from the subsequent screening protocol were then used again in the subsequent screening protocol until this had been carried out for 12 rounds of amplification.

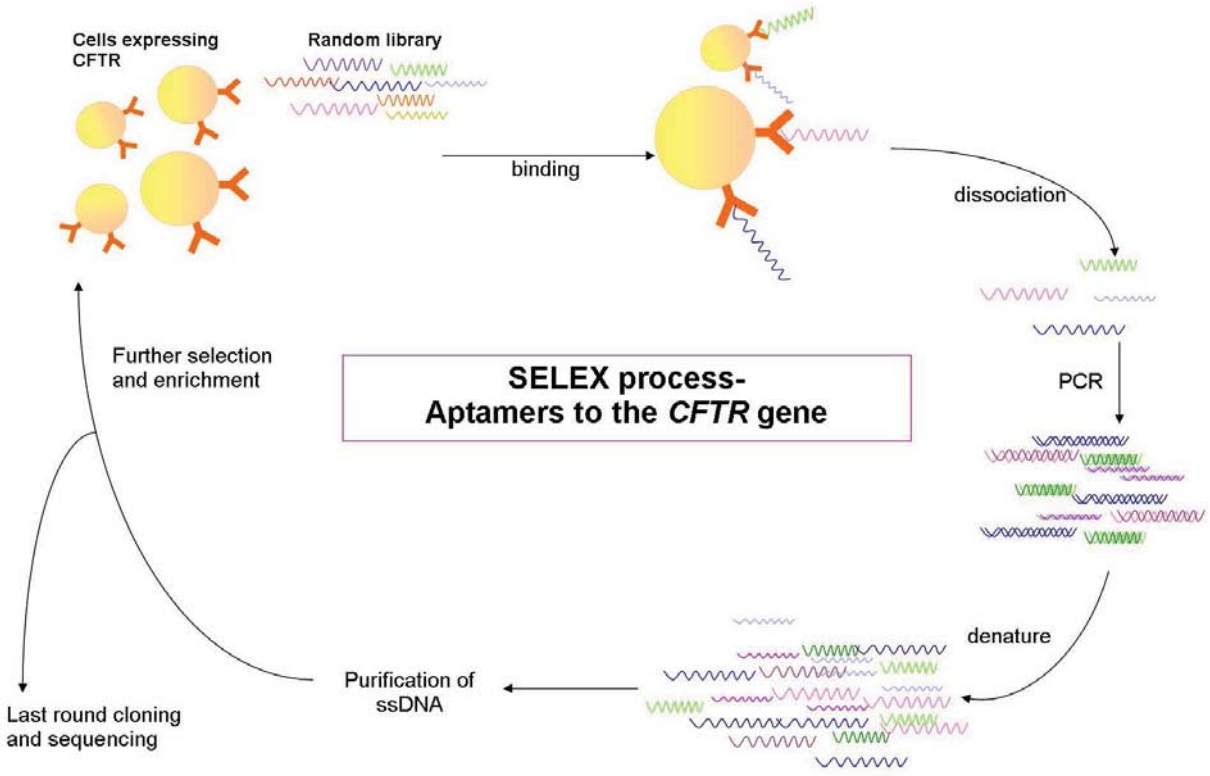
Attempts were made to monitor aptamer binding and elution by measurement of the fluorescence in samples taken following the binding step and samples after the PCR elution. Once the sample taken following elution reaches a plateau, this indicates the end of aptamer selection (as no further selection is taking place). This was attempted; however, the levels of fluorescence were too low to be measured

accurately. Radioactive markers are mostly used for the quantification of the enriched target-binding oligonucleotides as well as the amount of non-binding olidonucleotides of each selection round. This is a very sensitive method enabling the detection of slightest amounts of nucleic acids. Radioactive materials do however pose a health risk to employees and is very cost intensive [203].

A diagrammatic view of the SELEX selection process is shown in Figure 6-1.

Figure 6-1: SELEX method of aptamer selection

The Systematic Evolution of Ligands by Exponential Enrichment process by which aptamers to the CFTR gene were selected, isolated and enriched, ready for cloning into vectors to be sequenced and analysed for CFTR binding abilities.


Cells expressing CFTR
Random library
binding
dissociation
PCR
denature
Purification of ssDNA
Last round cloning and sequencing
Further selection and enrichment
SELEX process-
Aptamers to the CFTR gene

6.3.3 Cloning of amplified aptamer sequences into pBCKS cloning vector

Aptamers were amplified in 5 x 100 µL PCR reactions using forward and reverse primers without the HEGL_A20 and FITC modifications. PCR reactions were pooled and purified using the Qiagen MinElute PCR purification kit (Catalogue #28004) as per manufacturer's instructions.

To allow cloning into pBCKS, restriction sites for SacI and BamHI were incorporated into the primers, and aptamers were restricted with these two enzymes. Restricted aptamers were run on a 10% native acrylamide gel, and stained with 1 mg/mL ethidium bromide. The gel was visualized under UV light, and a distinct band was present at approximately 88 bp. The band was cut from the gel, and DNA aptamers were extracted *via* incubation in 0.3 M sodium acetate at 80 °C for 45 minutes, and then precipitated with absolute ethanol.

Aptamers were ligated into pBCKS (as described in section 2.2.9) that had also been restricted with SacI and BamHI. Aptamer ligations were electroporated into DH10β cells (as described in section 2.2.3.1). Cultures of aptamers were harvested, plasmid DNA prepared and sequenced using the T7 reverse primer. Sequencing was carried out by the sequencing centre at the IMVS using the big dye terminator chemistry.

A flow chart of the aptamer cloning process is shown in Figure 6-2.

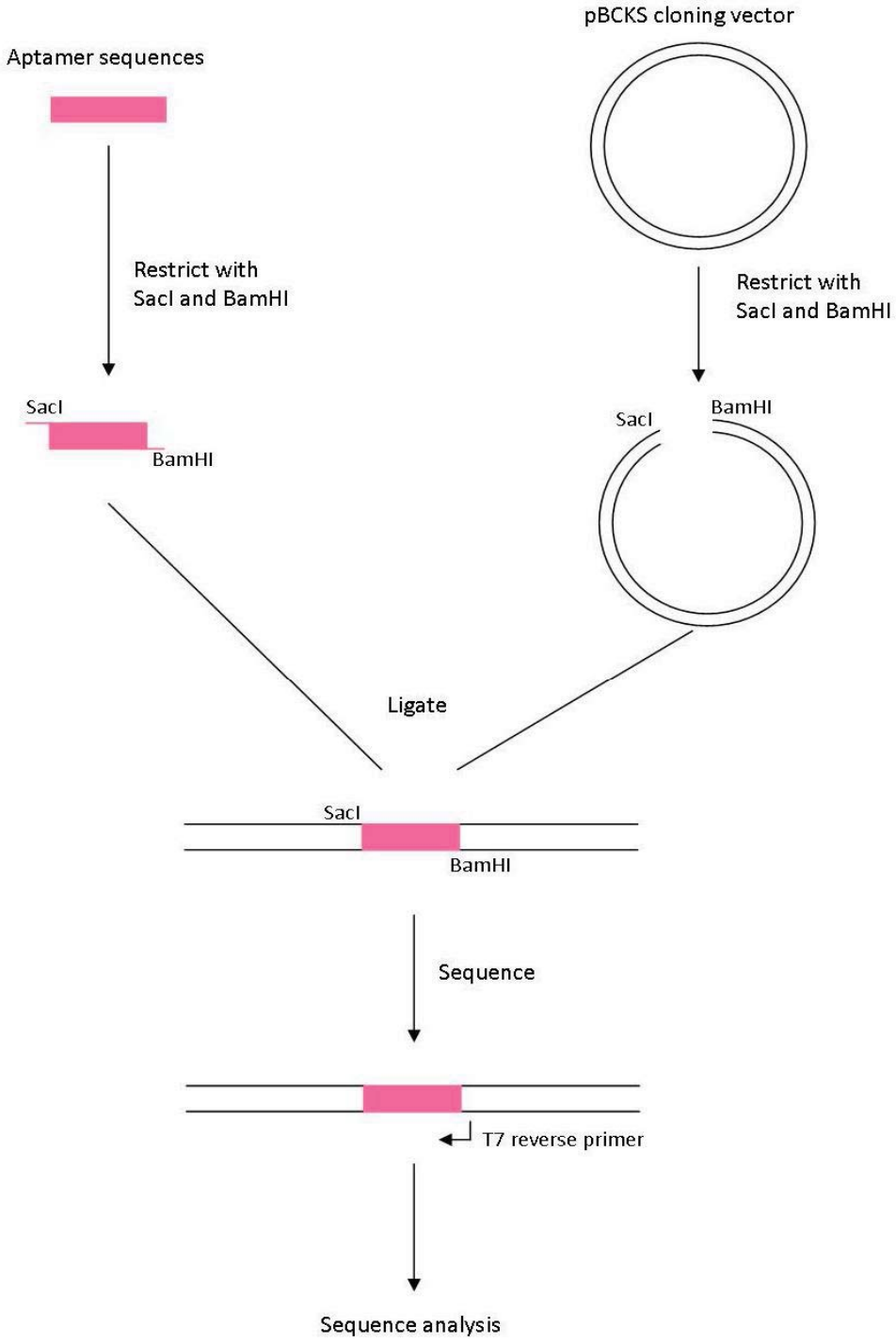


Figure 6-2: Flow chart of aptamer cloning into pBCKS cloning vector.

Analysis of the sequences was carried out by the construction of the reverse complement of each sequence. The sequence external to the SacI and BamHI restriction sites was deleted and the remaining sequence used for further analysis, as this represents the aptamer sequence itself.

This section of sequence was aligned with the aptamer forward primer and the reverse complement of the aptamer reverse primer sequences to show the primer sequences and the aptamer sequences between the two primer sites. Alignments of all sequenced aptamer clones are shown in Table 6-1, with no duplicate clones found. It was interesting to note that all aptamer sequences were found to be smaller than the original library. This could be due to the unique binding abilities of aptamers and the complex structure of the CFTR molecule, which allows small molecules to be generated to bind to specific regions of CFTR. This could also be due to an inherent bias in the amplification and selection procedure used.

Although there are segments of sequence that show similarities to each other, each has its own unique 3-dimensional structure. These structures were determined using the program RNAstructure version 5.0 © 1996-2009 Mathews Lab. The unique structures are shown inFigure 6-3.

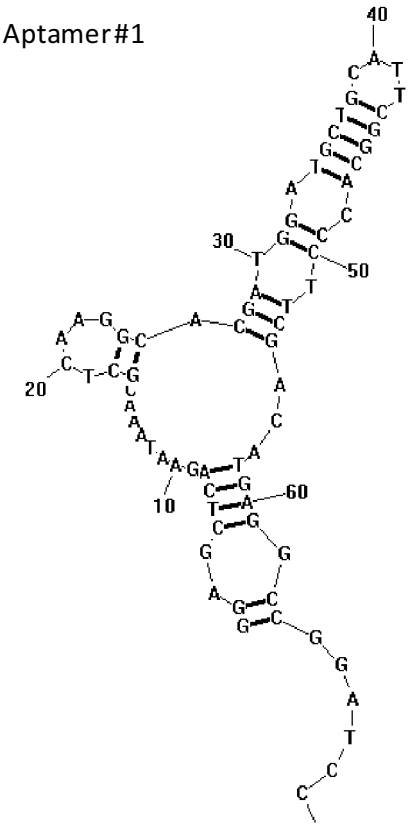
Table 6-1: Alignments of sequenced aptamers with forward and reverse primer sequences

Aptamer forward primer:	GGAGCTCAGAATAAACGCTCAA
Aptamer reverse primer rc:	GTTCGACATGAGGCCGGATCC
Aptamer #1 rc:	GGAGCTCAGAATAAACGCTCAAGGCACGATGGATGCTGCATTCGGCAC CCGTTCGACATGAGGCCGGATCC
Aptamer #2 rc:	GGAGCTCAGAATAAACGCTCAACGGGGCAGCAGACGTC GCGTG GTTCGACATGAGGCCGGATCC
Aptamer #3 rc:	GGAGCTCAGAATAAACGCTCAACGTGGACAAGTAGATGC TGTGTGG G CCGTTCGACATGAGGCCGGATCC
Aptamer #4 rc:	GGAGCTCAGAATAAACGCTCAAGGTGCACACACATCCTTA TGGCAGCACCTTGTTCGACATGAGGCCGGATCC
Aptamer #5 rc:	GGAGCTCAGAATAAACGCTCAACAGGCCATGCTCACACCG TGATGCTAC GTTCGACATGAGGCCGGATCC
Aptamer #6 rc:	GGAGCTCAGAATAAACGCTCAAGGGGGCATCCCGA TGTGG GTTCGACATGAGGCCGGATCC
Aptamer #7 rc:	GGAGCTCAGAATAAACGCTCAACACAGCGCACTACAACATCACTC GTTCGACATGAGGCCGGATCC
Aptamer #8 rc:	GGAGCTCAGAATAAACGCTCAAGCCCCAAGTTGACACATTATGAGCT GTTCGACATGAGGCCGGATCC
Aptamer #9 rc:	GGAGCTCAGAATAAACGCTCAACCCATGTACGGAGCCT TGGTGGTG TGGTTCGACATGAGGCCGGATCC
Aptamer #10 rc:	GGAGCTCAGAATAAACGCTCAATGTGCACATGC TGTCG GTTCGACATGAGGCCGGATCC
Aptamer #11 rc:	GGAGCTCAGAATAAACGCTCAATGCCGCTTGGAGTGGTCACGTGGC CCGTTCGACATGAGGCCGGATCC
Aptamer #12 rc:	GGAGCTCAGAATAAACGCTCAATGCGACTGGATGCTAGGTTGATCGTGC TTCGACATGAGGCCGGATCC
Aptamer #13 rc:	GGAGCTCAGAATAAACGCTCAACACAGCGCACTACAACATCACTC GTTCGACATGAGGCCGGATCC
Aptamer #14 rc:	GGAGCTCAGAATAAACGCTCAATGGGGGTGCATACCA TGCGTCGT GTTCGACATGAGGCCGGATCC
Aptamer #15 rc:	GGAGCTCAGAATAAACGCTCAACGTGGGCTGTGTCAA TGTCATGCA TGGTTCGACATGAGGCCGGATCC
Aptamer #16 rc:	GGAGCTCAGAATAAACGCTCAACCACAGGGCAGCATAAACAGAG TGGTTCGACATGAGGCCGGATCC
Aptamer #18 rc:	GGAGCTCAGAATAAACGCTCAATGTTGTGTACTGTGCTGGGGGTATG TGGTTCGACATGAGGCCGGATCC

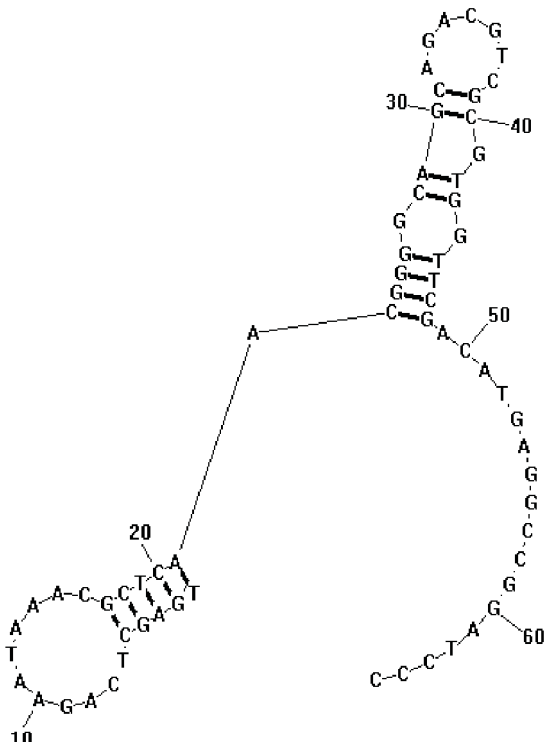
Figure 6-3: Structures of the CFTR aptamers isolated using the SELEX procedure

(see the following 5 pages for aptamer structures)

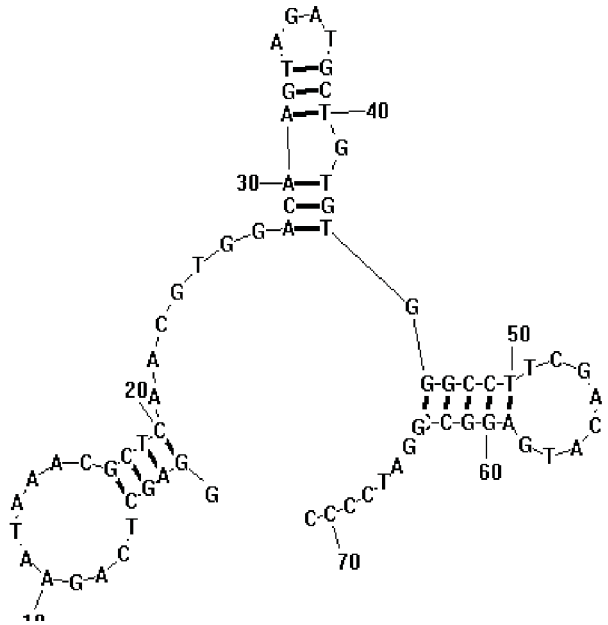
Aptamer#1



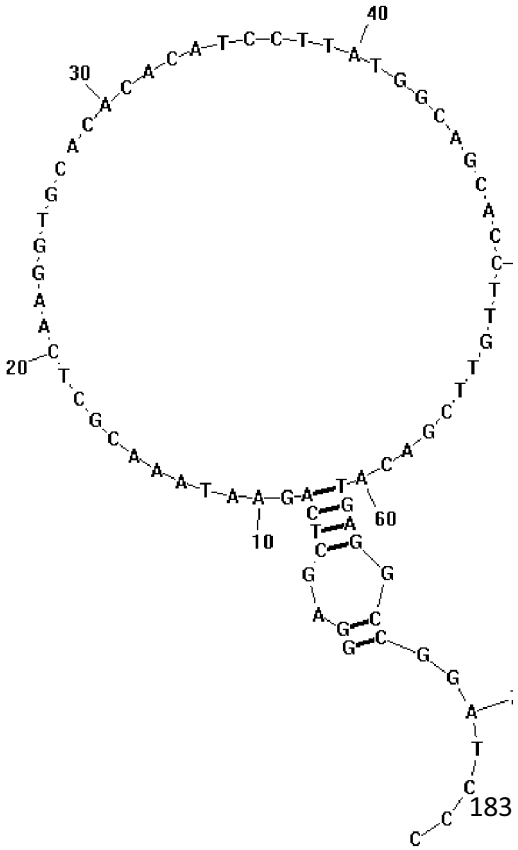
Aptamer#2



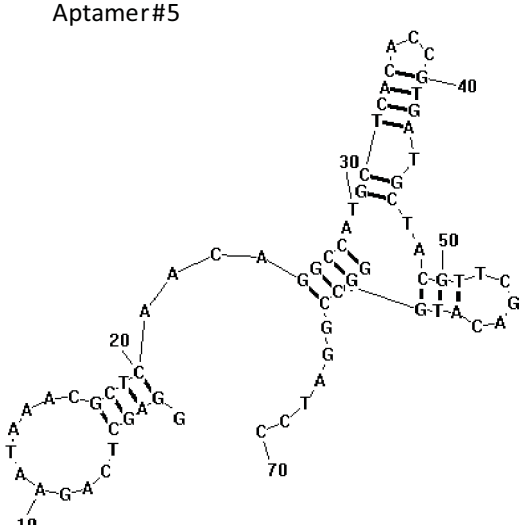
Aptamer#3



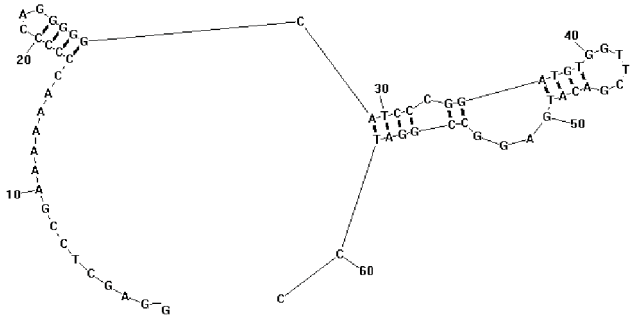
Aptamer#4



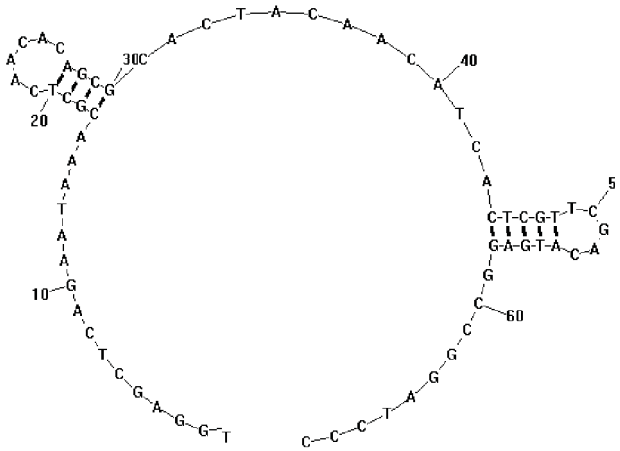
Aptamer #5



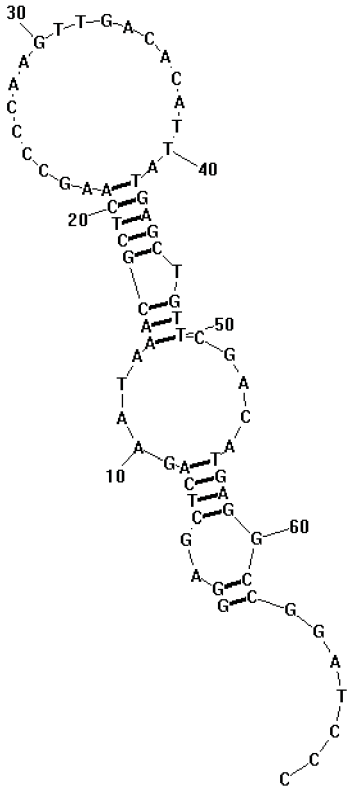
Aptamer #6



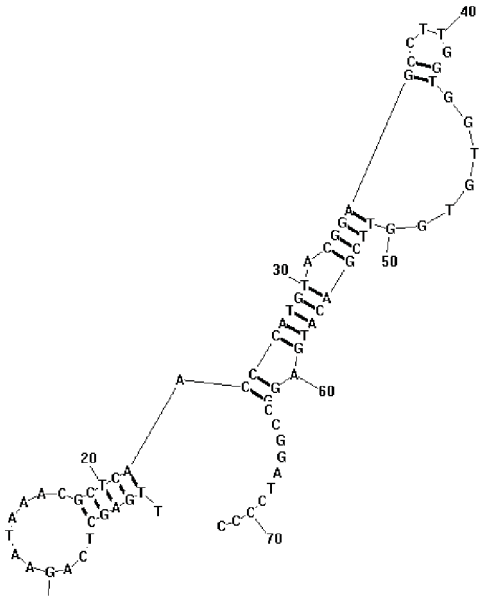
Aptamer #7



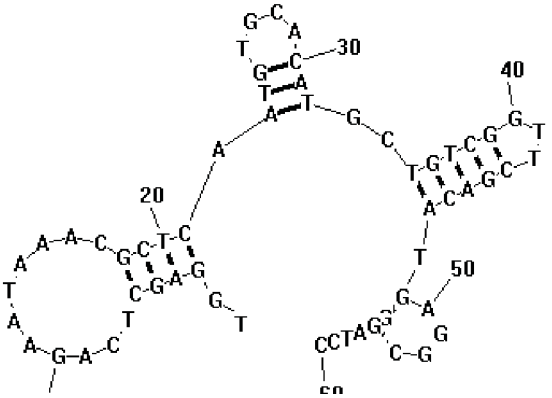
Aptamer #8



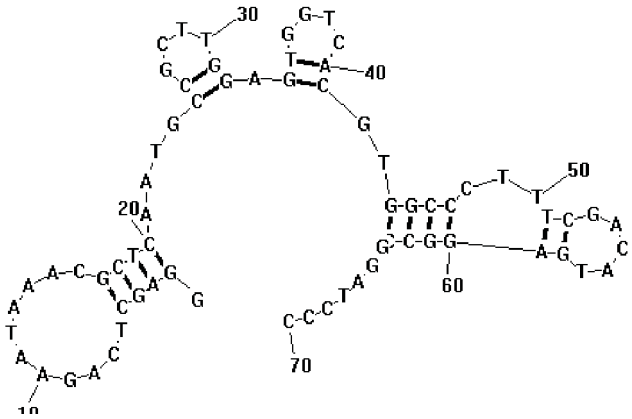
Aptamer#9



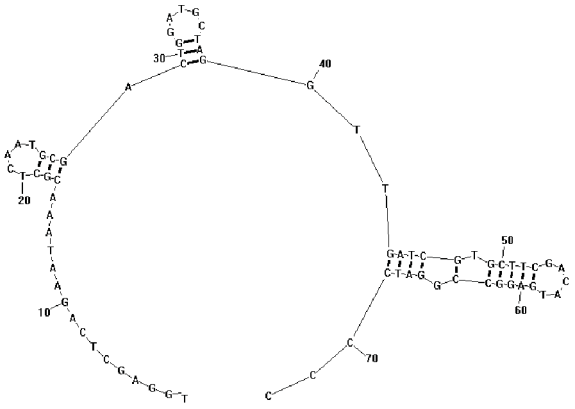
Aptamer#10



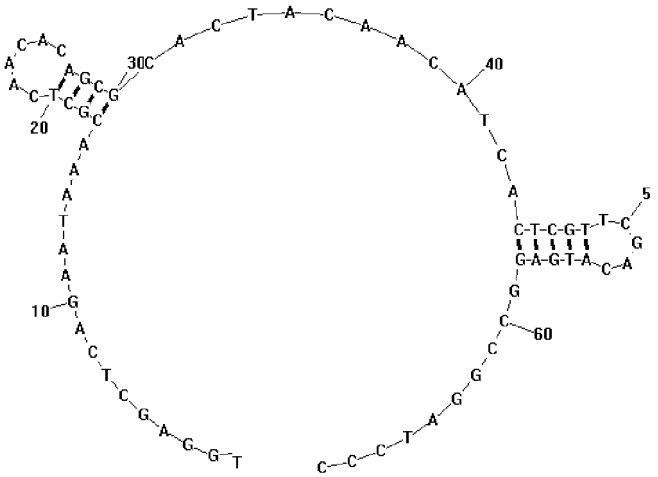
Aptamer#11



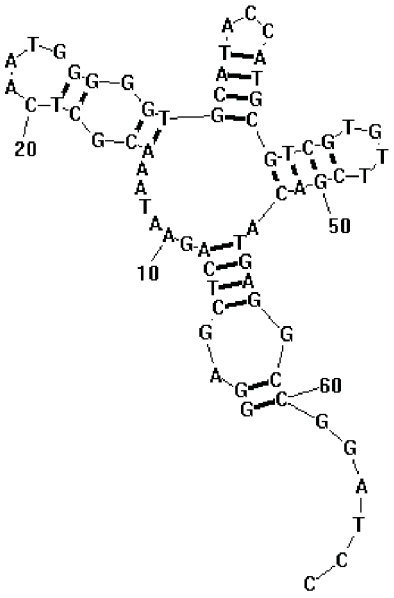
Aptamer#12



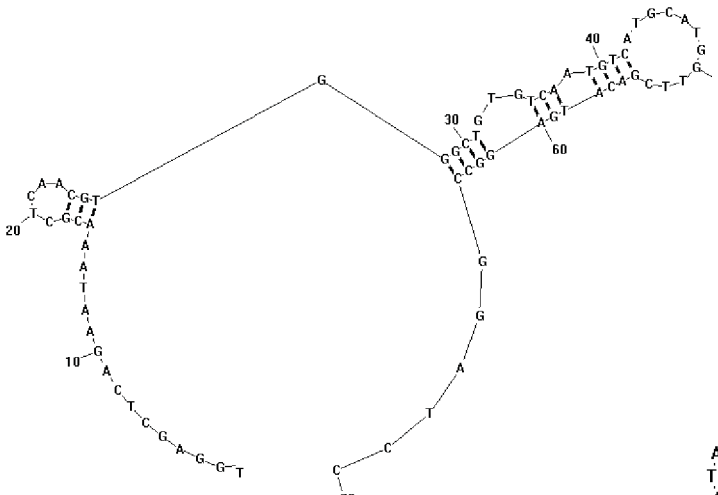
Aptamer#13



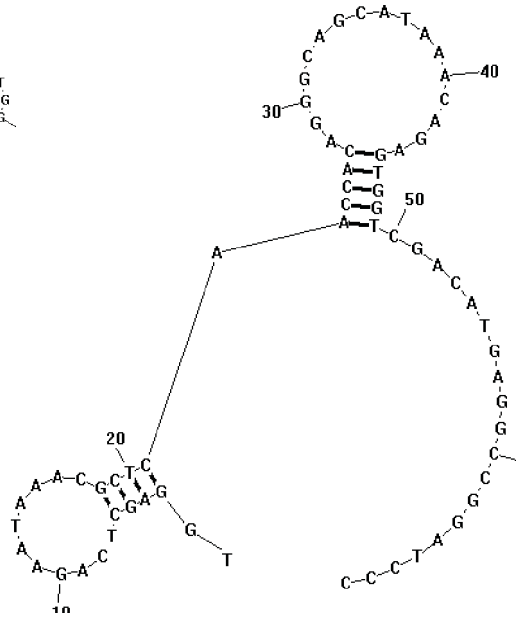
Aptamer#14



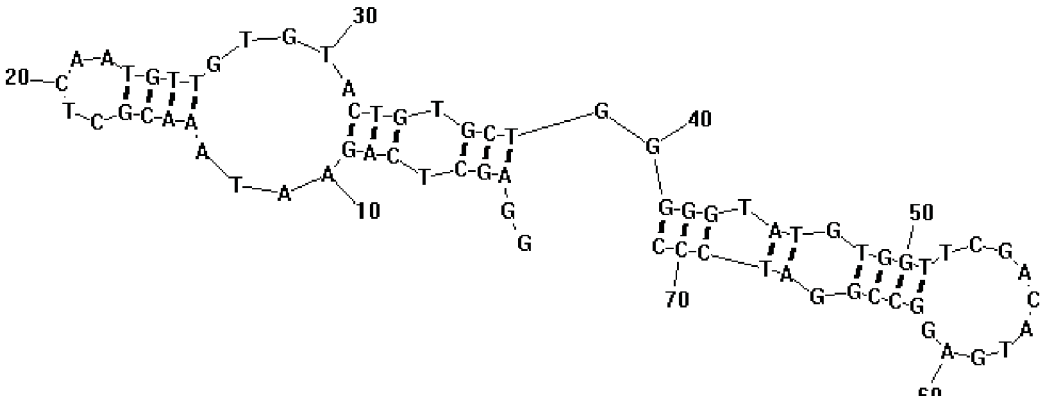
Aptamer#15



Aptamer#16



Aptamer #18



6.3.4 Screening and testing of aptamer isolates

Aptamer sequences were used to generate pure aptamer species with a 5' biotin label *via* PCR using the biotinylated Apt-For primer and the HEGL-A_{20}-Rev primer. Following PCR amplification, aptamers were heat denatured and run on a denaturing polyacrylamide gel and aptamers eluted from the gel with 0.3 M sodium acetate and precipitated with absolute ethanol. Precipitated aptamers were resuspended in a total volume of 20 µL 0.5x TE buffer. The concentrations of each purified aptamer were determined using 2 µL of sample, measured on a nano-spectrometer at absorbance 260 nm.

Table 6-2: Characteristics of purified isolated aptamers

Aptamer clone	Concentration ng/mL	OD 260	OD 260/280	OD 260/230
1	2.6	0.052	0.94	0.40
2	33.5	0.67	1	0.84
3	35.5	0.71	1.044	0.70
4	33	0.66	1.031	0.65
5	29.5	0.59	1	0.69
6	31.5	0.63	1	0.84
7	35	1.0	1.045	0.67
8	18.5	0.37	1.028	0.62
9	6.6	0.132	1	0.50
10	25	0.5	1.042	0.64
11	15.7	0.314	1.043	0.33
12	23.5	0.47	1.044	0.37
13	21	0.42	1	0.43
14	30	0.6	1.017	0.39
15	29	0.58	1	0.50
16	20.5	0.41	1.051	0.46
17	41	膀.82	0.988	0.76
18	18.5	0.37	0.974	0.52

Aptamers were not screened for their binding abilities to the CFTR gene in time for inclusion into this thesis. However, subsequent assessment did not identify any aptamer that recognised NIH3T3 cells transduced with a lentiviral vector expressing CFTR (DS Anson, personal communication).

6.4 Discussion and Conclusions

Aptamers are a solution to many of the problems associated with the use of antibodies, in terms of their specificity, sensitivity and raising antibodies to non- immunogenic and toxic targets as they negate the need for animals. As described earlier, a drawback of antibodies is the technology is only well established in the mouse, which makes the development of reagents for murine proteins difficult. Traditional monoclonal antibody technology requires the use of large numbers of animals and the process can be slow and laborious.

As outlined in this chapter, the attempted development of aptamers to target the CFTR gene was carried out. The process of developing and isolating aptamers is advantageous as their short DNA sequences (50-100 base pairs) bind with high affinity and specificity to their target as they adopt a specific three-dimensional structure [200] [201].

The use of the SELEX process of binding, dissociation, expansion, denaturation, purification and further selection and enrichment to develop aptamers to the CFTR gene was described in this chapter. Twelve stages of selection and enrichment were undertaken to obtain 18 different aptamer clones shown in Figure 6-3.

Problems that arose during the aptamer selection process were most prominently the inability to monitor selection by measuring the post-binding fluorescence. This meant there was not a measure to

indicate when the selection process was successful or when it had been completed. A number of 12 selection rounds was chosen as this appeared to be a common number of rounds in the literature.

Another problem faced was the inability to show the selection cell line expressed CFTR. This was due to the lack of a suitable antibody to detect the expressed CFTR. Real time PCR was carried out on cell lysates to confirm the presence of the CFTR transgene, however, it is not known whether the cells expressed the CFTR which was used for aptamer selection.

It is interesting to consider whether smaller aptamer sequences were inadvertently selected for *via* gel elution in the selection process, as evidenced by all aptamers being found to be smaller than the aptamer selection library.

It is also unfortunate that aptamer clones were not able to be tested for their binding abilities to CFTR in CHOK-1 cells for inclusion in this thesis. Testing was carried out by DS Anson on NIH3T3 cells as transduced CHOK-1 cells were unavailable at the time of testing. Given the negative result, no real conclusion can be drawn and testing of these aptamers on transduced CHOK-1 cells should be carried out. Testing of the aptamers was carried out by applying 100 ng of the aptamer clone to a CHOK-1 cell line expressing CFTR. The aptamers were fluorescently labeled using a biotinylated forward primer, and streptavidin-FITC to amplify the signal, and tested for histochemical detection of CFTR. Initially this was carried out on cell lines expressing CFTR, and clones showing positive results (fluorescence *via* binding of the aptamer to the CFTR expressing cells) will then be used to detect CFTR in sections from experimental animals, in similar methods to those described in chapter 4 with the 596 CFTR monoclonal antibody.

There is a need for development of new aptamers beyond the therapeutic market. Assays and analytical systems for medical diagnostics offer a wide range of applications. Some advantages of

aptamers in comparison to antibodies, like the chemical synthesis of aptamers, their easy chemical modification without loss of function and their easier immobilization compared to antibody immobilization should bring the replacement of antibody assays by aptamer based assays.

Aptamers appear to be a highly useful and required technology for the detection and visualization of target molecules in diseases such as ocular vascular disease and lysosomal storage diseases, and for use in CF gene therapy as an alternative to antibodies to the CFTR gene.

7 Development of Real Time PCR for determination of virus titre

7.1 Aim

The determination of virus titre can be carried out by a number of techniques dependent on the viral transgene. FACScan analysis can only be used to determine viral titre for virus expressing any fluorescent protein and β-galactosidase staining can only be carried out on virus expressing the LacZ gene. For virus expressing a therapeutic gene such as CFTR, Real Time PCR is one assay to determine viral titre that is of general applicability. The real time PCR can be designed to assay either a vector sequence, giving an assay that can be used on all vectors, or a vector specific sequence that can be used to titre a given vector if more than one vector is present.

A Real time PCR based on TaqMan® chemistry was developed to titre all viruses independent of their transgene. TaqMan® chemistry utilizes a fluorogenic probe in addition to the standard PCR primers. The probe contains a fluorogenic molecule at one end and a non-fluorescent quencher, which quenches the signal of the fluorogenic molecule at the other. The probe binds in between the primers and through the action of the polymerase; it is digested during the synthesis of the PCR product releasing the fluorogenic molecule allowing it to fluoresce. The advantage of Taqman ® chemistry is the specific hybridization between the probe and target that is required to generate a fluorescent signal, reducing the background and false positives. To apply this technology to the determination of virus titre, two assays are required, one to measure the amount of virus sequence and another that corrects for the number of cell equivalents of genomic DNA in the assay sample. An assay to determine viral titre in NIH3T3 (mouse embryonic fibroblast cell line) and A549 (carcinomic human alveolar basal cells) cell lines was developed in our laboratory, with the use of a vector sequence corresponding to the start of the *gag* coding sequence and a single copy gene chosen to correct for the number of cell equivalents per reaction. The gene chosen was transferrin, as both the human and mouse transferrin genes contain sequences that are unique within their respective genomes.

This assay can be used to detect the gag sequence from our virus in NIH3T3 and A549 cell lines. Titering a virus containing the LacZ gene has traditionally been done *via* a staining assay (outlined in section 2.3.5.1) in a CHOK-1 cell line (Chinese hamster ovary cells). To determine if the titre calculated *via* this staining assay was comparable to the titres achieved by RT-PCR, a RT-PCR assay was developed to assess the vector in CHOK-1 cells.

7.2 Development of a CHOK-1 Real Time PCR assay

As the same assay for the vector sequence (gag) can be utilized for this assay also, the gene chosen to correct for the number of cell equivalents per reaction was the partial slc35a1 gene for CMP-sialic acid (Accession number: AJ810300). A region of unique sequence was identified by BLAST searches and TaqMan® Gene Expression Assays developed. The assays were developed by Applied Biosystems. Sequences for the desired targets were chosen and given to Applied Biosystems, where algorithms utilizing heuristic design rules generated primers and probe sequences with matched melting temperatures, accounting for binding contribution of the dyes, MGB and specific di-nucleotide pairs. Additional rules included the minimization of the effect of base runs, reduction of secondary structure formation and primer-dimer formation. The sequences of the probe and primer concentrations are shown below, Table 7-1.

In order to determine the copy number per cell, a standard of known copy number is required. To obtain a standard of known copy number, CHOK-1 cells were infected with low amounts of virus carrying the neomycin resistance gene. The resulting clones were selected by culture in Hams F12/10% FCS supplemented with 1 mg/mL active G418. When distinct colonies had formed, they were isolated and transferred to a well of a 96 well plate. Once confluent, the cells were expanded and gDNA was prepared using the Qiagen Blood and Cell Culture gDNA Midi Kit, following the frozen cells protocol. The copy number of vector in each clone was confirmed by Southern blot analysis.

Table 7-1: Probe and Primer sequences for Real time PCR assays designed by Applied Biosystems

All sequences written 5' → 3'. NFQ = Non-Fluorescent Quencher

Gene	3' primer sequence	5' primer sequence	Probe sequence
CHO-CMP-sialic acid	CTATGTAATGTTGTTCTTGTTGACTTGCT	CTACTACATCAGACACAGTTACTACTAACATTAGA	FAM-TCCCAGAGAAAATTTAC-NFQ
NLS-LacZ	GCCACTTCTTGATGGACCACTT	CCGCCACCGACATCATCT	FAM-CACGCGGGCGTACAT-NFQ
Luciferase	TTGTCGATGAGAGTGCTCTTAGC	GCGCAGCTTGCAAGACTATAAG	FAM-CTGGTGCCCACACTAT-NFQ
CFTR	CCTTTAGAGAGAAGGCTGTCCTT	CGCTGATGCGAGGCAGTAT	FAM- CCCTGCTCAGAATCT-NFQ

The standards created were used to determine the reaction efficiencies of the assays. Genomic DNA was diluted in 10 fold serial dilutions until a 1:1,000,000 fold dilution was reached. Five microlitres of each dilution was assayed as outlined in section 2.3.5.2.

From this the reaction efficiency- the slope of the standard curve- and the R_2 values were determined using the Applied Biosystems 7300 system software. The R_2 values for gag and CHOK-1 slc35a1 were 0.998 and 0.999 respectively. The reaction efficiency of gag and CHOK-1 were -3.746 and -3.782. As the reaction efficiencies of both assays were within 10% of each other, the ΔCT method was applied. This method requires only one dilution of standard to determine the copy number of the gene of interest in a sample. Once standards and samples have been run and the CT is determined, the following formulas can be applied to determine viral titre.

ΔCT sample = Ct gag – Ct CHOK-1

$\Delta\Delta$CT = ΔCT of sample – average ΔCT of standards

Copy number per cell = $1/2^{\Delta\Delta CT}$

$$\text{Titre} = \frac{\text{copy number per cell x number of cells plated initially}}{\text{Volume virus assayed (µL)}}$$

To validate the CHOK-1 Real Time PCR assay, a sample of concentrated LV-MPSvNLSLacZ virus (0.05 µL) was assayed on CHOK-1 cells, with titre determined by LacZ staining assay and Real Time PCR. The results showed titreing LacZ virus using the staining method appeared to underestimate virus titre by 49-fold (Figure 7-1). This underestimation of titre by cell counting is not unexpected due to the human error involved in counting LacZ positive cells in culture, however, a 49-fold difference is too much to be solely explained by the human error involved in counting the transduced cells. A possible explaination is that the stained cells do not express the transgene at high enough levels to be counted. It is unlikely that the RT-PCR result is inaccurate as the cells were grown in culture for 4 weeks to remove possible plasmid DNA contamination. The copy numbers (shown in Table 7-2) are less than 1 copy per cell,

which means the RT-PCR titre is not an underestimate of actual titre, as, if the copy number was high, doubly infected cells would only be accounted for once.

When comparing titres of the same virus carried out by Real Time PCR on two different cell lines, the results were much tighter, with the A549 cells giving a 2.5-fold higher titre than that calculated using the CHOK-1 cell Real Time PCR assay (See Figure 7-2). This result can be explained by the different infectibilities of the two different cell lines utilized here, with A549 showing marginally higher infectibility than the CHOK-1 cells.

Table 7-2: Copy number per cell of LacZ transcripts in transduced CHOK-1 cells

Virus	Volume assayed	Copy number/cell
LV-MPSvNLSLacZ	0.5 µL	0.5176
LV-MPSvNLSLacZ	0.5 µL	0.4829
LV-MPSvNLSLacZ	0.5 µL	0.4413

Figure 7-1: Comparison of LacZ titre determined by staining assay and CHOK-1 Real Time PCR (n=3)

CHOK-1 cells were transduced with LacZ expressing virus and the transducing units/ mL were determined by both LacZ staining assay and Real Time PCR. Error bars represent standard error.

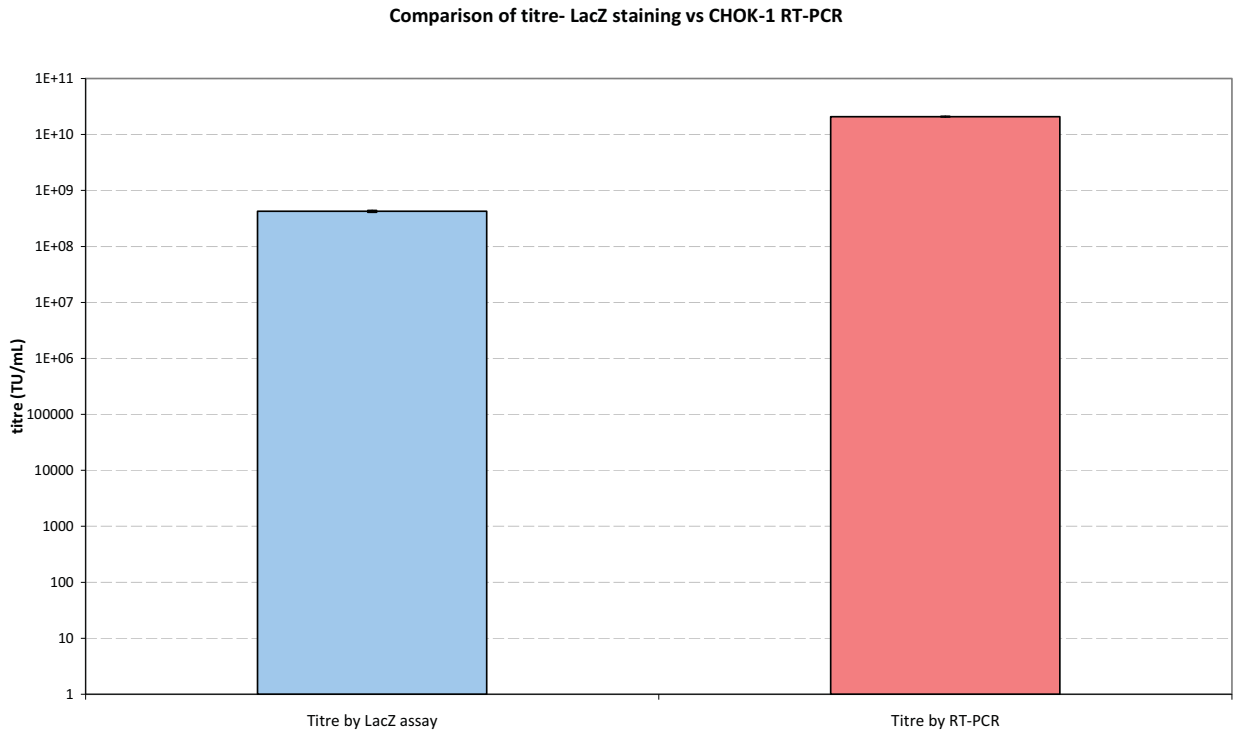
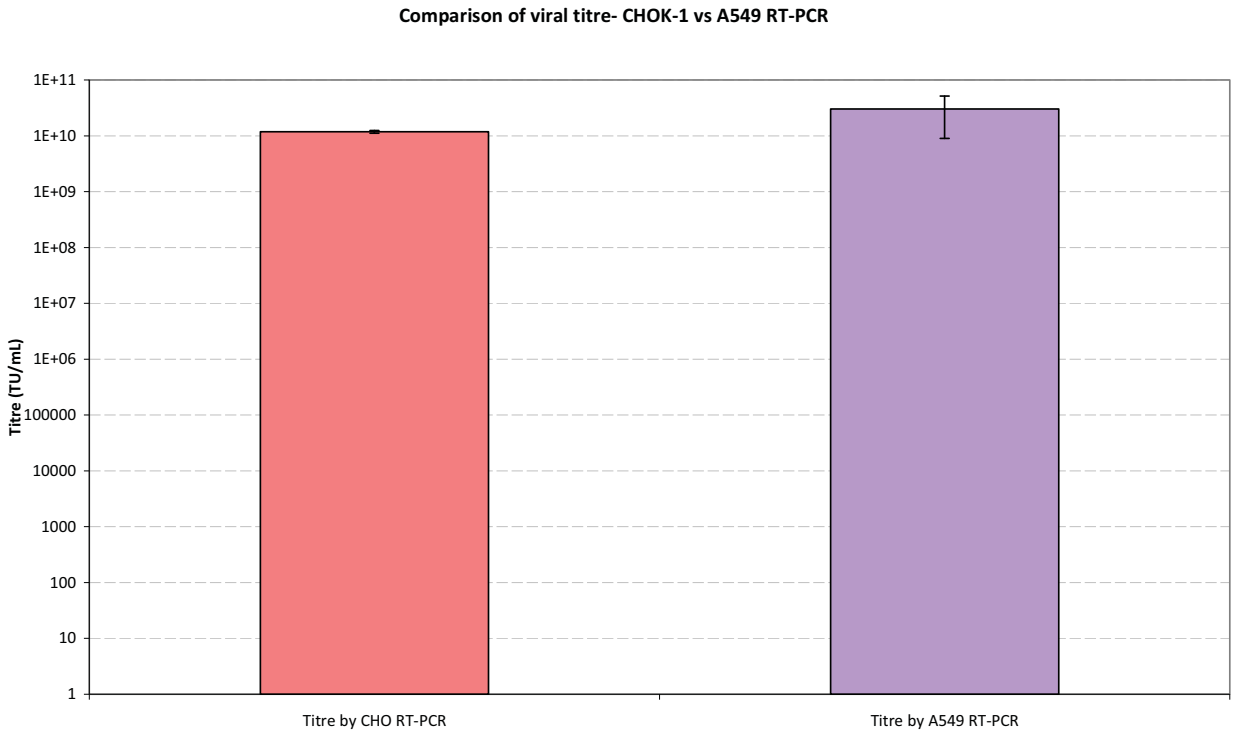

Comparison of titre- LacZ staining vs CHOK-1 RT-PCR
titre (TU/mL)
1E+11
1E+10
1E+09
1E+08
1E+07
1E+06
100000
10000
1000
100
10
1
Titre by LacZ assay
Titre by RT-PCR

Figure 7-2: Comparison of titering virus using CHOK-1 and A549 Real Time PCR (n=3)

CHOK-1 and A549 cells were transduced with the same volumes of lentivirus.

Titre was determined on both CHOK-1 and A549 cell lines using Real Time PCR.


Comparison of viral titre- CHOK-1 vs A549 RT-PCR
Titre (TU/mL)
1E+11
1E+10
1E+09
1E+08
1E+07
1E+06
100000
10000
1000
100
10
1
Titre by CHO RT-PCR
Titre by A549 RT-PCR

7.3 CFTR, Luciferase and LacZ Real Time PCR assays

Real Time PCR assays were also designed by Applied Biosystems to the CFTR, Luciferase and the nuclear localized LacZ genes used in our vector systems. The primers and probe sequences are shown in Table 7-1.

The premise behind designing assays to these transgenes is for titreing virus such as that used in section 8, where multiple transgenes are used. This virus was made as per the method outlined in section 2.3.4, however, when the transfection was carried out, either two (Luciferase and LacZ) or three (CFTR, Luciferase and LacZ) vectors were transfected into the cells in varied proportions. As all virus made, whether it contains LacZ, Luciferase or CFTR, will be detected by our gag reaction as it recognises a unique sequence in our vector, to distinguish between each transgene, an alternative assay was required. The assays developed for these three transgenes can be used in the same way the gag reaction is used, with a CT value determined for each reaction, and the titre for each different virus can be determined using the standard formula shown above. The titre results of virus containing multiple transgenes can be found in section 8 promoters section.

7.4 Discussion and Conclusions

The Real Time PCR assays developed here have allowed for more sophisticated virus preparations to be made, containing mix of different viruses that carry different transgenes, that within a single PCR assay can each be detected to obtain the titre of the three transgenes of interest- CFTR, LacZ and Luciferase.

The development of a Real Time PCR assay in CHOK-1 cells, has also allowed us to obtain a comparison for titering LacZ viruses via real time PCR and the previously used staining method in the same cell line. It was thought that the real time PCR method may be more accurate and eliminate the error involved

in manually counting blue cells, however, the 49-fold difference in titre is greater than would be expected by human error in counting the assay. The error involved in counting may account for a portion of the 49-fold increase, it is more likely that the assay itself does not express the LacZ transgene at a level where it can be visualized.

With the development of real time assays to all of the transgenes used- LacZ, Luciferase and CFTR, the titreing of viruses can now be standardized across all the different types of viruses made- whether they contain marker genes (Luciferase/LacZ) or therapeutic genes- CFTR, and all assays can be carried out in the same cell line, as there is a difference in the infectibility of different cell lines. The creation of these assays also allows for the development of more sophisticated virus preparations to be made, with titres for each individual transgene able to be measured in the one RT-PCR assay.

8 Assessment of the level of gene expression on the phenotypic correction of the CFTR defect

Chapter 8 - Assessment of the level of gene expression on the phenotypic correction of the CFTR defect

8.1 Aim

Experiments outlined in this chapter were designed to assess the effect of the level of gene expression of CFTR on phenotypic correction of the CFTR defect.

8.2 Effect of promoter on gene expression

Cystic fibrosis, as described previously, is the most common autosomal recessive disorder affecting Caucasian populations. However, the level of gene transduction and the optimal level of gene expression needed to correct or reduce disease severity and phenotype *in vivo* remains unknown. It is unclear what proportion of epithelial cells would be required to be transduced with a therapeutic gene to obtain a clinically beneficial correction of the underlying electrophysiological defect in CF. It has been suggested that as little as 5% of normal CFTR levels significantly ameliorates the severity of lung disease in patients [204]. It is also not certain that over expression of CFTR will compensate for inefficient transduction. *In vitro* experiments showed over expression of CFTR in normal epithelial cells does not increase the level of chloride transport in the epithelium [59].

Early studies show the endogenous CFTR promoter to be weak, producing approximately 1-2 transcripts per cell [204]. Even less CFTR expression may be sufficient for normal function, because studies of humans with splice site variations suggest as little as 8% of normal transcripts preserve normal lung function [205]. Similarly, mouse intestine expressing 5% of normal CFTR levels showed no intestinal disease and retained approximately 50% of normal Cl^- transport [206]. In all cases, the endogenous CFTR promoter drove expression in most if not all of the epithelial cells. In contrast, gene transfer vectors express CFTR in a smaller fraction of cells, governed by the transduction efficiency of the vector.

This raises considerations of what promoter strength might be required in a gene transfer vector that targets <100% of airway cells and whether CFTR over expression might have unexpected consequences.

Previous reports have focussed on the percentage of cells expressing CFTR that were required to correct the CF Cl^- transport defect *in vitro* [60]. That study used a Moloney murine leukemia virus retroviral vector that used the strong long terminal repeat (LTR) promoter to stably express CFTR in a CF epithelial cell line. As few as 6-10% of vector corrected cells was sufficient to achieve Cl^- transport at levels close to those seen in totally corrected cell lines. Previous studies suggested the MoMLV promoter generates expression at a level comparable to that of the cytomegalovirus (CMV) promoter [207-209]. CMV drives CFTR expression at very high levels in airway epithelia [210-212]. The percentage of cells required to repair Cl- transport with a weaker promoter driving CFTR is unknown.

In a later in vitro study, gene correction in 25% of cells was sufficient to restore mucus transport to normal, again a vector that expressed CFTR at supranormal levels was used [61] but again the effect of varying the level of CFTR expression was not assessed.

Many gene transfer vectors have utilised viral promoters because they generate high level expression in numerous different cell types. There is some evidence to suggest that over expression of CFTR may have a deleterious effect, as over expression of CFTR in cultured cell lines reduced the rate of cell proliferation in NIH-3T3 fibroblasts, a monkey kidney cell line and in a CF bronchial epithelial cell line [213-215]. However, *in vivo*, over expression of the human CFTR in the gastrointestinal organs by the fatty acid binding promoter [198] has no apparent adverse effect.

Studies have been carried out previously *in vivo* using lentivirus expressing LacZ under the control of a promoter that drives a high level of ubiquitous expression- the elongation factor 1 α gene promoter

(EF1α), a promoter that induces low level ubiquitous expression- phosphoglycerate kinase gene promoter (pgk), and the cytokeratin 18 gene promoter (K18) which is reported to drive a low level, ciliated airway epithelial cell specific pattern of expression which mimics the endogenous expression of CFTR [216]. These studies showed EF1α to give the highest level of LacZ expression, followed by pgk and then K18, as would be expected by the description of the promoters. However, these studies utilising LacZ only provide a qualitative indication of relative promoter strength. Interestingly, these studies also showed that in the context of the lentiviral vector used in this study, the K18 promoter was not restricted to ciliated cells, as previously described when delivered as plasmid DNA to a human CF bronchial cell line [216], but was expressed in both ciliated and non-ciliated epithelial cells in the same ratio as with the two other promoters.

8.3 Assessing relative promoter activity in vivo

To provide a quantitative measure of promoter strength the Luciferase transgene was utilised.

Four groups of mice (n=4) were instilled using the inhalation driven method of LPC/LV as described in section 2.4.1 with 4 µL 0.3% LPC followed by 20 µL of saline (control group) or LV expressing Luciferase under control of 3 different promoters- EF1α, pgk and K18. The Luciferase marker gene was used to analyse the promoter strength. A second LV expressing the LacZ gene under the control of the MPSV promoter was also included in the virus (≈20% of transfected transgene was the LacZ gene, and ≈80% was Luciferase) to analyse tissues post-mortem for cell type analysis, and give a quantitative measure of the rate of transduction, allowing the Luciferase results to be normalised for transduction efficiency.

Mice instilled with the Luciferase constructs were imaged one week post instillation using the Xenogen IVIS 1000 system as described in 2.4.2.5.

Immediately following imaging, mice were sacrificed and stained for LacZ expression as described in section 2.4.2. Cell counts were carried out on cut sections from all mice. The cell counts were found to correspond with the *in vitro* LacZ virus titre of each virus preparation. The mice treated with LV-K18Luciferase/LacZ had the highest LacZ titre of 5.75 x 10^8 TU/mL (as assayed on A549 cells) and also showed the highest LacZ cell count average of 121 LacZ positive cells. The pgk group had a LacZ titre of 3.77 x 10^8 TU/mL and had the second highest average cell count of 95.25 LacZ positive cells. The LV-EF1αLuciferase/LacZ group had the lowest LacZ titre out of all the virus preparations made at 2.86 x 10^8 TU/mL. However, this group of mice showed no LacZ positive cells. It remains unknown why this group showed no LacZ cells at all and this result means that the Luciferase results for the EF1αLuciferase/LacZ group cannot be normalised, which makes it difficult to give a definitive result for the relative strength of this promoter in the nasal airway epithelium. However, to provide an alternative analysis, the Luciferase data was also normalised to the relevant *in vitro* Luciferase titres. This confirms the relative strengths of the 3-promoters, and suggests that EF1α is the strongest followed by pgk and K18. The ratios of each promoter shown under Table 8-1 suggest that normalising Luciferase results to Luciferase titre may be a valid method of analysis as the values for pgk:K18 agree closely with those obtained after normalising to LacZ. In addition, the LacZ data from the pgk and K18 groups confirms that transduction was limited to the respiratory and transitional epithelia.

Figure 8-1 a and b show the raw Luciferase data and Luciferase data normalised for LacZ for the pgk and K18 treated groups. After normalisation for LacZ titre and transduction efficiency the difference the Luciferase results for the K18 and pgk groups is accentuated, increasing from 1.59-fold to 2.62-fold. However, significance does not alter before and after normalisation to LacZ even though a bigger difference between the two groups is seen, as large standard deviations are observed. A scatter plot for both pgk and K18 promoter groups (see Figure 8-1 c and d) shows low R^2 values for each promoter, indicating a low level of correlation between the LacZ and Luciferase results. The pgk group was similar, with the highest Luciferase response also showing the largest LacZ response.

The low R^2 values undermine the basis of the experiment somewhat. Reasons for such low R^2 values may be due to the LacZ data not sampling enough tissue to be representative. This may be true given that some of the LacZ cell counts are very low or zero. A change that could be made to adjust for this is to increase the proportion of LacZ plasmid in the virus preparation mixture. Another method to compensate for this may be to cut and assess more sections to gain a more representative sample- closer to what was measured with the Luciferase imaging. There may also be something affecting one of the transgenes post-transduction in a variable way, which could be the beginnings of an immune response, however unlikely this may be only one week post-transduction. The data most likely indicates an increase in 'n-value' to gain a significant and clearer outcome.

Mice treated with Luciferase have been shown to exhibit an immune response to Luciferase (P. Cmielewski, personal communication), with expression decreasing over time, correlating to the increasing level of Luciferase antibodies. This experiment should be repeated with assessment of immune response to the two transgenes (Luciferase and LacZ), so that mice who exhibit an immune response can be excluded from the study. It is unlikely however for an immune response to be present at just one week post treatment, but this additional step may add more insight into the issues involved in the variability in the gene transfer process.

Table 8-1: Determination of normalised Luciferase data

Luciferase construct	Luciferase (Photons/Sec)	LacZ cell count	Ratio Luc to LacZ titre	Normalised Luciferase	Average Norm Luciferase	Luciferase normalised to Luc titre	Average Norm to titre
EF1α	1539000	0	1.65	-		326059	
EF1α	6376500	0	1.65	-		1350953	
EF1α	2204100	0	1.65	-		466970	
EF1α	3297700	0	1.65	-	-	698665	710662
pgk	106800	178	0.326	2598		86829	
pgk	458500	77	0.326	2578		372764	
pgk	195130	121	0.326	698		158642	
pgk	340110	5	0.326	29454	8832	276512	22368
K18	442210	169	1.66	4213		46450	
K18	281290	0	1.66	0		29547	
K18	245290	163	1.66	2423		25765	
K18	326220	152	1.66	3455	3364	34266	34007

Ratio of pgk:K18 (normalised to LacZ count) = 6.63

Ratio of EF1α:pgk (normalised to Luciferase titre) = 3.17

Ratio of EF1α:K18 (normalised to Luciferase titre) = 20.89

Ratio of pgk:K18 (normalised to Luciferase titre) = 6.577

Figure 8-1: Luciferase gene transfer in normal mice treated with LV-Luciferase under control of the three test promoters EF1α, pgk and K18

a) Raw Luciferase measurements for each promoter group measured in photons/second, ANOVA p=0.367, Mean +/- SE

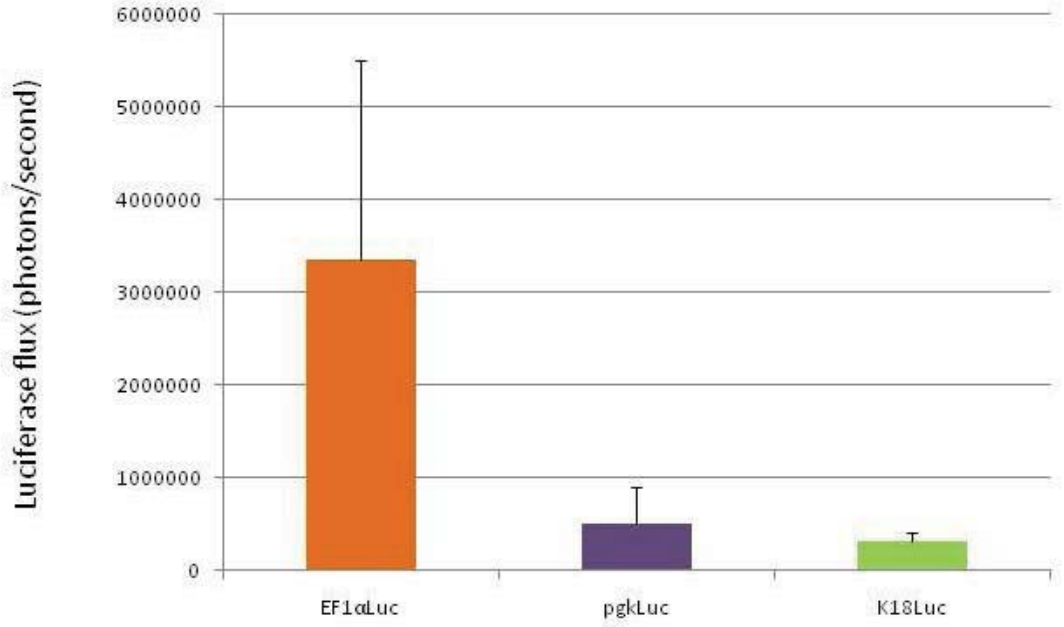
b) Luciferase data normalised for LacZ and *in vitro* titre for pgk and K18-Luciferase treated mice, t-test p=0.857, Mean +/- SE

c) Scatter plot of Luciferase data compared to LacZ result for each individual mouse in the pgkLuciferase/MPSVLacZ group, R^2= 0.2325

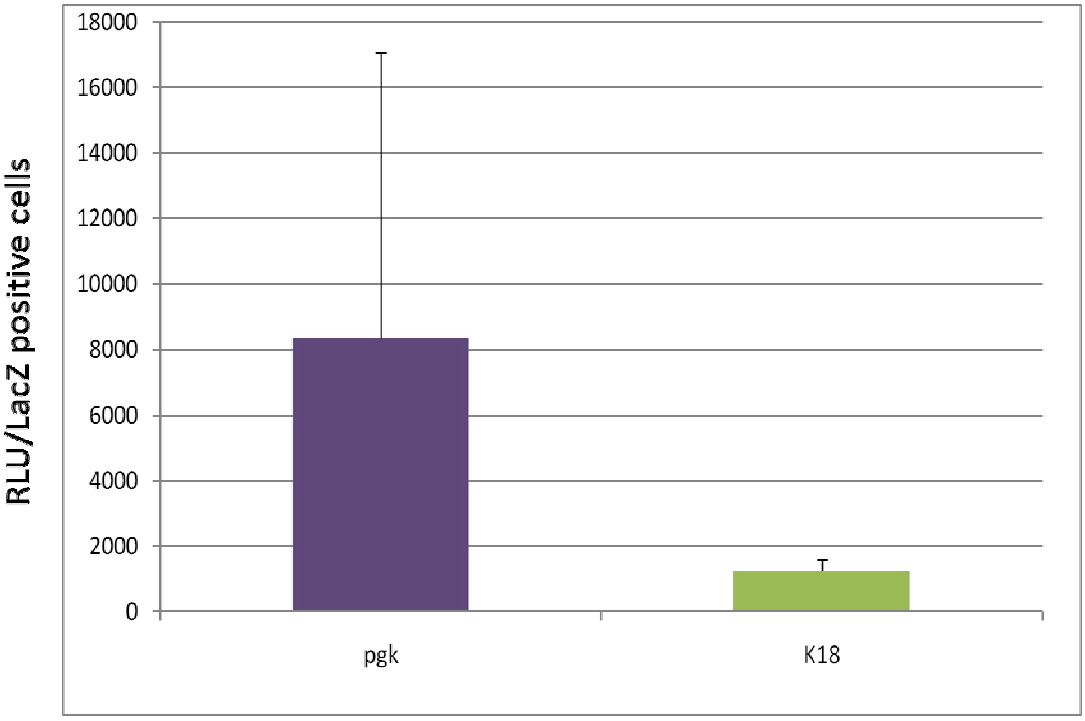
d) Scatter plot of Luciferase data compared to LacZ result for each individual mouse in the K18Luciferase/MPSVLacZ group, R^2= 0.1337

Chapter 8 - Assessment of the level of gene expression on the phenotypic correction of the CFTR defect

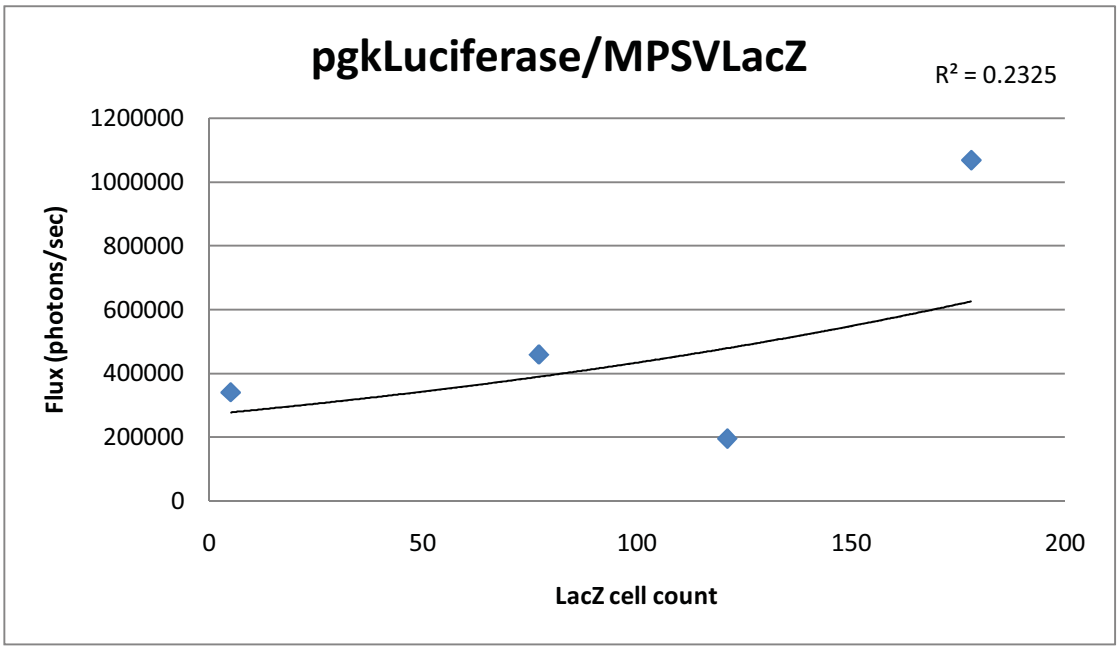
a)



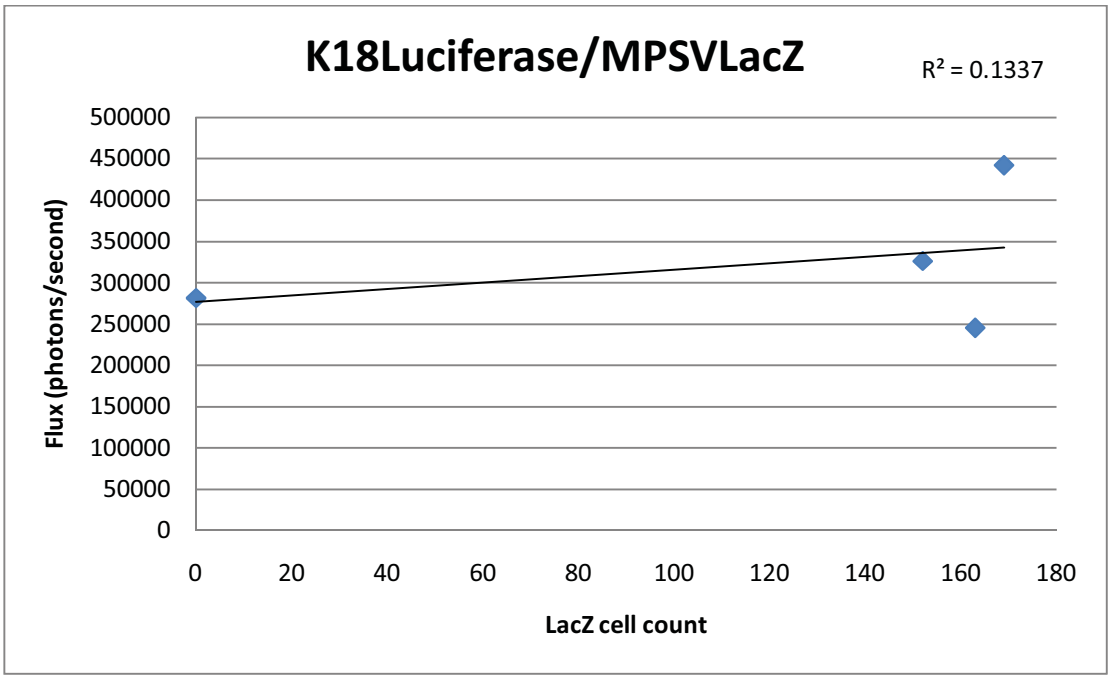
b)



c)


pgkLuciferase/MPSVLacZ
R² = 0.2325
Flux (photons/sec)
1200000
1000000
800000
600000
400000
200000
0
0
50
100
150
200
LacZ cell count

d)


K18Luciferase/MPSVLacZ
R² = 0.1337
Flux (photons/second)
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0
20
40
60
80
100
120
140
160
180
LacZ cell count

The same promoters driving expression of CFTR were tested in FABp-CFTR knockout mice (as described in chapter 5). Each CFTR vector (EF1a-CFTR, pgk-CFTR and K18-CFTR) were co-packaged with constructs expressing Luciferase (EF1a-Luc) and LacZ (MPSV-LacZ) to provide two independent measures of *in vivo* transduction efficiency (Luciferase imaging and X-gal staining). In each case the transfection for virus generation used 80% by mass of the relevant CFTR vector and 10% by mass of the other two vectors. The rationale for having the three constructs in the virus preparations was to use CFTR for assessing phenotypic correction of the CFTR defect, Luciferase to provide an ongoing real time quantitative measure of gene transfer efficiency and LacZ for a second quantitative measure of gene transfer post-mortem. For each virus preparation, the relative transduction efficiencies of each transgene was measured *in vitro* using real time PCR analyses specific to each transgene (see section 2.3.5.2).

8.4 Testing the effect of promoter on functional CFTR expression

Four groups of mice were treated as per section 2.4.1 with 4 µL of 0.3% LPC followed one hour later by 80 µL LV-CFTR under control of the 3 test promoters, EF1α, pgk and K18 with a control group receiving saline in place of vector. Mice were imaged for Luciferase expression at 1 month post vector instillation to provide a quantitative measure of transduction and TPD analysis was then performed immediately after to determine functional Cl^- channel correction.

The results from the Luciferase imaging (Figure 8-2) showed significant expression at the 1 month experimental period. The results in Figure 8-2 are broken down into the different promoter/CFTR groups, however, all mice received the same EF1αLuciferase LV with the only variable in the Luciferase viruses being the titre of the virus. The flux (photons/sec) measured from each treatment group corresponds with the *in vitro* Luciferase titres of each virus, with the EF1αCFTR/EF1αLuciferase/MPSvLacZ virus having the highest Luciferase titre of 1.05 $x10^9$ TU/mL,

K18CFTR/EF1αLuciferase/MPSvLacZ having the second highest Luciferase titre of 3.27 x 10^8 TU/mL and the pgkCFTR/EF1αLuciferase/MPSvLacZ virus having the lowest Luciferase titre of 1.07 x10^8 TU/mL.

Data from Figure 8-2 shows that when compared to virus titre, there was not a linear relationship present, as a 10-fold decrease in titre, only decreases gene transfer by 50%. The virus titres are better, although not wholly reflected by the lung transduction, as nasal transduction appears to reach a point of saturation with respect to vector dose, whereas lung transduction appears to better represent the titres of each virus.

Following Luciferase imaging, TPD measurements were taken to assess the functional correction of the CFTR Cl^- channel. Figure 8-3 shows the ΔPD and Basal PD results seen at 1 month following LV instillation.

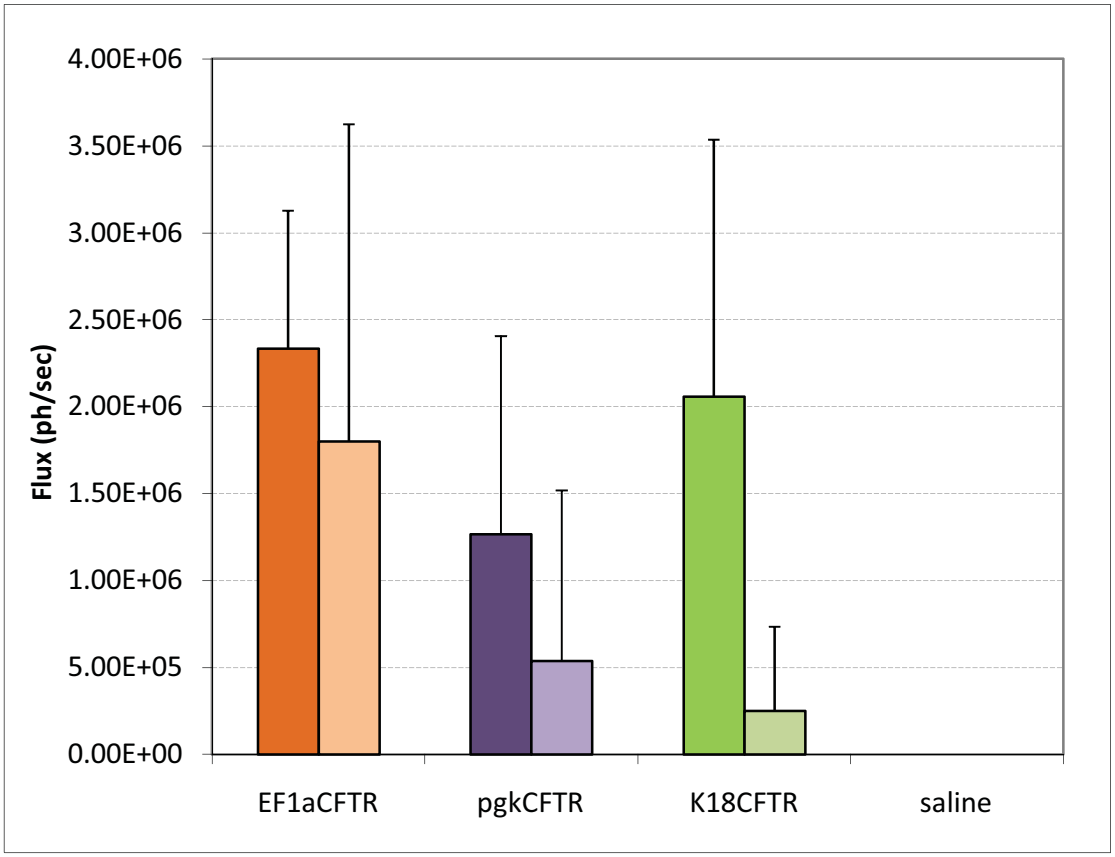


Figure 8-2: Luciferase flux measured in each CFTR promoter group

Luciferase gene expression measured in photons/sec for each of the promoter groups at 1 month after vector delivery. *note all groups expressed Luciferase under control of the EF1α promoter. Solid bars represent transduction in the nasal airways; faded bars represent transduction in the lung. Mean, +/- SEM, ANOVA (nasal) p = 0.096, ANOVA (lung) p = 0.012.

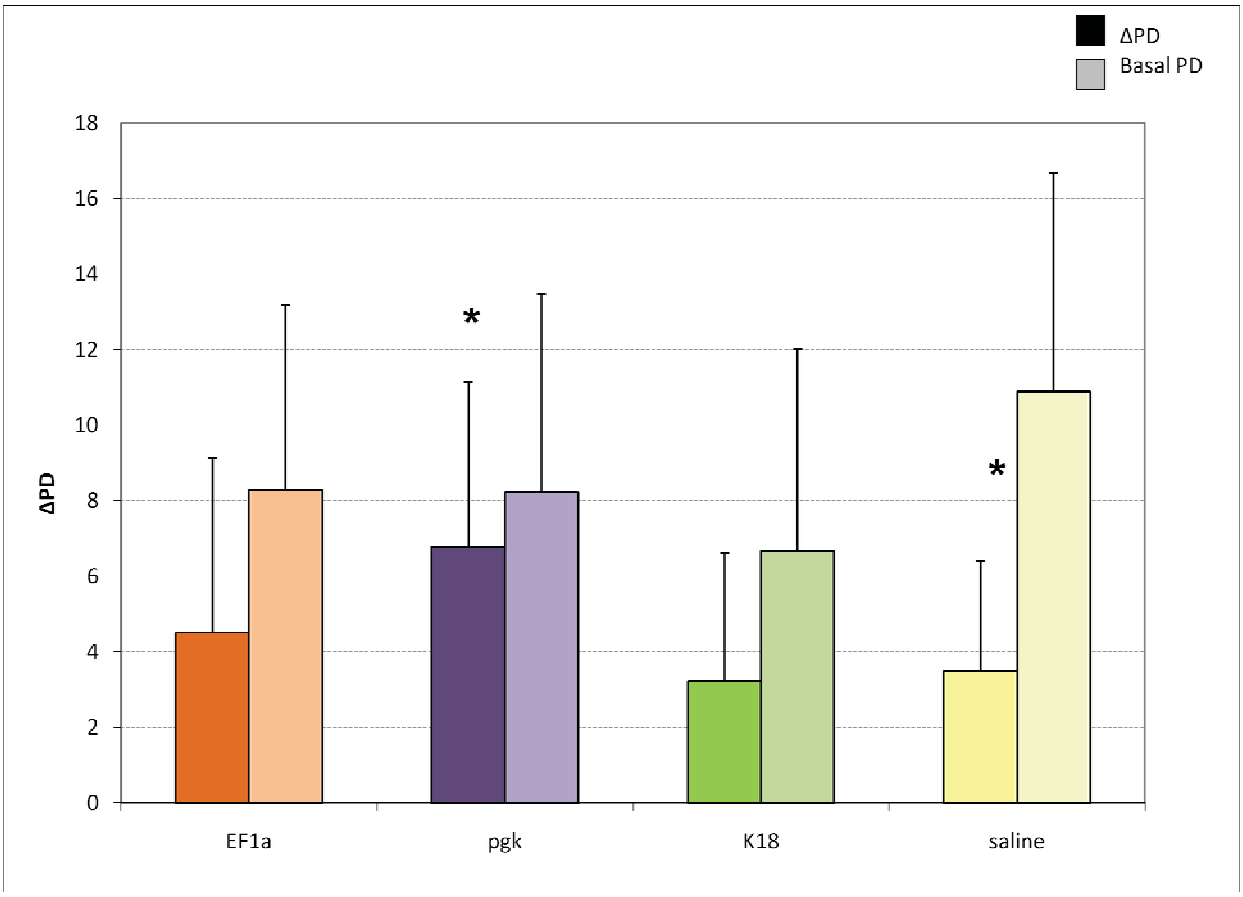


Figure 8-3: ΔPD measurement and Basal PD measurement at 1 month post treatment

Data showing the average ΔPD measurement for each promoter group at 1 month post LV instillation. Normal ΔPD value is -5 to -7, normal Basal PD value (average) +3. n= 9 per group +SEM. Solid bars represent ΔPD values, transparent bars represent Basal PD values.

*t-test p=0.047 (pgk ΔPD v saline ΔPD)

There were no significant changes towards correction of the electrophysiological defect- either ΔPD or basal PD measurement- seen with any of the CFTR treatment groups. There was a small trend towards normal for ΔPD seen in mice treated with K18CFTR, however this was absent in the other treatment groups. This change towards normal may not reflect K18 as the best promoter to use for correction of the CFTR defect, but instead reflect the higher CFTR titre seen with this vector compared to both the EF1α and pgkCFTR viruses (see Table 8-2). All treatment groups showed a trend towards normalisation of the Basal PD, however, again the greatest effect was seen with the K18CFTR vector, most likely explained by the higher CFTR virus titre seen in this virus. The results suggest transduction efficiency for the CFTR vectors was too low to achieve significant normalisation of the electrophysiological parameters. The experiment should be repeated with virus preparations that show a higher titre, as this would seem to be the likely reason for the lack of ΔPD normalisation seen in the EF1α and pgkCFTR groups.

Figure 8-3 also shows the Basal PD measurement (this is the difference between Basal solution reading and Basal + amiloride reading) which measures sodium channel activity. CF untreated mice have a large positive change in basal PD, and normal mice have been shown to have a small positive change between the two basal readings. As expected, the saline (untreated) group show a large positive change in the basal PD reading, and there is a trend seen with the treated groups to a smaller positive change. K18CFTR treated mice showed the largest decrease in basal PD- this meaning it trended towards what would be seen in normal mice. However, the differences seen in basal PD were not significantly different. It is interesting to note the basal PD appears to return closer to normal values than the ΔPD. This suggests a lower level of CFTR gene transduction may be required for the regulation of ENaC than for correction of Cl^- ion transport. As sodium transport is thought to be a more important issue in the development of CF pathophysiology, this result can be seen as somewhat encouraging.

One week following Luciferase and TPD analysis of gene transfer, mice were sacrificed as per section 2.4.2 and LacZ analysis was carried out.

All groups showed low levels of LacZ gene transfer (Figure 8-4). This low level of gene transfer is not entirely surprising as each of the viruses delivered was made up of only approximately 10% LacZ. The results from the LacZ cell counts show the K18 group to give a level of gene transduction as would be expected for this composition of virus. This LacZ result appears to correlate with the ΔPD results achieved by the K18CFTR, as it reflects high efficiency of gene transfer rather than the level of expression. There is no significant difference seen in this LacZ data. It is however somewhat surprising that the level of gene transfer was seen to be so low in the EF1α and pgk virus preparations, as the experiment carried out in normal C57Bl/6 mice using virus containing both the Luciferase and LacZ marker genes, with the virus containing 20% LacZ vector gave much higher cell counts than those seen in the FABp-CF knockout mice, while LacZ titres were similar (as shown below in Table 8-2. From previous experience, virus expressing only LacZ with a similar titre in normal C57Bl/6 mice would produce a result of approximately 150-200 LacZ positive cells, which is much higher than the results achieved here. It would indicate a difference between LacZ transduction in normal and FABp-CF mice or as experienced previously there may be something causing the LacZ to be affected post-transduction as the LacZ data is in disagreement with the Luciferase data, as there was a level of Luciferase expression measured that reflects the Luciferase titre.

Table 8-2: Virus titres (*in vitro* titres measured on A549 cells)

Virus	LacZ titre (transducing units / mL)	Luciferase titre	CFTR titre (if applicable)
EF1αLuciferase/MPSvLacZ	2.86×10^8	4.72×10^8	-
pgkLuciferase/MPSvLacZ	3.77×10^8	1.23×10^8	-
K18Luciferase/MPSvLacZ	5.75×10^8	9.25×10^8	-
EF1αCFTR/EF1αLuciferase/MPSLacZ	3.43×10^8	1.05×10^9	9.10×10^{10}
pgkCFTR/EF1αLuciferase/MPSLacZ	3.63×10^8	1.07×10^8	8.44×10^{10}
K18CFTR/EF1αLuciferase/MPSLacZ	3.54×10^8	3.27×10^8	1.02×10^{11}

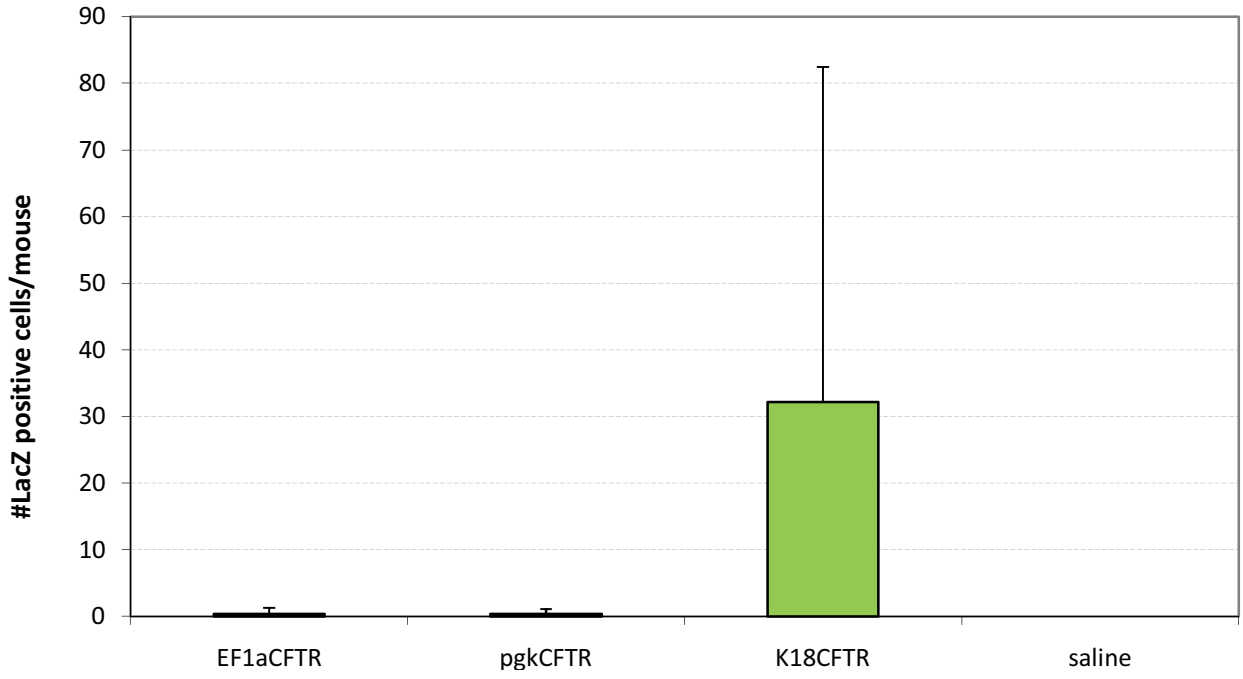


Figure 8-4: LacZ gene transfer in mice treated with LV-CFTR/Luciferase/LacZ

Average LacZ cell counts for each promoter treatment group in FABp-CFTR mice. *note all LacZ was under control of the MPSV promoter

This difference in LacZ gene transfer and the apparent lack of correction seen in the FABp-CFTR mice raises the question as to whether there is a difference in gene transfer between normal C57Bl/6 mice and the FABp-CFTR mice.

8.5 Comparison of LacZ gene transfer in C57Bl/6 and FABp-CFTR mice

Two groups of C57Bl/6 mice and two groups of FABp-CFTR mice were instilled using the standard instillation protocol described in section 2.4.1, with n=8 in each treatment group. One group of each breed of mice were instilled with 20 µL of virus and the other group instilled with 80 µL of virus. The LV-LacZ titre was 3.93×10^9 TU/mL.

Mice were sacrificed one week post virus instillation and stained for the presence of LacZ (section 2.4.2.2).

Shown in Figure 8-5, FABp-CFTR mice, whether they received 20 µL or 80 µL of LV-LacZ demonstrated very low levels of gene transfer, 0.625 and 6.25 LacZ positive cells/mouse respectively. The normal C57Bl/6 mice that received the exact same treatment using the same stocks of LPC and LV-LacZ showed much higher gene transfer, with the 20 µL group having an average of 108.25 LacZ positive cells/mouse and the 80 µL group having 146.125 LacZ positive cells/mouse. The difference between groups was shown to be significant (ANOVA, $p < 0.001$).

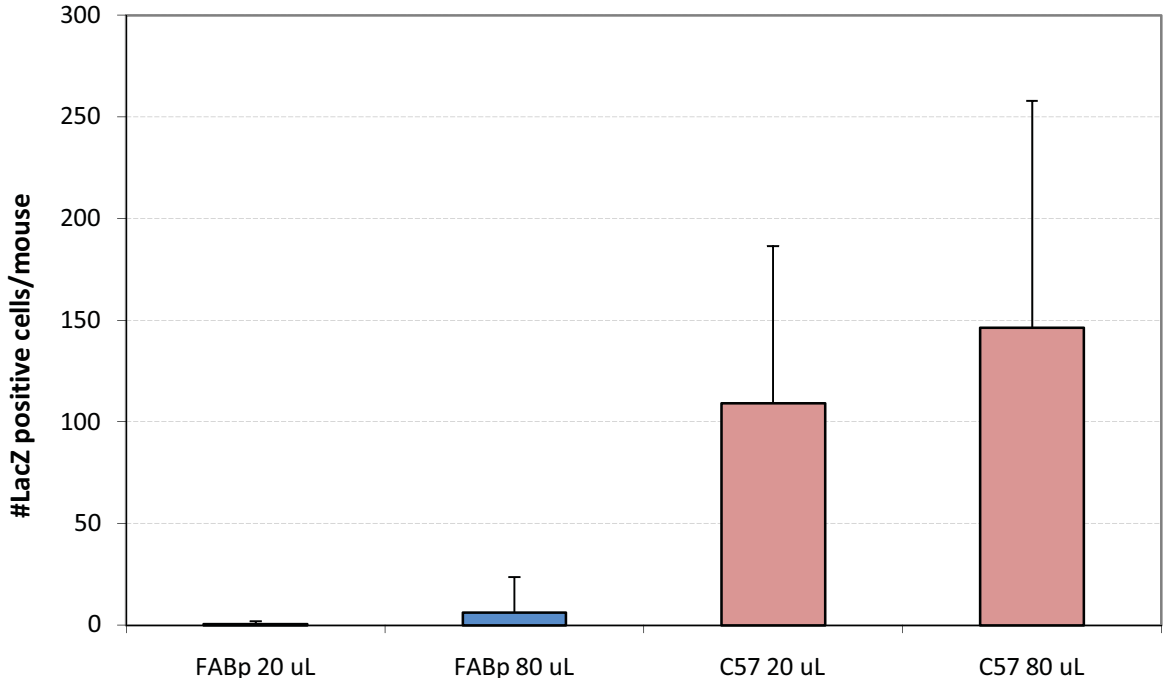


Figure 8-5: Comparison of LacZ gene transfer in FABp-CFTR and C57Bl/6 mice

Comparison of average LacZ cell counts in FABp-CFTR and C57Bl/6 mice treated with 20 µL and 80 µL of LV-LacZ. n =8, ANOVA, $p < 0.001$

8.6 Discussion and Conclusions

Initial studies looking at the effect of different promoters on gene expression showed that in marker gene studies (Luciferase) the EF1α promoter drove a significantly higher level of gene expression in the nasal airway compared to the pgk and K18 promoters. The design of the experiment utilising the two reporter genes- Luciferase and LacZ – was to allow the normalisation of the Luciferase data to gene transduction efficiency (ie the LacZ cell counts) for each mouse, taking into account the *in vitro* titres of both transgenes. Although it was thought that this would improve the data, smaller standard deviations were not seen after normalisation. The EF1αLuciferase group could not be analysed in this way as surprisingly there was no LacZ gene transfer seen in any of these mice. Immune responses to Luciferase have been measured after lentiviral vector mediated gene transfer to the mouse nose (P. Cmielewski, personal communication). It may therefore be possible that there was an immune response seen to the LacZ transgene in this cohort of mice. However, this would seem unlikely given that the same LacZ vector is used in each case, especially given that the animals were analysed only one week after gene delivery. A further experiment should be carried out as described in this chapter, with the additional steps of blood collection prior to treatment and in the weeks post treatment to allow analysis of antibody formation to the transgenes. This would allow mice who show an antibody response to be excluded from the data set.

However, looking at only the raw Luciferase data, it closely resembles the data obtained in the LacZ studies carried out utilising the same three promoters with EF1α giving the highest level of gene transduction, followed by pgk and K18, indicating that the experimental design was fundamentally sound. Normalising the Luciferase results for *in vitro* Luciferase vector titre leads to the same conclusion, although this approach not without issues of its own given that the relationship between titre and gene transfer does not seem to be a simple linear one.

The lack of close correlation between Luciferase and LacZ in each cohort of mice, is potentially a problem. There are possible explanations for this, however, it makes it difficult to assess whether either of the reporter genes can provide an accurate surrogate for gene transduction efficiency in the CFTR experiments, which is an integral part of the experimental design. Apart from the possibility of an immune response to one or both gene products (Luciferase/LacZ), this may indicate that gene transfer is highly non linear relative to titre or to an unknown factor. Luciferase provides a global analysis of gene transfer, while LacZ is a local analysis. More exhaustive sampling of LacZ cell counts and a larger number of animals per group may be beneficial to determine a relationship between the two reporter genes and their corresponding titre values.

Similar experiments using the FABp-CFTR mice and the CFTR transgene- with Luciferase and LacZ also incorporated into the virus- were also conducted. The data obtained from these experiments measuring phenotypic benefit of CFTR *via* ΔPD measurement at 1 month post treatment, showed markedly different results from those seen in the Luciferase/LacZ study.

The results showed that the level of gene transfer with all vectors was too low to give significant electrophysiological correction of phenotype. The K18 promoter did appear to show a trend towards correction of the defect, however this is likely due to the higher titre of this virus compared to the EF1α and pgkCFTR viruses.

The LacZ data from this experiment indicates that gene transduction in the EF1α and pgk groups is low compared to the K18 group, despite the 3 virus preparations having almost identical LacZ *in vitro* titres. LacZ data shows the same pattern as seen with the CFTR results with the K18 group showing the best phenotypic correction when compared to the other two promoter groups. This could indicate three things; (1) gene transfer using LV-K18CFTR was efficient when compared with the other two viruses (2)

there is an unexplained environmental factor interfering with LacZ gene transfer or (3) the viruses are behaving differently *in vivo* compared to the *in vitro* titre assays.

The low level of LacZ gene transfer seen in the LacZ analysis of the CFTR experiment in FABp-CFTR mice raised questions over the efficiency of gene transfer in this mouse strain. This led to the requirement to examine whether there is a difference in the levels of gene transfer achieved in the two mouse strains used in this set of experiments- the FABp-CFTR and the C57Bl/6 normal strain.

The subsequent experiment to test any differences between the two groups of mice showed a distinct difference between the two groups. The only variables in this experiment were the volumes of LV-LacZ delivered (either 20 µL or 80 µL) and the strain of mouse. The mice were age matched as well as possible to avoid any possible effect age may have on gene transfer. Mice were treated with the same vial of LPC and the same aliquots of virus, again to avoid the variability of making solutions on different days that may affect gene transfer.

The results indicated a distinct difference in the level of gene transfer seen in the two groups of mice, with the normal mice exhibiting high levels of gene transfer, which were representative of mice treated with this virus previously, at both treatment volumes and the FABp-CFTR mice showing extremely low levels of LacZ gene transfer. This experiment was then repeated (by another person at a later date) with another group of FABp-CFTR mice, again comparing to normal C57Bl/6 mice. In this experiment, the results showed the FABp-CFTR mice exhibited gene transfer of the same order of magnitude as the C57Bl/6; however it was seen to be at lower levels (approximately 250 LacZ positive cells) when compared to the normal mice (600+ LacZ positive cells) (P. Cmielewski, personal communication). Although a different batch of virus was used in the two studies, this is unlikely to be the cause of the differences in the results is as only one batch of virus was used in each study. However, it may be noteworthy that all FABp-CFTR mice in the first study outlined were housed in the same cage, whereas

in the subsequent study mice were not housed in this same way. It is possible that with the commonly caged mice colony there was a nasal infection or similar environmental factor that may have been present in the FABp-CFTR colony, but not in the control (C57/Bl6) mice, which did not allow efficient gene transfer event to occur. However, it remains unclear what caused the difference between what appear to be two identical studies. The inconsistent results do however seem to suggest that certain environmental factors may have a large influence on the efficiency of gene transfer.

The result of comparing the FABp-CFTR mice to normal mice is important to consider when analysing the previous experiment utilising the 3 promoters and CFTR gene delivered to the FABp-CFTR mice. The lack of complete phenotypic correction may be due to the low level of gene transfer achieved by delivery of our lentivirus into the FABp-CFTR mice. However, the transduction by Luciferase in the FABp-CFTR mice cannot be disregarded. There may be complications in measuring the electrophysiological response to CFTR gene delivery in FABp-CFTR mice that is not found in the CFTR^{tm1Unc} mice. It is also interesting to note the lack of published data to report successful CFTR gene transfer in the FABp-CFTR mouse strain [217]. There have however been reports of successful CFTR gene transfer in the CFTR^{tm1Unc} mouse strain, which has been bred on a C57Bl/6 background [93] [218].

This set of experiments is a commencement into understanding the relationship regarding the level of gene expression most optimal for phenotypic correction of the CFTR defect. The analysis of efficiency of gene expression from each promoter *via* Luciferase imaging was established with the EF1α promoter giving the highest level of gene expression. However, when this was transferred into the FABp-CFTR mice, the results obtained showed no phenotypic correction as the levels of gene transfer were too low to give a result.

As with the Luc/LacZ studies, a repeat of the CFTR/Luciferase/LacZ studies should be carried out with the additional step of blood collection and analysis for antibody formation to any of the transgene. This

is important to determine if an antibody response is being mounted against the treatment, and eliminate mice that do exhibit an antibody response from the data set. An additional change to the experimental design to rule out the potential influence of an immune response may be to carry out all analysis (Luciferase, TPD and LacZ) 1 week post instillation as this would make it unlikely that a significant immune response would be mounted. In addition, if it could be determined which marker gene provided the best surrogate measure of CFTR gene transfer, then the experimental design could be simplified to use only one marker gene, rather than both.

It would also be beneficial to carry out the CFTR arm of the experiment in both the FABp-CFTR mice and CFTR^{tm1Unc} colonies to examine any differences in phenotypic response in these two strains of mice.

9 Development of aerosolised delivery of lentiviral vectors *in vivo*

9.1 Aerosolised delivery of therapeutics

Delivery of therapeutics to the airways is most easily done using an inhalable aerosol as this allows simple, widespread and effective delivery. The broad area of deposition (large airways, small airways, bronchioles and alveoli) can be controlled by altering the aerosol droplet size. Part of the development of airway gene therapy is to examine the potential for delivery of lentiviral particles as an aerosol.

Aerosol delivery of therapeutics such as gene therapy vectors is ideal due to the non-invasive delivery, targeting of the area of interest- the lungs- and the reduced systemic toxicity when compared to other methods of gene delivery [219].

The most commonly used aerosol devices are jet or ultrasonic nebulisers which have showed poor *in vivo* results when used with non-complexed plasmid DNA ranging in size from 4.7-7.2 kbp [220]. These atomisation and spray devices are however regularly utilised in the delivery of non-viral, nucleotide based therapies to the lungs with good success. To achieve micron sized droplets for delivery to the lower airways, a significant increase in surface energy must be applied to a fluid stream. For this reason, high strain rates are often applied.

Several options exist for atomisation of liquid formulations in a clinical setting including pressure driven, pneumatic, ultrasonic and electrostatic devices. Each of these devices imparts strain on the fluid steam in a unique manner to create the desired droplet size [220]. Metered dose inhalers use a high pressure propellant or simplex atomiser geometry to break fluid streams into small droplets [221-223]. Lower pressures can be employed if centrifugal forces are used in conjunction with the applied pressure, as occurs with pressure-swirl atomisers [224]. Energy provided by compressed gas flow is used in pneumatic jet nebulisers in order to generate an aerosol from a reservoir containing the therapeutic [225]. Electrostatic sprays apply significant voltages that are large enough to overcome the surface tension forces of the fluid, resulting in the generation of an aerosol [226].

To date, most studies looking at the aerosolisation of gene therapy agents have been focused on the use of non-viral therapies. The studies outlined in this thesis, investigate the aerosolisation of a lentiviral vector using the Aerogen nebuliser device.

The Aerogen nebuliser contains a vibrating mesh or plate containing multiple holes which uses electricity to vibrate the element at low frequency, forming droplets of solution that can inspired [220].

The advantages of the Aerogen system are the low sheer forces created, therefore there are no alterations or damage to the test compounds and molecular integrity and concentration are maintained. The Aerogen produces a low velocity, fine, controlled droplet size aerosol without compressed air. This system has also been shown to have high efficiency, with small to no wastage of input material, which is a significant advantage when using small volumes and costly materials such as gene therapy vectors.

Initially *in vitro* experiments were undertaken to assess the nebulisation technology with lentivirus to ensure any loss of viable virus was minimal. The Aerogen piezoelectric nebuliser with a pore size of 3.7 µm was deemed best for the aerosolisation of lentivirus following tests assessing the recovery of virus following nebulisation with the Aerogen. Tests were also conducted using protein stabilisers to enhance the recovery of nebulised virus. Foetal calf serum, Bovine Serum Albumin, Pluronic F68 and Maltodextran were added to the virus prior to nebulisation and their effect on recovery of viable virus measured. Bovine Serum Albumin at a concentration of 0.3% when added to the final virus consistently gave the best recoveries of nebulised virus. Four tests using 0.3% BSA were carried out with the following recoveries: 93.89%, 73.64%, 66.55% and 100%. This *in vitro* data was used when translating to *in vivo* use. (Chuan K. Tan, unpublished data).

A rat model using the SCIREQ Flexivent small animal ventilator in conjunction with the Aerogen was used to deliver the LPC pre-treatment and lentivirus to the lungs of this small animal model.

The SCIREQ small animal ventilator is designed to be used in conjunction with the Aerogen nebuliser and allows targeted and controlled delivery of aerosol directly to the airways *via* intubation. The controlled delivery *via* the SCIREQ ventilator allows nebulisation into the lungs to be co-ordinated with inspiration.

Using the SCIREQ Flexivent, lung compliance, airway tissue resistance and tissue elastance can be continually monitored through the dosing procedure to monitor the effect of the procedure on the lungs.

Experiments were carried out using a LV expressing the LacZ marker gene to allow the visualisation and quantification of gene therapy.

9.2 Nebulisation of airway gene therapy into rat lung

Rats were anaesthetised with 75 mg/kg Domitor and 0.5 mg/kg Ketamine *via* intra-peritoneal injection.

Intubation of the rats was carried out by laying the rat in a supine position. The nose of the rat was immobilised by a piece of cotton thread. The trachea was visualised using a hook-shaped pen light which was used to gently lift up the bottom jaw. The trachea was located by the transient opening and closing of the epiglottis. Once the trachea was located, a 16G catheter was slowly and gently inserted into the trachea. To check the rat was correctly intubated, the catheter was connected to the Flexivent machine. If correctly intubated, the lungs will visibly expand, whereas if inserted incorrectly (into the stomach) the stomach will expand in which case the catheter was removed.

Rats were allowed to adjust to ventilation for 30 minutes prior to pre-treatment. Lung mechanics were also measured to gain an understanding of lung function prior to gene therapy treatment.

Three rats were initially tested in the nebulisation model.

9.2.1 Rat 'A'

The first rat received 500 µL of 0.1% LPC in PBS nebulised into the lungs on inspiration. The nebulisation of LPC took 6 minutes. The nebuliser was detached from the Flexivent setup and was cleaned with PBS as LPC has negative effects on the stability of lentivirus (unpublished observation). It was vital that any remnants of LPC were removed as to not cause loss of viability of the lentivirus to be delivered to the lungs. Lung mechanics were measured during the hour between LPC delivery and the LV delivery. One millilitre of LV-LacZ formulated in PBS was then delivered *via* the Aerogen nebuliser on inspiration to the lungs of the rat. The delivery of LV-LacZ took 12 minutes to be delivered to the lungs. Lung mechanics were measured for10 minutes after the completion of LV-LacZ delivery. Following the measurement of lung mechanics, the rat was removed from the Flexivent and woken with antisedan and allowed to recover from anaesthesia in an incubator.

9.2.2 Rat 'B'

Rat 'B' was treated in the same manner described in 9.2.1, but with the following changes.

The LPC concentration was increased to 0.3%. This concentration was chosen as this is the concentration of LPC used in mouse experiments and has shown the highest levels of gene transfer when compared to other concentrations [218]. As the concentration of LPC was increased, the volume delivered was decreased to 250 µL. The nebulisation of the LPC *via* the Aerogen nebuliser took 3 minutes to be totally delivered to the lungs. One hour post administration of the LPC the same volume of LV-LacZ was delivered- 1 mL- however, the virus was made to a final concentration of 0.3% BSA as a

means of protection for the virus during the nebulisation process. The nebulisation of the virus took 15 minutes, with the increased time likely due to the increased viscosity of the virus solution due to the addition of the 0.3% BSA.

9.2.3 Rat 'C'

The treatment given to rat three was identical to that given to rat 'B'. However, testing of lung mechanics was undertaken every 5 minutes post LPC delivery for the hour between LPC and LV-LacZ delivery. It should also be noted that rat 'C' died 3 days post treatment.

9.2.4 Lung mechanics

The mechanics of the lung were measured prior to gene therapy treatment, following LPC delivery and following LV-LacZ delivery to note the effects of treatment on the lungs.

Rn: Newtonian resistance, which is indicative of the resistance of the central airways

I: Inertance, which is indicative of the inertive properties of the gas in the airways, this is generally close to 0 or slightly negative in intubated rats

G: Tissue damping, is closely related to tissue resistance and reflects the energy dissipation in the lung tissue

H: Tissue elastance, which shows energy conservation in the lung tissues

Eta (η): Hysteresivity, represents the ratio of energy dissipation to energy conservation (G/H), an increase in η shows a structural change in the lungs

COD: Coefficient of determination: a quality control parameter measuring the "fit" of the test model (a value of 1.0 indicates a perfect fit of the model)

PEEP: Positive End Expiratory Pressure, is the pressure applied when breathing out. This keeps the lungs from collapsing while on ventilation. (The PEEP parameter is a set value at the beginning of testing which is then measured when lung mechanics are measured)

The lung mechanics data from each of the three test rats is shown in the tables below (Table 9-1 through to Table 9-4).

Table 9-1: Rat 'A' and Rat 'B' lung mechanics

Rat 'A'					
	Baseline	**Post LPC**	**Pre LV**	**Pre LV**	**Post LV**
Rn	0.126	0.112	0.127	-6.04	-0.133
I	0.001		-0.0002	0.001	0.001
G	1.022	1.226	0.786	6.971	1.313
H	5.484	1.218	3.895	0.230	0.702
eta	0.186	1.006	0.202	30.356	1.870
COD	0.574	0.250	0.236	0.021	-0.057
PEEP	3.605	6.349	5.28	3.652	7.035

Rat 'B'					
	Baseline	**Post LPC**	**Pre LV**	**Pre LV**	**Post LV**
Rn	0.171	0.053	-0.030	-0.037	-0.018
I	0.00019	.0003	0.00006	0.0004	0.0005
G	0.655	1.347	1.216	1.116	2.481
H	6.551	4.378	1.115	1.228	5.126
eta	0.099	0.307	1.090	0.987	0.484
COD	0.791	.867	0.403	0.313	0.270
PEEP	2.713	2.868	2.943	1.103	6.957

Table 9-2: Rat 'C' - Baseline

Rat 'C' - Baseline				
	1	**2**	**3**	**4**
Rn		0.077	0.066	0
I	1.74E-05	2.14E-05	-0.00016	0
G		0.006	-8.49E-05	0
H	0.0002	0.017	-0.0003	0
eta		0.348	0.284	0
COD		-0.084	-0.151	0
PEEP	2.619	2.625	2.634	2.614

Table 9-3: Rat 'C' – Post LPC

Rat 'C' – Post LPC ***(Data points 1-14)***														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Rn	0.125	-24.78	0.105	0.115	0.089	-82.877	0.072	0.069	0.065	0.064	0.063	0.062	0.056	0.058
I	0.0003	0.0001	0.0003	0.0002	7.23E-05	1.93E-05	5.42E-05	5.97E-05	7.06E-05	7.38E-05	2.54E-05	6.48E-05	3.86E-05	2.48E-05
G	0.261	25.021	0.383	0.110	0.125	82.978	0.049	0.057	0.053	0.057	0.052	0.048	0.047	0.034
H	1.358	0.014	1.388	1.228	0.651	0.001	0.118	0.436	0.288	0.304	0.155	0.255	0.062	0.178
eta	0.192	1760.7	0.275	0.090	0.193	65812	0.416	0.130	0.184	0.189	0.338	0.188	0.761	0.195
COD	0.815	0.052	0.923	0.705	0.672	-0.068	0.149	0.424	0.281	0.298	0.142	0.203	0.047	0.046
PEEP	3.015	2.812	2.783	2.674	2.852	2.755	2.532	2.476	2.453	2.468	2.572	2.499	2.587	2.487

Rat 'C' – Post LPC ***(Data points 15-27)***													
	15	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
Rn	0.057	0.053	-0.292	0.073		0.058	0.059	0.062	0.071	0.068	-0.045	0.072	0.418
I	2.57E-05	1.77E-06	2.30E-05	1.27E-05	1.64E-07	2.05E-05	1.83E-06	7.51E-06	1.01E-05	-4.50E-07	0.0001	4.08E-05	-1.10E-06
G	0.053	0.033	0.377	0.014		0.026	0.016	0.034	0.026	0.017	0.178	0.022	-0.339
H	0.142	0.084	0.004	0.067	0.001	0.122	0.01	0.059	0.105	0.099	0.022	0.165	-0.001
eta	0.373	0.392	830499	0.218		0.218	1.825	0.570	0.248	0.175	8.125	0.135	448.23
COD	0.113	-0.0485	-0.065	-0.125		-0.045	-0.119	-0.015	0.005	-0.023	0.220	0.098	-0.151
PEEP	2.55	2.517	2.528	2.59	2.55	2.63	2.625	2.557	2.657	2.662	2.630	2.569	2.591

Table 9-4: Rat 'C' – Post LV-Lac Z delivery

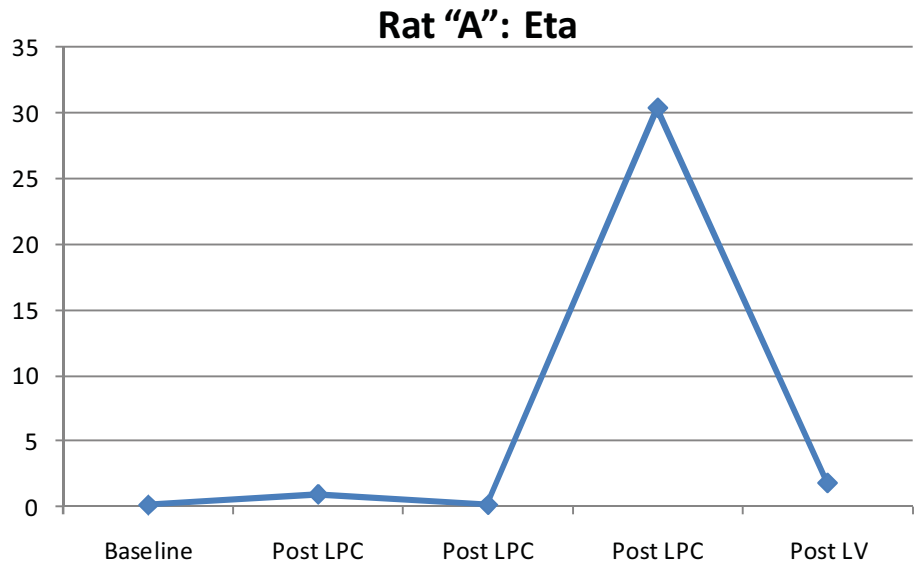
Rat 'C' – Post LV-Lac Z delivery															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Rn	-4.712	0.018	0.070	-1.398	-26.291	0.033	0.039		0.074	0.078	0.077	0.074	0.081	0.073	0.074
I	2.76E-05	6.93E-05	3.64E-05	3.13E-05	3.10E-05	3.43E-05	1.59E-05	-4.10E-07	1.73E-05	1.82E-05	9.89E-06	5.12E-05	5.22E-05	1.53E-05	2.96E-05
G	4.808	0.071	0.012	1.481	26.375	0.052	0.042		0.006	0.008	0.010	0.042	0.029	0.022	0.023
H	0.003	0.006	0.164	0.002	0.002	0.004	0.002	-0.002	0.002	0.013	0.049	0.203	0.282	0.170	0.029
eta	1711.7	11.05	0.078	627.09	16444	12.586	21.847		2.575	0.640	0.216	0.208	0.104	0.131	0.795
COD	-0.046	-0.049	0.056	-0.067	-0.085	-0.064	-0.109		-0.113	-0.092	-0.047	0.207	0.198	0.075	-0.008
PEEP	2.630	2.613	2.595	2.846	2.757	2.687	2.721	2.729	2.711	2.567	2.589	2.610	2.667	2.631	2.612

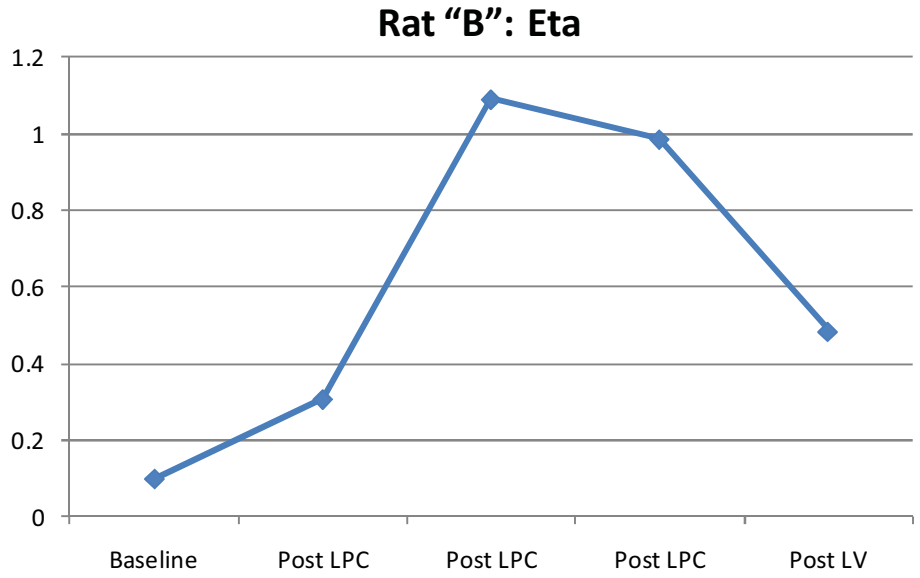
The lung mechanics measured in each of the three rats cannot be considered reliable due to the "fit" of the model measure being below the desired threshold of 0.9 (90% fit). In all of the three rats to undergo this treatment, the initial measurement of the model fit was below the desired 0.9 value. A series of baseline measurements once the rat has been ventilated for an amount of time and adjusted to the anaesthesia and ventilation may be a better indication of the "fit" of the model. This was tested for rat 'c', however COD values were quite different at each time point. The COD measurement may also be low due to the rat trying to breathe against the Flexivent, or in the initial stages due to light anaesthesia. The COD measurement is likely to reduce during the treatment protocol due to the changes occurring in the lungs with the LPC and LV-LacZ volumes affecting the measurements being taken during the ventilation process.

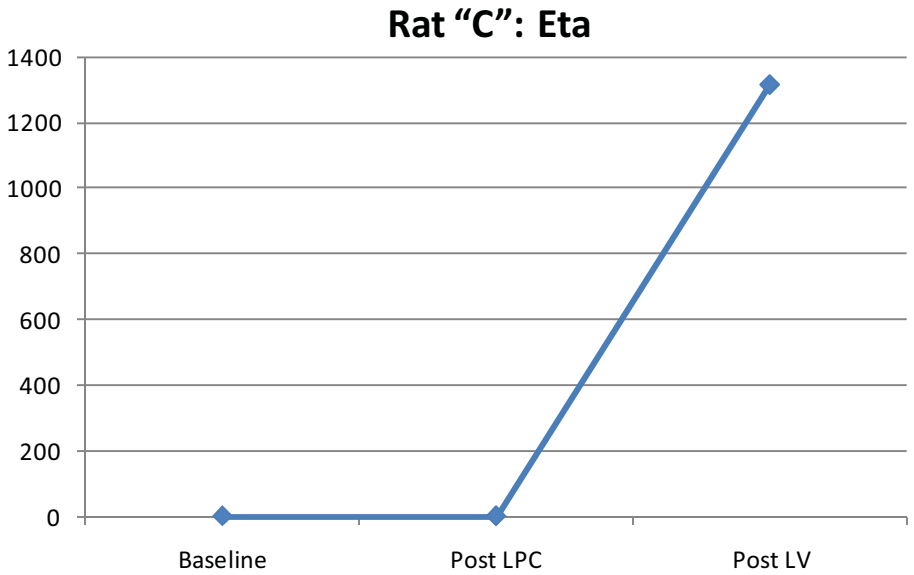
Lung mechanics from each rat were examined, however, as the COD- the fit of the model- prior to any treatment is below 0.9 which is considered a "cut off" for using the data, the results of the lung mechanics cannot be used with any confidence. Although the COD values are low, another important value to look at is the η value, as an increasing value indicates structural changes and damage to the lung tissue. Figure 9-1 shows graphically the η values measured prior to treatment, post LPC treatment and post LV treatment. In all rats, an increase is seen following LPC pre-treatment, indicating that there may be some structural damage to the lungs. It is however, interesting to note that there is a large change in η for rat C post LV delivery. This large increase in η is indicative of damage to the lungs, and may explain the unexpected death of this rat 2 days post treatment.

Figure 9-1: Eta (η) measurements for Rats A-C

Eta measurements for all experimental Rats, with data points taken prior to treatment and during the treatment process


Rat "A": Eta
35
30
25
20
15
10
5
0
Baseline
Post LPC
Post LPC
Post LPC
Post LV


Rat "B": Eta
1.2
1
0.8
0.6
0.4
0.2
0
Baseline
Post LPC
Post LPC
Post LPC
Post LV


Rat "C": Eta
1400
1200
1000
800
600
400
200
0
Baseline
Post LPC
Post LV

9.2.5 Measurement of gene transfer

One week post treatment Rats were sacrificed with an overdose of pentothal (140 mg/kg). The trachea was exposed *via* incision through the skin on the neck, and a small blunt needle was placed into the trachea, facing toward the lungs. The needle was secured with a suture, and the needle connected to a syringe containing 2% PFA/0.5% Glutaraldehyde. The fixative solution was allowed to run into the lungs until they were inflated to near capacity. The fixative flow and needle were removed and the suture used to seal the trachea and the fixed lungs. Lungs were then cut out from the chest cavity and placed in a pot containing fixative solution. The lungs then underwent staining for LacZ as described in section 2.4.2.2, and were sectioned following incubation in 10% NBF. Lungs were sectioned as follows:

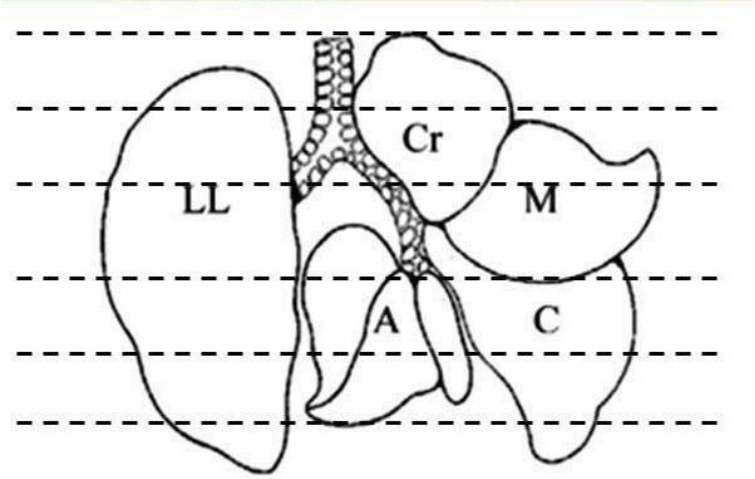


Figure 9-2: Diagramatic representation of rat lung dissection

LL= left lung, Cr= cranial lobe, M= median lobe, C= caudal lobe, A= accessory lobe. Rat lung was cut into sections represented by the dotted lines.

The six gross lung sections were embedded in paraffin and 8 µm sections taken and stained with Saf O for analysis of gene transfer.

9.2.6 Analysis of Rat lung sections

Rat lung sections prior to embedding were observed under high power magnification. Initially it appeared that gene transfer was present due to the large amount of blue staining in all treated lungs. See Figure 9-3 a-h. Fine sections were observed under 400 x magnification for identification of cellular level gene transfer. Gene transfer was not present in any sections of any levels of lung taken. There were cells found in the thymus gland that appeared to stain the blue of the LacZ gene. These cells however were identified as macrophages.

The thin sections of rat lung that were observed also showed areas of bronchitis or pneumonia. This is likely caused by the volumes of fluid entering the lungs, which caused damage to the bronchioles in the airway (see Figure 9-3 i-j).

As no gene transfer was seen in the Saf O sections, but there were what appeared to be distinct areas of gene transfer in the gross lung sections, lungs from an untreated rat were removed and stained using the protocol described earlier (section 2.4.2.2) and observed for background/non-specific staining. It was interesting to observe what appeared to be distinct areas of gene transfer (areas of blue staining-see Figure 9-3 g-h). When embedded and fine sections were cut, there was again no evidence of LacZ staining in the lung. It appears that the rat lung, and in particular the thymus has some endogenous LacZ activity-giving the lungs the appearance of gene transfer on gross section, but when fine sections are taken, there is no evidence of what appeared to be these distinct areas of gene transfer.

Rat “A” and “B” showed no gene transfer to be evident. Small amounts of damage were seen using high magnification, which is not surprising as when lung mechanics were measured an increase in η was seen following LPC delivery which is an indication of structural damage to the lungs.

Rat “C” died unexpectedly 2 days following treatment. Although looking at the lung mechanics data and in particular at the η data (showing structural damage) it is not surprising as this value increased enormously following LV-LacZ delivery. En Face section showed what appeared to be widespread destruction of the lung tissue, and following fine sectioning of the lungs, areas of bronchitis and pneumonia were present, which likely contributed to the unexpected death of this rat.

Figure 9-3: Gross and Histological Rat lung sections

a) Rat A, upper lung section, x 20 magnification

b) RatA, upper lung section, x 40 magnification

c) Rat B, upper lung section, x 20 magnification

d) Rat B, upper lung section, x 40 magnification

e) Rat A, lower left lung section, x 20 magnification

f) Rat A, lower right lung section, x 20 magnification

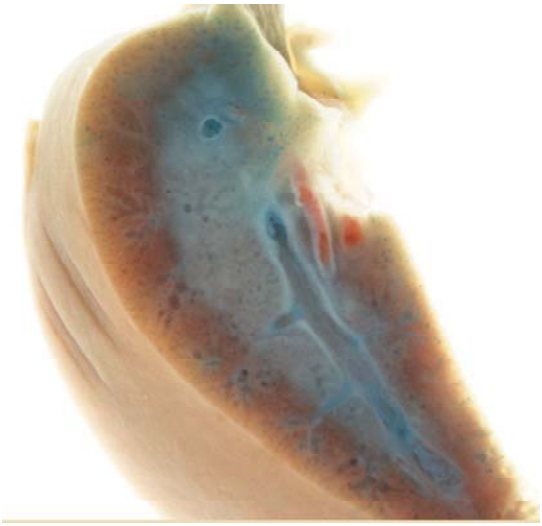
g) Control rat, lower right lung section, x 20 magnification

h) Control Rat, lower left lung section, x 20 magnification

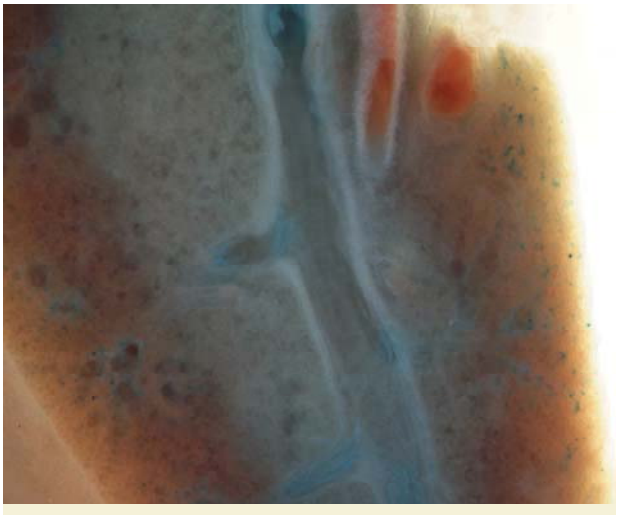
i) Rat A, x 400 magnification, bronchitis indicated by the black arrow

j) Rat A, x 400 magnification, pneumonia indicated by the black arrow

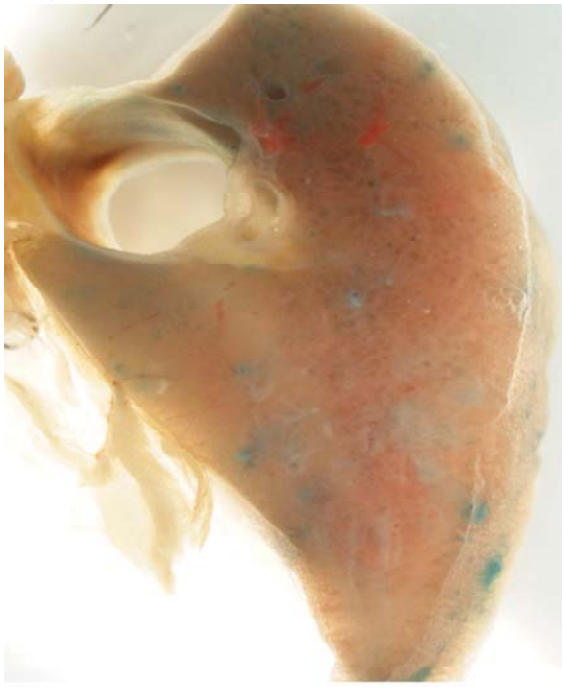
a)



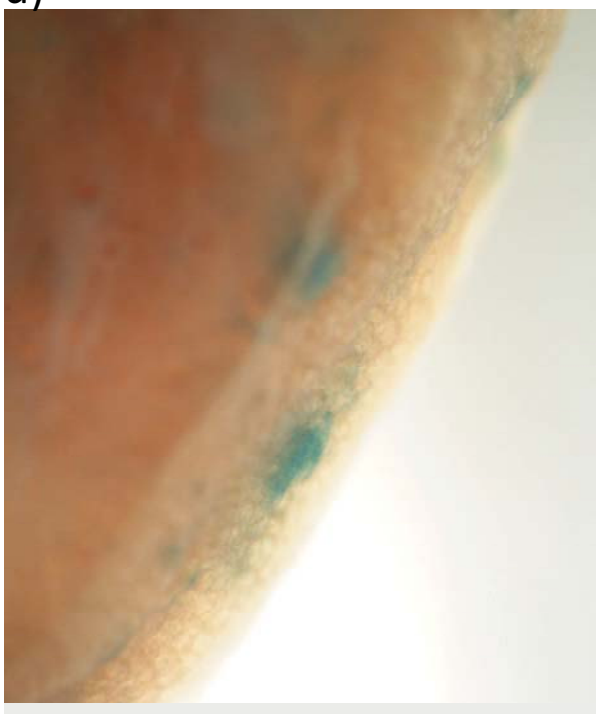
b)



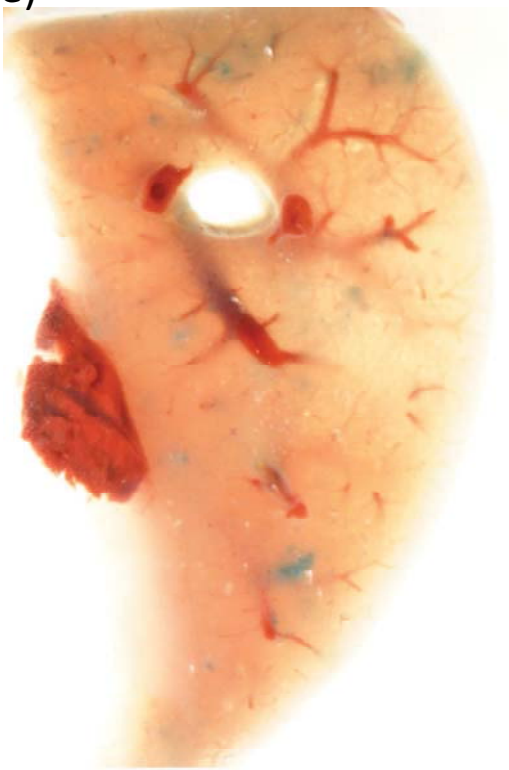
c)



d)

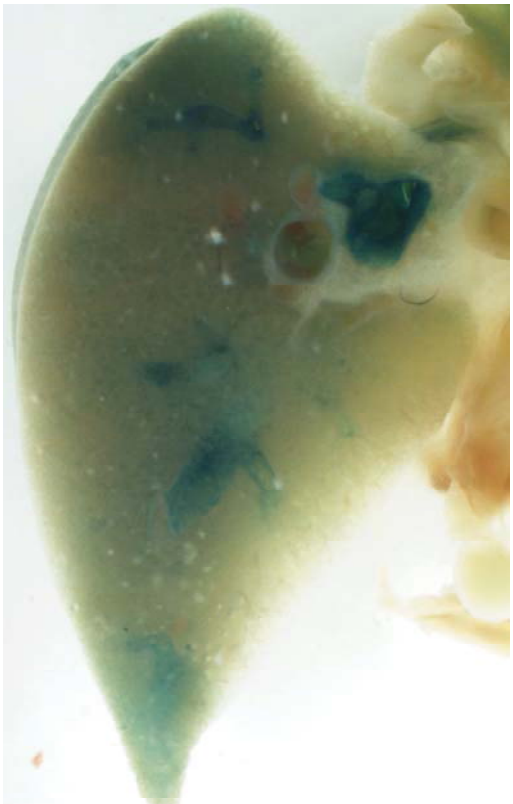


e)

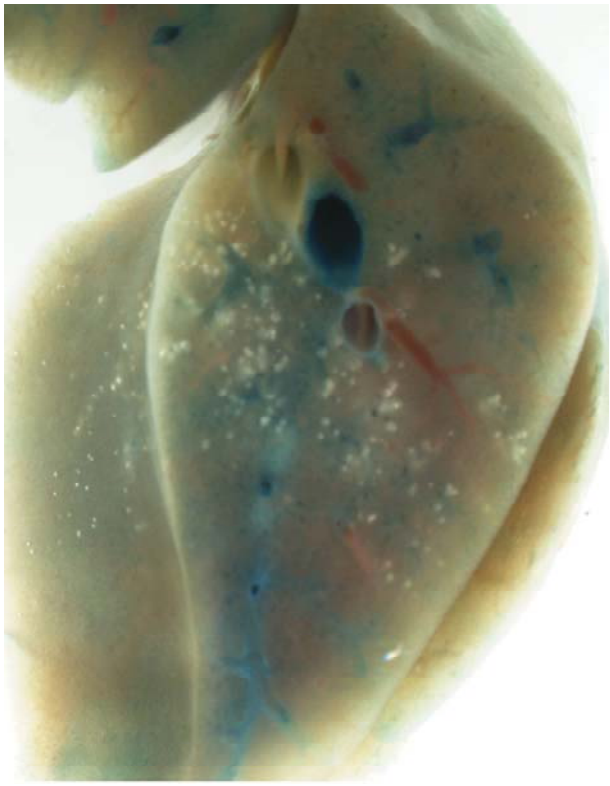


f)

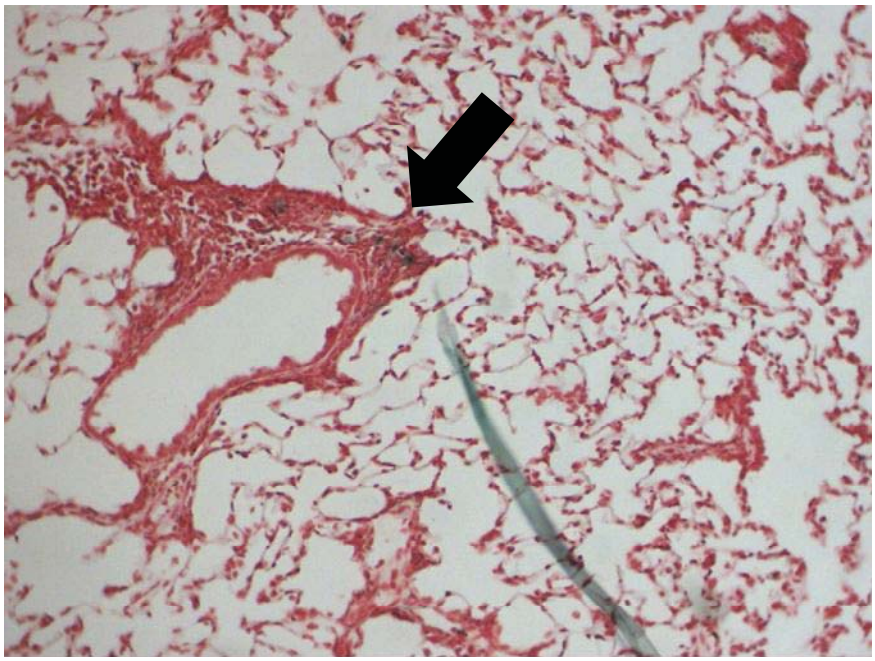
g)



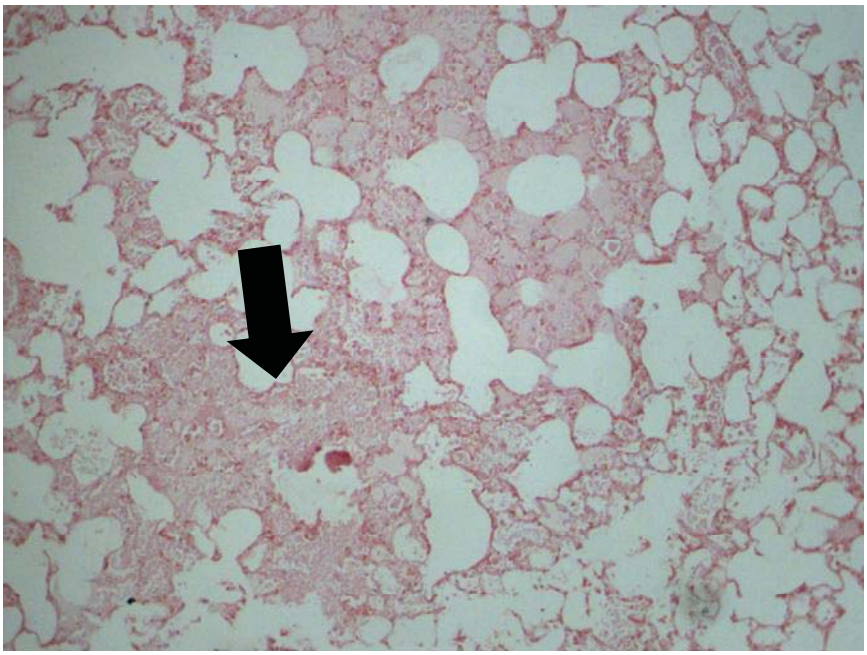
h)



i)



j)



9.2.7 Discussion and Conclusions

The aerosolisation of therapeutics into the lungs for direct delivery to the affected area in a disease such as CF is the eventual aim for many therapies such as gene therapy. Currently aerosol therapy is most commonly used to treat asthma and chronic obstructive pulmonary disease, and there are a large number of medications used or in development for other diseases. The development of more efficient aerosol delivery devices has allowed specific targeting of specific parts of the airways and lung. Efforts in the use of aerosols as gene transfer agents have largely centred on complementary DNA transfer of the CFTR gene in CF patients. For gene transfer to be effective as an aerosol, the vector and its package must be stable to shear forces during aerosolisation [227].

In vitro tests were conducted to ensure the recovery of the virus post nebulisation, however, it may be useful to conduct a similar *in vitro* experiment identical to the protocol used in vivo. It has been shown that LPC reduces the stability of lentivirus following co-incubation. Following nebulisation of LPC, the nebuliser needs to have all traces of LPC removed to ensure the viability of the virus for delivery into the lungs. *In vitro* testing to determine good cleaning procedures is essential to ensure that the highest amount of virus is being delivered to the lungs as possible.

The lungs when removed from treated rats were seen to be intact; however, there was injury to the lung, likely caused by effects of the LPC. There appears to be a delicate balance between the concentration and volume of LPC required to achieve gene transfer and the concentration and volume required to cause damage to the epithelium. The local effect of LPC on opening tight junctions appears too small for a gene transfer event; however, globally the effect of LPC is too high to be tolerated by the rat. A series of titration experiments with LPC alone nebulised into the rat lung testing a series of volumes and concentrations of LPC (0.1% and 0.3% as these concentrations have been shown to allow gene transfer in mouse nasal airway gene therapy) and then examining for the effects on the lung. Once the highest tolerated dose of LPC has been determined, an appropriate volume of concentrated

LV-LacZ can be determined, and added into the treatment protocol, and lungs observed for gene transfer. Time needs to be spent in a methodical determination of optimal concentrations and volumes to achieve a high level of gene transfer. It would be useful to measure lung mechanics in the days post treatment to ensure that lung function has returned to normal (baseline) values, also ensuring the well-being of the rat post treatment.

This nebulisation approach to virus delivery may also need to be adapted to treat only a small portion of the lung at a time, to reduce the impact on the whole lung. The use of lentiviral vectors pseudotyped with an envelope targeting the apical surface of the epithelium, such as GP64 or filovirus, may also be warranted to exclude the need for LPC pre-treatment as this may be the cause of the damage seen in the treated lung sections.

The experiments conducted here were a small step towards the understanding of using lentivirus as an aerosol in the lungs of rats. More investigation needs to be carried out to achieve gene transfer without causing damage to the lungs.

Although these experiments did not show gene transfer in the lungs, it has developed a new technique within the laboratory for the intubation of rats and their ventilation using the SCIREQ small animal ventilator. More work should be continued into developing this gene therapy protocol using LPC and lentivirus for the delivery of genes to the conducting airways, as this is the most effective and possibly non-invasive method of delivering the therapeutic gene CFTR to CF patients with targeted delivery to the direct area.

10 Concluding Remarks

The development of a gene therapy protocol for an inherited genetic disease such as Cystic Fibrosis is a complex process with many processes that need to be considered. In the case of cystic fibrosis, it is clear from previous work that the efficiency of gene transfer, and the resulting effects, are of primary concern. One factor that will impact the response to gene therapy is the immune response initiated to the gene delivery vector, in this study a Lentivirus, and whether this can be controlled to achieve better outcomes. Another important aspect to consider is the level of gene expression required to achieve a therapeutic outcome. The way in which the gene delivery vector is delivered to best target the affected organs must also be considered, as well as developing the technology in an appropriate animal model that represents the disease state while remaining robust enough to measure therapeutic outcomes for a significant period of time. An appropriate method of measuring gene transfer outcomes is also essential for critical analysis of the gene transfer process. This thesis has described work undertaken to further study these areas to optimize and achieve the best therapeutic outcomes for gene therapy for cystic fibrosis.

The body naturally initiates an immune response to foreign particles entering the body. This immune response involves organelles including the proteasome and lysosome, which act to degrade foreign proteins and eliminate them from the system. Proteasome and lysosome inhibitors Z-LLL, Doxorubicin HCl and Bafilomycin A1 were tested for their ability to inhibit the proteasome response to the introduction of foreign peptides- such as our lentiviral gene therapy. In all cases, topical application was not found to be beneficial for gene transfer, and in reality caused damage to the nasal epithelium. These results were not seen to be beneficial for our gene transfer protocol. When the inhibitors were delivered *via* I.P injection prior to gene transfer, the level of gene transfer was not seen to increase significantly compared to our standard gene transfer protocol. The use of proteasome inhibitors was not pursued further as no beneficial effect was seen when investigated.

As the studies described in this thesis used a HIV-1 derived lentivirus, an inhibitor of the innate antiretroviral immune response- Leptomycin B- was tested for any beneficial effects it may have on suppressing the immune response to our lentiviral vector. Initial testing showed a modest increase in gene transfer when coupled with our gene transfer protocol utilizing a LacZ marker gene in a normal mouse model. When transferred into a disease model results were not entirely replicated to what was seen in the normal models, and mice treated with Leptomycin B did not show increased correction of the CFTR defect when compared to mice that did not receive the Leptomycin B. Although it showed initial promise for increasing the level of gene transfer achieved, when tested in the disease model, no benefit of incorporating Leptomycin B was observed.

These results suggest that manipulating the immune response to increase the efficiency of gene transfer *in vivo* is not feasible, despite the many *in vitro* studies that provided the intellectual basis for this work, and that other means need to be considered. These could include improving pertinent characteristics of the vector such as titre, specific infectivity, purity and dose. In addition, manipulation of the acquired, rather than the innate, immune response to allow repeated dosing of vector may be beneficial in terms of achieving the desired outcomes.

Another problem with the experimentation undertaken to investigate the manipulation of the immune response to increase efficiency of gene transfer was the low levels of gene transfer observed in mice treated with the standard gene therapy protocol. With low starting gene transfer efficiencies, this may have impaired the feasibility of the studies (using proteasome and lysosome inhibitors and LMB) performed.

To develop a gene therapy protocol for Cystic Fibrosis or any genetic disease, it is important for the protocol to be tested on the best representative disease model, which for Cystic Fibrosis is a mouse model. The mouse model used previously at the WCH was the $CFTR^{tm1Unc}$ knockout model in which CFTR is knocked out. This CFTR knockout causes intestinal disease and so there is a requirement for the mice to have a modified diet to reduce the effects of these intestinal problems. The mice are also

noted to be less robust compared to normal mice which makes long term experimentation difficult- particularly with repeated testing for CFTR correction via TPD at regular intervals which requires multiple rounds of anesthesia. This led to a more robust model of the disease being sought and the *CFTR*^{tm1Unc}-Tg(FABPCFTR)1Jaw/J, which contains a knock-in of CFTR under control of the FABp promoter to prevent gastrointestinal problems, was obtained. Other advantages of this mouse model are that both female and male CFTR-knockout mice are fertile, which allows breeding of more affected mice compared to the CFTR^{tm1Unc} colony, which only allowed for breeding heterozygous females with affected males, producing only 50% affected progeny. The work in this thesis allowed a colony of the *CFTR*^{tm1Unc}-Tg(FABPCFTR)1Jaw/J mice to be set up that was homozygous for both the CFTR knockout and the FABP-CFTR knock-in, maximizing its productivity. It is hoped that this mouse colony will be more robust and allow for long term experiments to be carried out for the life of the mice more easily- which is crucial for developing a life long gene therapy cure for Cystic Fibrosis.

Antibodies to the CFTR gene for analysis of gene transfer in the disease model of cystic fibrosis have been often hard to obtain. Problems with low specificity have also been apparent meaning it can be difficult to get useful results with these reagents. This thesis described the development of aptamers to the CFTR gene as a potential method of visualizing CFTR in treated mouse tissues. These aptamers hold great potential in becoming tools for analyzing gene transfer, however, isolated aptamers require further testing to ensure specificity and binding to the CFTR gene.

Real time PCR assays are also described in this thesis as a method of streamlining the process of titering lentivirus with any transgene. The development of these assays allows more complex and sophisticated virus mixtures to be used, containing multiple transgenes with the titre of each individual virus being easily obtained using only one virus assay. This allows for more accurate and consistent titering of viruses. The development of these assays is not only beneficial for determining virus titre,

but can also be used for analysis of treated mouse tissues, with a reading of how many copies of the delivered transgene have been incorporated into the treated cells.

Although it is clear from natural experiments (mild CF) that only low levels of CFTR expression in all cells is sufficient to avoid severe disease *in vivo*, and that expression of normal or high levels of CFTR in as few as 5-10% of cells in *in vitro* models is sufficient to correct the Cl^- transport defect, it remains unclear how the level of gene expression and the efficiency of gene transduction interact *in vivo*. The ability to correct the electrophysiological defect *in vivo* with our vector system creates a unique opportunity to assess this issue. This thesis compared 3 different strength promoters and their effects on the level of correction of the CFTR defect observed. Unfortunately no definitive conclusions can be drawn from this data as experiments in the disease model showed no significant difference in the level of correction seen between the 3 different promoters. Testing in normal mice using marker genes under control of the 3 test promoters successfully showed the different strength of each promoter (Luciferase), and the cell type specificity (LacZ), but was not easily translated into the disease model. These experiments should be repeated with vector preparations that result in sufficiently high levels of gene transfer to achieve at least partial electrophysiological correction. In addition collection of blood samples pre and post treatment would allow testing for immune responses to the transgenes used that may influence the results obtained.

Finally, when treating a disease it is important to target the sites where disease is present. In cystic fibrosis, the major target organ is the lung, as it is lung disease that ultimately causes morbidity and mortality in cystic fibrosis patients. Aerosolisation of our gene therapy vector into the rat lung is described in this thesis. The technique is still very early in development, however, this work describes a significant starting point for a number of previously unknown methods in our laboratory, including the intubation of the rat and ventilation using the SCIREQ flexivent. The delivery of pre-treatment and lentivirus into the lungs using the Aerogen piezoelectric nebulizer was also developed. Further work to

determine concentrations of the LPC pre-treatment and volumes of both pre-treatment and virus that can be tolerated by the rat also need to be developed in order to begin larger studies to determine the areas of the lung that are targeted to achieve high level gene expression. Again, these experiments highlighted the complex issues raised by moving from model systems to more biologically relevant models.

In conclusion, the use of lentiviral vectors as gene delivery agents is not a trivial process. Many aspects of the gene delivery process need to be analysed to obtain the best distribution and level of expression in the target organ. Robust disease models are required for analysis using the therapeutic gene, and tools for analysis of this gene transfer are required. All of the issues described above need to be carefully addressed to obtain the level of gene transfer required to allow this treatment to be transferred to the clinic.