Towards Gene Therapy for Cystic Fibrosis Airway Disease: Development of Single-Dose Lentiviral Gene Transfer for Lifetime Airway Expression

Alice Stocker
School of Medical Sciences
The University of Adelaide
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I dedicate this thesis to
my family, particularly my husband Dave,
for their constant support and unconditional love.
I love you all dearly.
Chapter 1  A Review Of The Literature  01

1.1  Cystic Fibrosis: The Disease  02
1.1.1  A Brief History Of Cystic Fibrosis  02
1.1.2  Clinical Presentation Of Cystic Fibrosis  05
   a. Cystic Fibrosis Lung Disease  06
   b. Cystic Fibrosis Pancreatic Disease  07
   c. Cystic Fibrosis & Other Organs  07
   d. Liver Disease  08
   e. Infertility  08
   f. Cystic Fibrosis Related Diabetes  09
   g. Osteopenia & Osteoporosis  10
1.2  Cystic Fibrosis: The Gene  11
1.2.1  A General Overview  11
1.2.2  Mutations of CFTR  12
   a. Class I Mutation  13
b. Class II Mutation

c. Class III Mutation

d. Class IV Mutation

e. Class V Mutation

1.2.3 Assessment of Chloride Channel Function

1.2.4 Incidence of Cystic Fibrosis

1.2.5 Neonatal Screening for Cystic Fibrosis

1.2.6 Current Management of Cystic Fibrosis

a. Specialised Cystic Fibrosis Centres

b. New Antimicrobial Medications

c. Pseudomonas Vaccination

d. Organ Transplantation

e. Mucolytics

f. Airway Hydration

g. Nutrition

h. Pancreatic Enzymes

1.3 The Airway Epithelium

1.3.1 A General Overview

1.3.2 Antimicrobial Properties of Airway Surface Liquid

1.3.3 Innate Immune System

1.3.4 Structure of the Conducting Airways

1.3.5 Human Airway Epithelial Ion Transport

a. The Low-Volume Hypothesis

b. The High Salt Hypothesis

c. The Serous Cell Malfunction Hypothesis

1.3.6 Evaluating the Hypotheses

a. In Vitro Studies

b. Mouse Studies
c. Human Studies

1.3.7 Animal Models Of Cystic Fibrosis Airway Disease
   a. The Murine Model
   b. Development Of Other Animal Models

1.4 Therapies For Cystic Fibrosis Airway Disease
   1.4.1 Gene Therapy For Cystic Fibrosis Airway Disease
   1.4.2 Non-Viral Gene Transfer Vectors
   1.4.3 Viral Gene Transfer Vectors
      a. Adenovirus
      b. Adenovirus Vectors: Progress In Gene Therapy Clinical Trials
      c. Helper-Dependent Adenoviral Vectors
      d. Adeno-Associated Viral Vectors
      e. Adeno-Associated Viral Vectors: Progress In Gene Therapy Clinical Trials
      f. Retroviral Vectors
      g. Lentiviral Vectors

1.4.4 The Women's & Children's Hospital Lentivirus
1.4.5 Stem Cells & Cell Therapies For Cystic Fibrosis
1.4.6 Barriers To Viral Gene Transfer To The Airway Epithelium
1.4.7 Physical Barriers Present In Normal Airway Epithelium
1.4.8 Physical Barriers Present In Cystic Fibrosis Airway Epithelium
1.4.9 Progenitor Cells Of The Airway Epithelium
1.4.10 Mild Damage To The Epithelium Increases The Level Of Gene Expression
1.4.11 Lysophosphatidylcholine Pre-Treatment To Improve Gene Transfer

1.5 Research Aims
   1.5.1 Overarching Aim
   1.5.2 Specific Aims
Chapter 2  Materials & Methods  

2.1 Chemicals and Reagents  
2.2 Virus Production  
2.2.1 Cell Culture  
2.2.2 DNA Plasmid Preparation  
2.2.3 DNA Construction and Virus Production  
2.3 Large Scale Recombinant Lentivirus Production and Processing  
2.3.1 General  
2.3.2 CaPO₄ Co-Precipitation and Transfection  
2.3.3 Filtration & Concentration of the Lentivirus Vector  
2.3.4 Real Time-Polymerase Chain Reaction (RT-PCR) to Determine Virus Titre  
2.3.5 Sample Preparation  
2.3.6 Virus Titre Assay  
2.3.7 X-Gal Determination of LVLacZ Viral Titre  
2.3.8 Assay For Detection of Helper Virus  
2.3.9 Sample Preparation  
2.3.10 Virus Titre Assay  
2.3.11 X-Gal Determination of LVLacZ Viral Titre  
2.3.12 Assay For Detection of Helper Virus  
2.4 Mice  
2.4.1 General Information  
2.4.2 In Vivo Instillation of Lentiviral Vector into the Mouse Nasal Airway  
2.4.3 Processing of Mouse Heads For LacZ Gene Expression  
2.4.4 Safranin-O Histological Staining  
2.4.5 Haemotoxylin/Eosin Histological Staining  
2.4.6 Transduced Cell Type Identification within the Airway Epithelium  
2.4.7 Identification of Label-Retaining Cells (LRC)
Chapter 3  Airway Pre-Treatment Enhances Lentiviral Mediated Gene Expression In Living Mouse Airways

3.1 Introduction 94
3.2 Methods 95
   3.2.1 LPC Pre-Treatment to Enhance Airway Gene Expression 95
   3.2.2 Studies of Altered Time Delay Between LPC and LV/LacZ Delivery on Enhancement of Airway Gene Expression 95
3.2.3 Histological Analysis of the Effect of LPC on the Airway Epithelium 96
3.3 Results 96
   3.3.1 LPC Enhancement Can Be Optimised for Airway Gene Transfer 97
   3.3.2 The Delay Between LPC and LV Gene Vector Delivery Affects the Level of Airway Gene Expression 100
   3.3.3 Distribution of Gene Transfer 102
   3.3.4 Increasing LPC Concentration Increases The Histological Changes in the Epithelium 105
3.4 Discussion 108
   3.4.1 LPC Concentration Can Be Optimised To Enhance Gene 111
Chapter 4  A Lifetime of Airway Gene Expression After a Single Dose of Lentivirus Vector

4.1 Introduction  119
4.2 Method  121
4.2.1 Instillation of a Single Dose of LVLacZ & Transduced Cell Type Identification within the Airway Epithelium  121
4.3 Results  122
4.3.1 24 Months of Gene Expression is Produced After a Single Dose of LVLacZ  122
4.3.2 A Single Dose of LVLacZ Produces Extended Gene Expression in Ciliated Respiratory Cells  126
4.4 Discussion  129
4.4.1 24 Months of In Vivo Gene Expression After a Single LVLacZ Vector Dose  129
4.4.2 Appropriate Cell Types Transduced For Cystic Fibrosis Gene Therapy  137

Chapter 5  Correction of CFTR Function In Vivo  139

5.1 Introduction  140
5.2 Methods  141
5.2.1 Instillation of a Single Dose of LVCFTR into the Mouse Nasal Airway Epithelium

5.2.2 Transepithelial Potential Difference Assessment of Functional CFTR within Mouse Airway Epithelium

5.2.3 Immunological Detection of CFTR within Mouse Airway Epithelium

5.3 Results

5.3.1 Restoration of CFTR Function Following a Single-Dose of LVCFTR
   a. One Month Post-Treatment
   b. Three Months Post-Treatment
   c. Twelve Months Post-Treatment
   d. Greater Than Eighteen Months Post-Treatment

5.3.2 Immunohistochemistry

5.4 Discussion

Chapter 6 Identification of Progenitor Cells within the Mouse Airway Epithelium Using Bromodeoxyuridine Incorporation

6.1 Introduction

6.2 Methods

6.2.1 Repeat Damage by Lysophosphatidylcholine and BrdU Labelling

6.2.2 BrdU Immunohistochemistry

6.2.3 LacZ Immunohistochemistry

6.2.4 Heat-Induced Epitope Retrieval

6.3 Results

6.3.1 BrdU Immunohistochemistry
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3.2 LacZ Immunohistochemistry</td>
<td>159</td>
</tr>
<tr>
<td>6.4 Discussion</td>
<td>160</td>
</tr>
<tr>
<td>Chapter 7 General Discussion</td>
<td>165</td>
</tr>
<tr>
<td>7.1 Overview of the Work Described in this Thesis</td>
<td>166</td>
</tr>
<tr>
<td>7.2 Future Directions</td>
<td>170</td>
</tr>
<tr>
<td>7.2.1 Immune Response to Gene Therapy</td>
<td>170</td>
</tr>
<tr>
<td>7.2.2 Gene Delivery to the Lung</td>
<td>172</td>
</tr>
<tr>
<td>7.2.3 Animal Models to Assess the Efficiency of Lentivirus Gene Therapy to the Airway Epithelium</td>
<td>173</td>
</tr>
<tr>
<td>7.2.4 Improved Assessment of CF Gene Therapy Success</td>
<td>175</td>
</tr>
<tr>
<td>7.3 How Much CFTR Transduction is Required to Achieve Clinical Benefit</td>
<td>177</td>
</tr>
<tr>
<td>7.4 The Ethical Considerations of Gene Therapy</td>
<td>180</td>
</tr>
<tr>
<td>7.4.1 Treatment Verses Enhancement</td>
<td>181</td>
</tr>
<tr>
<td>7.4.2 Somatic and Germline Gene Therapy</td>
<td>183</td>
</tr>
<tr>
<td>7.5.3 In Utero Gene Therapy</td>
<td>187</td>
</tr>
<tr>
<td>7.6 Concluding Remarks</td>
<td>189</td>
</tr>
<tr>
<td>Appendices</td>
<td>190</td>
</tr>
<tr>
<td>Appendix 1: Company Addresses</td>
<td>191</td>
</tr>
<tr>
<td>Appendix 2: Solutions And Buffers</td>
<td>193</td>
</tr>
<tr>
<td>Appendix 3: Animal Monitoring Sheet</td>
<td>200</td>
</tr>
<tr>
<td>Appendix 4: Challenges Associated With Mouse Nasal TPD Recordings</td>
<td>202</td>
</tr>
<tr>
<td>Appendix 5: Publications</td>
<td>208</td>
</tr>
<tr>
<td>Bibliography</td>
<td>226</td>
</tr>
</tbody>
</table>
Abstract

Cystic Fibrosis (CF) is the most common, fatal autosomal recessive disorder affecting the Caucasian population with a frequency of 1 in 2500 live births and has a current median survival age of approximately 33 years. Characteristics of CF include abnormalities in sweat glands, malnutrition, pancreatic disease and infertility. It is however, severe and chronic lung disease that currently accounts for greater than 95% of morbidity and mortality in CF patients. The CF transmembrane conductance regulator gene was discovered in 1989 and in vitro correction of the defect soon followed, providing the basis for gene therapy as a potential cure for CF lung disease. To date, the lack of an efficient gene transfer vector system combined with the physical barriers of the airway epithelium limit the successful application of CF gene therapy.

The work described in this thesis utilised a unique gene therapy approach developed by the CF Gene Therapy Research Group, which involved airway pre-treatment followed by gene delivery. Pre-treatment was with the natural detergent lysophosphatidylcholine (LPC), followed by a single-dose of a HIV-1 based lentivirus (LV) vector in vivo. Previously studies found significant gene expression within airway tissues, but areas of cell damage were also sometimes evident.

Initial work included examining the relationship between gene transfer, LPC dose and timing parameters, and airway epithelial damage. This study found that 0.3% LPC followed 60 minutes later with the LV produced significant gene expression within the airway, with only mild airway epithelial disturbance observed.

The longevity of LV-mediated gene expression was then evaluated in the nasal airway of C57Bl/6 mice using the LacZ marker gene. Treatment of mouse nasal airway
epithelium with the LPC prior to instillation of a single dose of an LVLacZ vector produced significant LacZ gene expression in many mice for at least 18 months. The finding of gene expression in one mouse after 24 months indicated essentially lifetime gene expression had been achieved.

We found that a single dose of LVLacZ produced immediate as well as lifetime mouse airway expression, confirming our hypothesis that use of an integrating vector extends transgene expression. Importantly, LVCFTR dosing achieved at least 12 months of CFTR expression, representing partial functional correction of the CFTR defect in CF knockout mice. These findings provide evidence that a single-dose Lentiviral gene transfer method may offer a novel in vivo therapeutic paradigm in the pursuit of a cure for CF airway disease.
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Alice Stocker and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Alice Stocker
17 April 2010
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back for all those long boozy lunches in the sunshine. No more social isolation by me!!

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Publications


A Stocker, P Cmielewski, DS Anson, DW Parsons. “Disturbance of airway epithelium is reduced, whilst Lentivirus-Mediated Gene Transfer is maintained, after Low Dose Lysophosphatidylcholine Pre-Treatment in vivo.” American Society For Gene Therapy, June 2005

A Stocker, P Cmielewski, DS Anson, DW Parsons. “Lentivirus-Mediated Gene Transfer Efficiency in vivo is maintained when using Low Dose Lysophosphatidylcholine Pre-Treatment.” European Cystic Fibrosis Conference, June 2005


A Stocker, P Cmielewski, DS Anson, DW Parsons "Lentivirus-Mediated Gene Transfer Efficiency in vivo is maintained when using Low Dose Lysophosphatidylcholine Pre-Treatment.” Journal of Cystic Fibrosis, (2005), 4 (Supplement):29 Abstract 107


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Australian and New Zealand Cystic Fibrosis Conference
“New Investigator” Award (2005)

Semi-finalist for the Women's & Children's Hospital Young Investigator of the Year Award (2005)
List of Figures

Figure 1.1: Example of a typical TPD trace from a cftr

Figure 1.2: Structure of Airway Epithelium

Figure 1.3: The ASL in healthy (A) and CF (B) airways

Figure 1.4: Ion Transport across the Airway Epithelium:

Figure 1.5: Women’s & Children’s Hospital Lentiviral Vector Expression Constructs

Figure 1.6: Molecular Structure of Lysophosphatidylcholine

Figure 2.1: The Structure of the LacZ Lentivirus Vector Construct

Figure 2.2: Filtration set-up for large scale Recombinant Lentivirus Production & Processing

Figure 3.1: Effect of Anaesthesia and Exposure to LPC / LV Vector on Mouse Weight

Figure 3.2: Airway LVLacZ gene expression in nasal airway after pre-treatment with increasing concentrations of LPC.

Figure 3.3: LVLacZ gene expression in nasal airway after different time-dosing regimes.

Figure 3.4: Enface view of LVLacZ gene expression in mouse nose.

Figure 3.5: Transduced cell types after 0.3% LPC pre-treatment, 60 minutes before LVLacZ vector delivery.

Figure 3.6: Histological effects on airway epithelium.
Figure 3.7: Effect of LPC on the Airway Epithelium.

Figure 4.1: Diagrams of the Cross-Sections of the Murine Nasal Airways.

Figure 4.2: Gene Transfer at Different Time Points after a Single Dose of the LVLacZ vector into the Mouse Nasal Airway.

Figure 4.3: LVLacZ Gene Expression Over 24 Months.

Figure 4.4: Transduced Cell Types after a Single Dose of LVLacZ Vector.

Figure 4.5: Percentage of LacZ Positive Cells after a Single Dose of LVLacZ Vector.

Figure 4.6: An example of real time bioluminescence imaging in a mouse after delivery of 0.3% LPC one hour prior to delivery of an HIV-1 derived LV vector containing the EF1a luciferase gene.

Figure 5.1: TPD Measurement set-up.

Figure 5.2: TPD Measurements of CF Mouse Treatment Groups.

Figure 5.3: The Number of TPD Experiments Rejected and Included at Each Time Point.

Figure A.1: Examples of Rejected TPD Traces.
List of Tables

Table 1.1: The five functional classes of CFTR mutations. 
Table 2.1: Volume of plasmids used to construct LV vector 
Table 2.2: Transfection Medium Composition. 
Table 5.1: CFTR Immunohistochemistry Antibody Dilutions. 
Table 6.1: BrdU Immunohistochemistry Antibody Dilutions. 
Table 6.2: LacZ Immunohistochemistry Antibody Dilutions.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB/PAS</td>
<td>Alcian Blue-Periodic Acid-Schiff</td>
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<tr>
<td>AdV</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-Associated Virus</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ASL</td>
<td>Airway Surface Liquid</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>β-Gal</td>
<td>Beta-Galactosidase</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celcius</td>
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<tr>
<td>cAMP</td>
<td>Adenosine 3', 5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy DNA</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>CFRD</td>
<td>Cystic Fibrosis Related Diabetes</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
</tr>
<tr>
<td>cftr&lt;sup&gt;tm1UNC&lt;/sup&gt;</td>
<td>CFTR knock-out Mouse Model</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Chloride Ion</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>CYWHC</td>
<td>Child, Youth &amp; Women's Health Service (formally the Women's &amp; Children's Hospital)</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ΔF508</td>
<td>In frame deletion of phenylalanine at position 508 of exon 10 of the CFTR gene</td>
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ATPD The difference between the TPD value recorded under basal + amiloride conditions and the TPD recorded under low Cl⁻ + amiloride conditions

dL Decilitre

DMEM Dulbecco's Modified Eagle's Medium

DNA Deoxyribonucleic Acid

DNase Deoxyribonuclease

DPX Distyrene-tricresyl-phosphate-xylene

ELISA Enzyme-Linked Immunoabsorbent Assay

EnaC Amiloride Sensitive Epithelial Sodium Channel

ER Endoplasmic Reticulum

FCS Foetal Calf Serum

FITC Fluoroscein Isothiocyanate

H&E Haematoxylin and Eosin

HDAdV Helper-Dependant Adenovirus

HIV Human Immunodeficiency Virus

hr Hour

HS Hypertonic Saline

HRP Horseradish Peroxidase

i.m. Intramuscular

i.p. Intraperitoneal

IRT Immuno-Reactive Pancreatic Trysonogen

K⁺ Potassium

kb Kilobase

L Litre

LacZ Beta-Galactosidase

LPC Lysophosphatidylcholine

LRC/s Label Retaining Cell/s

LV Lentivirus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MCC</td>
<td>Mucociliary Clearance</td>
</tr>
<tr>
<td>Min/s</td>
<td>Minute/s</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MLV</td>
<td>Murine Leukaemia Virus</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>MQ-H2O</td>
<td>Milli Q Water</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolts</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium Ion</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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<tr>
<td>ng</td>
<td>Nanograms</td>
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<tr>
<td>NSS</td>
<td>Normal Swine Serum</td>
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<tr>
<td>o/n</td>
<td>Overnight</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCL</td>
<td>Periciliary Liquid</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PD</td>
<td>Potential Difference</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Pancreatic Insufficiency</td>
</tr>
<tr>
<td>RCR</td>
<td>Replication Competent Retrovirus</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>Saf-O</td>
<td>Safranin-O</td>
</tr>
<tr>
<td>SCID-X1</td>
<td>Severe Combined Immunodeficiency, X linked</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junction</td>
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<tr>
<td>TPD</td>
<td>Transepithelial Potential Difference</td>
</tr>
</tbody>
</table>
TU  Transducing Units
UNC  University of North Carolina
v  Volume
VSV-G  Vesicular Stomatitis Viirus Glycoprotein G
w  Weight
X-Gal  5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Chapter 1
A Review of the Literature
1.1 Cystic Fibrosis: The Disease

1.1.1 A Brief History of Cystic Fibrosis

Cystic Fibrosis (CF) is the most common life threatening genetic disorder effecting 1 in 2500 Australians (George, Jones et al. 2009). While CF was not recognised as a disease until the 1930s, certain aspects of it were identified much earlier. Literature from Germany and Switzerland in the 1700s warned “Wehe dem Kind, das beim Kuß auf die Stirn salzig schmeckt, er ist verhext und muss bald sterben” (“Woe is the child who tastes salty from a kiss on the brow, for he is cursed, and soon must die”), recognising the association between salt loss in CF and illness. In 1905 Landsteiner described the neonatal intestinal obstruction meconium ileus, which affects about 15% of infants with CF (Landsteiner 1905). Additionally, in 1912 Garrod et al identified a possible recessive mode of inheritance in families whose children suffered from steartorrhoea and died from bronchopneumonia (Garrod and Hurley 1912). In 1936 Fanconi et al described some children suffering from a “coeliac syndrome” also having pancreatic changes and named the disease cystic pancreas fibromatosis and bronchiectasis (Fanconi, Uehlinger et al. 1936). Although some consider Fanconi’s paper to be the first clear description of CF, it was Dorothy Andersen, in the Babies’ Hospital New York 1938 report “Cystic Fibrosis of the Pancreas and its Relation to Celiac Disease: A Clinical & Pathological Study”, who clearly characterised CF of the pancreas and correlated it with the lung and intestinal disease (Anderson 1938).

A study conducted at the Children’s Hospital in Boston in 1943 by Sidney Faber recognised that CF was a generalised disease and named it “mucoviscidosis” (Faber 1943) a term still widely used throughout Europe. Faber et al concluded “the respiratory tract damage therefore depends on primary obstruction by thick mucus, failure of proper lubrication of ciliated epithelium and secondary staphylococcal infection”. However,
there was no agreement in the field as to the primary cause of the lung disease, a debate that would continue until the early 1980s. It was also during the 1940s that a study conducted by Anderson and Hodges investigated 103 families and concluded that the familial incidence indicated a recessive mode of transmission (Andersen and Hodges 1946). However, they remained convinced that the disease required another factor for its expression. Thus, the condition was considered to be primarily a nutritional problem, with vitamin A deficiency being an important component. In 1946 the use of antibiotics to treat CF lung disease was reported by Paul di Sant’Agnese, another leading clinician of the time. In 1953, Di Sant’Agnese also reported the abnormalities in sweat glands and the sweat test was developed to assist in the diagnosis of CF during this time (Di Sant’Agnese, Darling et al. 1953).

Through the 1960s sweat tests became more generally available and the accuracy of diagnosis was considerably improved, but despite the ability to diagnose the disease, the outlook for CF patients was still poor, with very few surviving into adulthood.

The 1970s saw the rise of a new, potentially dangerous pathogen, *Pseudomonas aeruginosa*. The severity of *Pseudomonas aeruginosa* infections and the increased frequency of infection throughout first North America (Isles, Maclusky et al. 1984) then the UK (Simmonds, Conway et al. 1990) led to a radical change of both clinical practice and the social habits of patients. This increased infection rate lead to the cancellation of the popular Cystic Fibrosis Holiday Camps that were designed to help with CF children’s socialisation and peer support. This also marked the introduction at many clinics, though not all, of three-monthly intravenous antibiotics as a treatment and preventive plan of action against *Pseudomonas aeruginosa* infection (Szaff, Hoiby et al. 1983; Jensen, Pedersen et al. 1989).
Evidence of epithelial dysfunction in patients with CF was identified by Michael Knowles of the University of North Carolina (Knowles, Gatzy et al. 1981). Significantly, these electrophysiological abnormalities identified were present in neonates, before any infection, and by 1987 Eric Alton had developed a clinical method of measuring nasal potential difference as a marker for CF (Alton, Hay et al. 1987). From the early 1980s scientists were trying to identify the defective CF gene and the first mutation for CF F508, was discovered by Francis Collins, Lap-Chi Tsui and John R. Riordan on the seventh chromosome (Tsui, Buchwald et al. 1985). The discovery of the CF gene soon followed and was termed the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene (Kerem, Rommens et al. 1989; Riordan, Rommens et al. 1989; Rommens, Iannuzzi et al. 1989) and the three co-discoverers, Lap-Chi Tsui, Collins and Riordan, were awarded the Paul di Sant’Agnese Prize at the 1989 North American CF meeting. Since that significant discovery, more than 1604 different mutations have been described (Zielenski, Sang et al. 2007). The 1980s also saw the first heart-lung transplants and the first heart-lung-liver transplants provided as a treatment option for those who were previously in the terminal stages of the disease.

After the discovery of the CFTR gene, the 1990s began full of expectation that a cure was now possible, and indeed two papers reported correction of the CFTR defect in vitro using retroviral gene transfer (Drumm, Pope et al. 1990; Rich, Anderson et al. 1990; Rich, Anderson et al. 1990) and the vaccinia-T7 hybrid expression system (Rich, Anderson et al. 1990). Following this initial encouraging progress, three mouse models were developed, the \textit{cftr}^{tm1Unc} knockout mouse (from North Carolina) (Snouwaert, Brigman \textit{et al.} 1992), the \textit{cftr}^{tm1Cam} knockout mouse (from Edinburgh) (Dorin, Dickinson \textit{et al.} 1992) and the \textit{cftr}^{tm1Hgu} partial knockout mouse (from Cambridge) (Ratcliff, Evans \textit{et al.} 1993), for further \textit{in vivo} investigations of CFTR function and correction. The 1990s also saw a continued improvement in clinical care and,
subsequently, the median life expectancy of a child born in 1990 had reached about 23 years (Elborn, Shale et al. 1991).

A recently published study from the UK CF Registry highlighted the continued improvement in prognosis over the past 30 years. From the current survival calculations they estimated that with current range of treatments available, most CF patients born from 2007 onwards will reach the fourth or fifth decade of life (Dodge, Lewis et al. 2007). While still less than that of the general population, this is a dramatic improvement from even 20 years ago. Continued efforts in both the scientific and clinical communities see regular advancements, which aim to culminate in an effective treatment plan and the eventual cure of this devastating disease.

1.1.2 Clinical Presentation of Cystic Fibrosis

The original presentation of what is now known as Cystic Fibrosis was neonatal intestinal obstruction and intestinal and respiratory complications. Interestingly, pancreatic histology was similar to vitamin A deficiency, and it was thought that the persistent respiratory infections seen were due to a reduction in vitamin A absorption resulting from the inability of the diseased pancreas to secrete enzymes necessary for absorption of fat.

Cystic Fibrosis is now characterised by

> viscous mucus secretions in the airways,
> pancreatic insufficiency caused by mucus blocking the pancreatic ducts,
> excessive salt secretion in sweat,
> male infertility and
> reduced fertility in females.
As these manifestations appear over time, failure to thrive in the first few weeks of birth was often the first clinical sign seen. The introduction of the neonatal screening program in Australia means that CF babies are identified at birth, before these clinical signs become obvious. In more recent times, where management regimes have increased life expectancy, CF patients have begun to contend with added complications, such as diabetes, osteoporosis and liver disease. The most serious of these clinical manifestations are CF lung and pancreatic disease below.

### a. Cystic Fibrosis Lung Disease

Chronic and progressive lung disease in CF is caused by recurrent airway infection and inflammation (Armstrong, Hook *et al.* 2005), which is attributed to over 95% of the morbidity and mortality now associated with CF. This is characterised by a self-perpetuating cycle of airway obstruction, chronic bacterial infection, and inflammation resulting in structural damage to the airway leading to a progressive loss of lung function and ultimately, respiratory failure. The major pathological manifestations of CF lung disease, mucus obstruction and infection, are initially confined to the conducting airways (Welsh, Tsui *et al.* 1995; Holder, Griesenbach *et al.* 2006). It is evident that airway disease is acquired sometime after birth with infants displaying no obvious abnormalities, however if untreated CF babies die within 12 months of birth (Welsh, Tsui *et al.* 1995). While mucus obstruction is thought to be the primary initiator of CF lung disease, the chronic infection of the respiratory tract appears to be the more destructive process (Chmiel, Berger *et al.* 2002). Excess mucus present in the airways provides a supportive environment for the growth of pathogenic organisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Haemophilus influenzae* and, once established, these organisms are virtually impossible to eradicate. Thus begins the cycle of repeated obstruction, infection and inflammation resulting in bronchiolectasis.
(chronic dilation of the bronchioles) followed by bronchiectasis (chronic dilatation of the bronchial tubes) which progressively destroys the tissue of the lung (Welsh, Tsui et al. 1995).

**b. Cystic Fibrosis Pancreatic Disease**

Symptoms related to the gastrointestinal tract may predominate, but they are rarely life threatening if treated properly. Pathological changes of the pancreas of CF patients were noted as early as 1936 when Dorothy Anderson called the disease "cystic fibrosis of the pancreas". About 85% - 90% of CF patients present with exocrine pancreatic insufficiency (PI) (Welsh, Tsui et al. 1995), and between 10% - 15% of these individuals also display meconium ileus at birth (Hodson and Geddes 1995). The thick mucus secretions seen in the lung are also present in the pancreatic ducts, blocking the movement of lipolytic and proteolytic pancreatic enzymes into the gut, resulting in irreversible damage to the pancreas, often with painful inflammation. The insufficient levels of these pancreatic enzymes within the gut leads to malabsorption due to difficulty in digesting and absorbing fats and protein. This pancreatic dysfunction leads to severe malnutrition, evidenced as failure to thrive by infants and young children with CF. Fortunately, current management with pancreatic enzyme supplements means PI is rarely associated with mortality.

c. **Cystic Fibrosis & Other Organs**

Cystic Fibrosis is a multi-system disease and with the increase in the number of adults with CF, and advances in medical testing techniques has allowed researchers to identify previously unrecognised chronic complications of CF. Obviously, dysfunctional
CFTR in individual organs results in complications, but in some cases this is also compounded by the effects of systemic inflammation (Shead, Haworth et al. 2006). For example, during lung exacerbations the inflammatory response has been shown to result in increased formation of osteoblasts, which contribute to an increase in the bone re-absorption seen in CF-related bone disease.

*d. Liver Disease*

Previously, CF liver disease appeared much less frequently than pulmonary and pancreatic disease, thus for many years liver involvement in CF received little attention. However, it is now considered as the third-leading cause of death after cardio-respiratory and transplantation complications (Lamireau, Martin et al. 2006), accounting for approximately 2% of overall CF mortality (Scott-Jupp, Lama et al. 1991).

Cystic fibrosis liver disease is characterised by focal biliary cirrhosis caused by mucous obstruction of the bile ducts. This is the most clinically relevant hepatic problem associated with CF, since chronic biliary damage may lead to multilobular biliary cirrhosis, followed by development of portal hypertension and eventually, liver failure (Colombo 2007).

*e. Infertility*

One of the many clinical manifestations of CF is infertility or sub-fertility in both males and females. The vas deferens, the thin duct that conveys sperm from the testes, is also a chloride-based fluid secreting organ. It is perhaps more vulnerable than any other organ to the destructive effects of CF lesions. A classic study by Oppenheimer
and Esterly established that the loss of the vas deferens in CF males is not the result of failure to develop, but is instead caused by degeneration secondary to obstruction (Oppenheimer and Esterly 1969). While 97% of male CF-related infertility is due to this vas deferens degeneration (Chan, Ruan et al. 2008), other causes, such as reduced sperm quality, are currently under intense debate (Slezak R 2007; Tamburino, Guglielmino et al. 2008).

In contrast to male CF-related infertility, females with CF show no anatomical abnormalities within the reproductive tract. However, it has been generally accepted that sub-fertility in females with CF is caused by thick cervical mucus that blocks the migration of sperm, and by the blockage of the fallopian tubes, again with thick mucus secretions, preventing egg-sperm fusion (Chan, Ruan et al. 2008). Additionally, mammalian sperm must be activated, or capacitated, within the female reproductive tract to fertilize eggs, a process driven by the presence of bicarbonate, a CFTR-dependent secretory process, (Wang, Zhou et al. 2003). Thus, the lack of bicarbonate within the female reproductive tract, which can prevent the activation of sperm and the possibility of fertilisation. However, the exact role of CFTR in reproductive physiology and in male and female infertility is not fully understood (Hodges, Palmert et al. 2008).

f. Cystic Fibrosis Related Diabetes

Cystic fibrosis-related diabetes (CFRD) is the most common extra-pulmonary complication of cystic fibrosis, occurring in 15-30% of adult cystic fibrosis patients (Alves Cde, Aguiar et al. 2007). However, as life expectancy increases, so does the incidence of CFRD. Dysfunction of chloride channel function results in viscous pancreatic duct secretions causing the development of obstructive lesions, fat cell infiltration and
progressive islet cell fibrosis. (Lombardo, De Luca et al. 2003; Mackie, Thornton et al. 2003) This leads to abnormally low secretion of both insulin and glucagon (Dobson, Sheldon et al. 2004). In addition, CF patients without CFRD still display a decrease in insulin secretion compared with the norm. Repeated infections and corticosteroid administration also contribute to this decrease and the eventual development of insulin resistance in many CF patients, (Dobson, Sheldon et al. 2004). Interestingly, female patients, homozygous for ΔF508, and patients with severe pancreatic insufficiency present a higher risk of developing CFRD (Alves Cde, Aguiar et al. 2007).

g. Osteopenia & Osteoporosis

Osteopenia (decreased bone mass and a precursor to osteoporosis) and osteoporosis (thinning of the bones to the point of brittleness) are more recently recognised complications faced by adolescents and adults with CF. The origin of bone disease in CF is multifactorial, with malabsorption of vitamin D, poor nutritional status, physical inactivity, glucocorticoid therapy, and delayed pubertal maturation or early hypogonadism all being important contributing factors (Aris, Merkel et al. 2005). The prevalence of bone disease appears to increase with the severity of lung disease and malnutrition (Laursen, Molgaard et al. 1999). Younger and healthier individuals are more likely to have normal bone mineral density suggesting that the bone disease is not directly related to the CFTR mutation, but occurs as patients get older because of the contributing factors mentioned above.
1.2. Cystic Fibrosis: The Gene

1.2.1 A General Overview

In 1989 the CF gene was mapped to chromosome 7q31.2 by Tsui et al. (Tsui, Buchwald et al. 1985) and through the use of positional cloning was isolated and sequenced later that year (Kerem, Rommens et al. 1989; Riordan, Rommens et al. 1989; Rommens, Iannuzzi et al. 1989). The CF gene is 250,000 base pairs long and encodes a membrane protein of 1,480 amino acids known as the CF Transmembrane Conductance Regulator (CFTR) protein. The CFTR gene belongs to the ATP-binding cassette transporter superfamily of proteins, collectively known as ABC transporters (Riordan, Rommens et al. 1989; Riordan, Rommens et al. 1989). These proteins are mainly involved in the APT-dependent transport of large molecules across cell membranes.

Located on the apical surface of epithelial cells in many organs, including the lung, liver, pancreas, digestive tract, reproductive tract, and skin, CFTR functions as a chloride (Cl-) channel, regulated by 3',5'-cyclic adenosine monophosphate AMP (cAMP) (Boat and Cheng 1989). As well as its role as a Cl- channel, CFTR influences the transport of other ions, such as sodium (Na+) (Boucher 1994). Specifically, via the down-regulation of an amiloride-sensitive Na+ channel (EnaC), CFTR regulates the levels of Na+ in the airway surface liquid layer (Stutts, Canessa et al. 1995). In a normal airway, a tightly regulated equilibrium exists, ensuring sufficient hydration of the epithelial surface, but when CFTR is mutated or absent defective ion transport ensues, producing surface secretions and reduced hydration levels, leading to increased infection and subsequent lung disease. A detailed review of the structure and function of CFTR is described by Riordan (Riordan 2008).
1.2.2 Mutations of CFTR

There are currently 1604 mutations of the CF gene that have been identified and logged on the CF Mutation Database (Zielenski, Sang et al. 2007). The most common is a nonsense mutation, of which 637 have been discovered, that produces a CFTR molecule which is synthesised and processed normally, but is abnormally regulated (Rosenstein and Zeitlin 1998). CFTR mutations are classified into five categories, based on the alterations to the CFTR protein (Table 1).

Table 1.1: The five functional classes of CFTR mutations (Zielenski and Tsui 1995)

NOTE:
This table is included on page 12 of the print copy of the thesis held in the University of Adelaide Library.
The correlation between the CFTR genotype and CF disease phenotype has been extensively studied, however the relationship is still not fully understood (Rowntree and Harris 2003). Many groups have described the connection between pancreatic manifestations of CF and CFTR genotype, but the severity of pulmonary disease appears to be highly variable and not specifically linked to CFTR genotypes (Rowntree and Harris 2003). However, it has been reported in some studies that Class I, II and III mutations result in a classic CF phenotype with pancreatic insufficiency, while Class IV and V mutations result in milder expression of the disease (Proesmans, Vermeulen et al. 2008).

a. Class I Mutation

In class I mutations there is a premature termination of the CFTR mRNA translation. This is caused by either base substitutions that create stop codons or by mutations that shift the reading frame or nonsense mutations which result in defects in protein production (Zielenski and Tsui 1995). An example of a class I mutation is G542X, which results from a nucleotide change (G to T) at base pair 1756 resulting in the production of a stop codon at position 542 in exon 11 in the gene (Zielenski, Sang et al. 2007). This mutation results in no protein being synthesised within the nucleus of the cell and is associated with severe PI (Zielenski, Sang et al.).

b. Class II Mutation

The most common CF gene mutation is ΔF508, a class II mutation which is found in more than 70% of CF Caucasian chromosomes (Rosenstein and Zeitlin 1998). This mutation results from a deletion of three base pairs in the CFTR nucleotide sequence.
(Kerem, Rommens et al. 1989), which causes an in-frame deletion of the amino acid phenylalanine located at position 508 in exon 10 in the gene (Kerem, Rommens et al. 1989). When the normal CFTR protein is synthesized, it is transported to the endoplasmic reticulum (ER) and Golgi apparatus for additional processing before being integrated into the cell membrane. When a CFTR protein with the ΔF508 mutation reaches the ER, the quality-control mechanism of this cellular component recognizes that the protein is folded incorrectly and marks the defective protein for degradation. As a result, ΔF508 never reaches the cell membrane (Kerem, Rommens et al. 1989). People who are homogenous for the ΔF508 mutation tend to have the most severe symptoms associated with CF, including severe lung disease and PI (Hodson and Geddes 1995). The ΔF508 deletion has been found in approximately 90% of CF patients in Australia, with approximately 51% homozygous for this defect (Goldblatt, Creegan et al. 1995).

c. Class III Mutation

Class III mutations are regulatory mutations in which protein reaches the surface of the cell but fails to respond consistently to cyclic AMP activation signals (Zielenski and Tsui 1995). An example of a class III mutation is G551D, which occurs as a result of a nucleotide change (G to A) at base pair 1784 (Zielenski, Sang et al. 2007). This causes an amino acid change (Glycine to Aspartic Acid) at codon 551 in exon 11. In this mutation the CFTR protein is normally synthesised and transported to the endoplasmic reticulum and is integrated into the cell membrane, but regulation of CFTR is defective and thus CF disease develops.
d. Class IV Mutation

As with class III mutations, class IV mutations result in protein reaching the plasma membrane, but produce altered channel properties that result in defective protein conduction. An example of a class IV mutation is R117H, which occurs as the result of a nucleotide change (G to A) at base pair 482 (Zielenski, Sang et al. 2007). This results in an amino acid change (Arginine to Histidine) at codon 117 in exon 4 of the gene. Class IV mutations are generally associated with a milder CF phenotype.

e. Class V Mutation

Class V mutations result in reduced synthesis of CFTR due to a reduction in the amount of RNA necessary for normal production. An example of a class V mutation is A455E, which occurs as a result of a nucleotide change (C to A) at base pair 1496 (Zielenski, Sang et al. 2007). This leads to an amino acid change (Alanine to Glutamic Acid) at codon 455 in exon 9. Class V mutations are generally associated with a milder CF phenotype and no PI (Zielenski, Sang et al. 2007).

1.2.3 Assessment of Chloride Channel Function

Although the exact pathogenic pathways in CF are still debated, defective epithelial ion transport has been a known consequence of CF since 1981 (Knowles, Gatzy et al. 1981). Epithelial cells absorb Na⁺ from, and secrete Cl⁻ onto, the apical surface of the cells and the net ion flux across the cells generates a transepithelial electrical potential difference (PD), which concomitantly promotes water movement across the airway mucosa (Welsh 1987; Widdicombe, Bastacky et al. 1997). This can be assessed in the airway
in vivo by measuring transepithelial potential difference (TPD), the voltage generated across an electrically tight epithelium by the active transport of charged sodium and chloride ions. The bioelectrical properties measured using the TPD assay are believed to reflect the function of the CFTR protein. It is known that the subcutaneous space is isoelectric at all locations within the human body (Kanoh, Tamaoki et al. 2001), thus the transepithelial voltage can be quantified by referencing the PD to ‘zero’ in the submucosal space. Additionally, a subcutaneous reference electrode anywhere in the body would be the equivalent to a reference electrode in the submucosal space of the airway epithelium. The voltage between the reference electrode and the nasal electrode is considered to accurately reflect the TPD.

By perfusing the epithelium with solutions containing high or low levels of Cl\(^-\), different aspects of the nasal ion transport characteristics related to the presence or absence of CFTR can be examined. First, a basal solution that closely mimics the ionic properties of the normal ASL is perfused to obtain a neutral reading. Once a steady voltage reading has been achieved, the perfusion solution is changed to include amiloride in the basal solution. Amiloride blocks Na\(^+\) channels in the apical membrane of the respiratory epithelium, thereby inhibiting cellular Na\(^+\) uptake. The membrane potential on the apical surface becomes hyperpolarised (more negative) and thereafter the changes in TPD voltage readings will be limited to predominantly reflecting changes in Cl\(^-\) movement across the epithelium.

The perfusion is then switched to a low Cl\(^-\) solution where Cl\(^-\) ions are replaced with gluconate, a membrane impermeable ion, (with amiloride still present). The increased Cl\(^-\) gradient across the apical side increases the efflux of Cl\(^-\) through cAMP-activated Cl\(^-\) channels. This increase efflux of Cl\(^-\) depolarises (becomes less negative) the apical cell membrane potential, which is reflected in the reduced TPD response across the epithelium. An example of both a normal and a CF TPD trace are shown in Figure 1.1.
In the $cfr^{tm1Unc}$ knockout mouse (A) the administration of a low chloride solution an environment for Cl$^-$ secretion is created, but as CFTR is dysfunctional there is no Cl$^-$ secreted (i.e. little or no change is measureable in the TPD reading). Thus in contrast to the large response in non-CF epithelia, there is no change from the initial basal + amiloride PD level after the administration of the low Cl$^-$ solution.

In a normal mouse (B), when amiloride is added, Na$^+$ absorption is blocked so the TPD becomes less negative. When the low chloride solution is administered a large hyperpolarisation of the TPD occurs which, in the presence of amiloride, is assumed to be chloride secretion, which causes the TPD to become more negative. The original basal TPD is much lower in the $cfr^{tm1Unc}$ knockout mouse compared to the normal mouse. This is due to the Na$^+$ hyperabsorption characteristic of CF airways.
Unfortunately, there is no internationally accepted method for performing the TPD assay in the mouse model, (Schuler, Sermet-Gaudelus et al. 2004) or standardisation of the solutions used. Generally, all protocols involve the use of a basal solution and a low chloride solution, but while many groups use amiloride to block the action of the sodium channel (Hofmann, Senier et al. 1997; Smuszkiewicz, Drobnik et al. 2006; Sweezy, Smith et al. 2007), (Knowles, Gatzy et al. 1981; Stutts, Canessa et al. 1995; Jiang, Akita et al. 1997; Wang, Zabner et al. 2000), other groups use one, or a combination of; the CFTR agonist forskolin (Pavenstadt, Lindeman et al. 1991; Brady, Kelley et al. 2001; Hardiman, Lindsey et al. 2001; Salinas, Pedemonte et al. 2004), a CFTR inhibitor, thiazolidinone (Salinas, Pedemonte et al. 2004), or isoproterenol, which stimulates chloride secretion (Salinas, Pedemonte et al. 2004; Sermet-Gaudelus, Dechaux et al. 2005). Additionally, the location of the reference electrode can vary significantly between groups. In humans, for example, different researchers have tended to measure different areas of the nasal epithelia, but while the method used may be clearly stated, it prevents direct comparison across studies. The USA Cystic Fibrosis Foundation has developed a standardized protocol to be across all clinical trial centres, however this is currently unique to the United States with other countries yet to adopt a standardised method.

Mice present different challenges for TPD readings. In mice, the location of the nasal electrode can only be assessed via how deep into the nasal cavity it extends as visual or other access is not possible. The orientation and positioning of the electrode can be significantly different in individual mice, resulting in the reading coming from the septum in one mouse, but from a nasal turbinates in another (Parsons, Hopkins et al. 2000). This is important as the epithelium along the septum is rich in ciliated epithelial cells, while the turbinate contains more transitional ciliated cells or olfactory cells, which may provide a different (non-respiratory) TPD reading. These placement issues will contribute significantly to the high variability in the TPD measurement between
mice and when repeat measures are conducted on the same mouse. Great care must therefore be taken in ensuring that TPD measurements adhere to stated protocols to limit variability, and cautious interpretation of results from this type of experiment is required. A study conducted by Uta Griesenbach and colleagues investigated intra-animal variability by assessing TPD measurements taken on three occasions (days 1, 14 and 28), in 21 mice. This study showed that while the basal perfusion TPD measurement did not vary significantly at the different time points, the low chloride response showed a large amount of variability (Griesenbach, Smith et al. 2008). Their study also investigated inter- and intra-operator variability and showed that there was very little variability when assessing basal TPD measurements, indicating very high reproducibility between operators, however when assessing the low chloride response, the variability was much higher.

Nasal TPD measurements clearly show a difference between CF and non-CF subjects, thus the numbers required in each group are small if complete (100% treatment effect) correction is achievable (Griesenbach, Sumner-Jones et al. 2009). In order to detect smaller changes towards non-CF values, however much larger n values are required due to the high level of variability with the assay.

It is also important to note that practice, some skill and a good deal of experience is required to achieve greater consistency with this method, however electrical measurements in the mouse nose are technically difficult, even for experienced operators, and the smallest of movements of the catheter can markedly influence the recording.

In CF the absence of CFTR is not the cause of disease, it is the absence of functional CFTR, thus it is crucial to be able to assess in vivo if any potential gene therapy treatments result in functional CFTR. Analyses such as immunohistochemistry (against CFTR)
and PCR (to detect the presence of the gene transfer vector) are valuable in assessing whether significant CFTR is present in the transduced tissue. However, these tests are only able to detect the presence of CFTR, not whether it is functioning correctly. At present, TPD measurements are the only way to assess CFTR function in vivo.
1.2.4 Incidence of Cystic Fibrosis

The genetic basis of CF was noted as early as 1945 when, after the investigation of 103 families, Anderson & Hodges concluded that the familial incidence indicated a recessive mode of transmission (Andersen and Hodges 1946). In Australia about one in 25 individuals are heterozygote carriers and there is a disease incidence of one in 2500 live births, with males and females equally at risk (Massie, Delatycki et al. 2005). The incidence of CF and the frequency of mutations vary greatly among different ethnic and geographic populations. For example, the highest incidence of CF carrier rates occurs in the Ashkenazi Jewish population, with a heterozygote carrier incidence of one in 19 (Orgad, Neumann et al. 2001), while only one in 46 of the Hispanic population (Kosorok, Wei et al. 1996), one in 65 African Americans (Macek, Mackova et al. 1997) and one in 90 of the Asian population are carriers of a CF disease mutation (see table 1.2). Interestingly, the Finnish population’s carrier rate is only one in 250, 10 times less than the general Caucasian population (Kere J, Estivill X et al. 1994). The remote location of Finland and that the population is considered as a homogeneous isolate (Palo, Ulmanen et al. 2009) gives a possible explanation for the rarity of CF (Kere, Norio et al. 1989; Kere J, Estivill X et al. 1994), but there has not yet been a definitive explanation.
Table 1.2: Worldwide Incidence of Cystic Fibrosis, modified from (Bobadilla, Jr et al. 2002).

<table>
<thead>
<tr>
<th>Ethnic Background</th>
<th>Frequency of CF (1 case per X live births)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ireland</td>
<td>1,800</td>
</tr>
<tr>
<td>Australia</td>
<td>2,500</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>2,600</td>
</tr>
<tr>
<td>United States</td>
<td>2,800</td>
</tr>
<tr>
<td>Germany</td>
<td>3,300</td>
</tr>
<tr>
<td>Spain</td>
<td>3,500</td>
</tr>
<tr>
<td>Russia</td>
<td>4,900</td>
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<tr>
<td>Sweden</td>
<td>7,300</td>
</tr>
<tr>
<td>Finland</td>
<td>25,000</td>
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</table>

The prevalence of CF in Caucasian populations has been attributed to the high incidence of the ΔF508 mutation. It has been argued that the frequency of CF without the ΔF508 mutation would not be different from that observed in other ethnic populations (Zielenski and Tsui 1995). Subsequently, many theories on heterozygous advantage have been proposed to account for the high incidence of CF among Caucasians. The development of the transgenic CF mouse (cftrtm1Unc) has been used to demonstrate that a reduced level of CFTR (i.e. the level in a heterozygote) produces a proportional reduction in chloride and water secretion in the small intestines, which theoretically would prevent the fatal intestinal fluid loss during cholera infection (Gabriel, Brigman 2002).
et al. 1994). Many experts in the field have since discarded this theory as it was discovered that in human studies the heterozygote state was indistinguishable from the non-carrier state (Hogenauer, Santa Ana et al. 2000). Additionally it has been proposed that heterozygotes for the ΔF508 CFTR allele may have increased resistance to both tuberculosis (Meindl 1987) and typhoid fever (Poolman and Galvani 2007) although there has been no in vivo studies to confirm this. The low level of ΔF508 mutations outside European ancestral populations, in places where cholera, typhoid fever and tuberculosis were endemic, is not currently explicable in terms of these hypotheses.

1.2.5 Neonatal Screening for Cystic Fibrosis

All newborn babies in Australia and New Zealand are tested for a range of over 30 genetic or metabolic conditions, including CF, within 72 hours of birth (Barlow-Stewart, 2007). The process involves a blood sample is obtained via a heel prick and measurements of serum immuno-reactive pancreatic trypsinogen (IRT) are made. High levels of IRT in circulating serum are indicative of an obstruction in pancreatic secretion, a typical symptom of CF (Ryley, Robinson et al. 1981). All infants with an IRT value in the highest 1% of the population (i.e. higher than 140 ng/dL) are further tested for the five most common CF gene mutations using polymerase chain reaction (PCR). This test has the benefit of not only identifying infants with CF, but also asymptomatic carriers of the disease as well. Once a CF gene mutation is identified a pilocarpine iontophoresis sweat test is performed to confirm the diagnosis (Ryley, Robinson et al. 1981). The pilocarpine iontophoresis sweat test, standardised in 1959 (Gibson and Cooke 1959), measures the concentration of chloride and sodium that is excreted in the sweat. Because of the defragment of sweat salt levels in those with CF this can provide final confirmation of a positive CF diagnosis.
Several studies have shown individuals diagnosed by newborn screening have improved growth and preservation of normal pulmonary function without increased risk of *Pseudomonas aeruginosa* colonization and, interestingly, some indication of improved pulmonary status later in life (Farrell, Lai *et al.* 2005; Collins, Abbott *et al.* 2008; McKay and Wilcken 2008).

Australia, New Zealand, Europe and Canada have had compulsory National newborn screening programs in place for a number of years. To date, 37 states in the United States have adopted similar programs, in the hopes of improving CF outcomes. Additionally, CF Foundation recently announced that all 50 American States, plus the District of Columbia, have passed legislation requiring that all newborns be screened for cystic fibrosis (CF) by the year 2010.

### 1.2.6 Current Management of Cystic Fibrosis

The primary objectives of CF treatment are to control infection, promote mucous clearance and improve nutrition. Current treatments are purely ameliorative and there is still no cure. Well established treatments are outlined in the following sections.

#### a. Specialised Cystic Fibrosis Centres

Patients who attend accredited CF centres for treatment, staffed with a multidisciplinary team of dedicated healthcare professionals who specialise in the treatment of CF across all organs, do significantly better than other patients (Pedersen, Jensen *et al.* 1986; Mahadeva, Webb *et al.* 1998; Mérelle, Schouten *et al.* 2001; Navarro, Rainisio *et al.*
2001; Schnabel, Grasemann et al. 2007). Intensive follow-up and treatment, in addition to multidisciplinary expertise, are very important in increasing survival in people with CF.

b. New Antimicrobial Medications

The most commonly isolated bacteria from the lungs of young CF patients are *Staphylococcus aureus* and *Haemophilus influenzae* while infection with *Pseudomonas aeruginosa* increases with age. Most patients with CF will ultimately be infected with *P. aeruginosa* during their lifetimes which leads to a more rapid decline in lung function than any other pathogen (Speert, Campbell et al. 2002) (Frederiksen, Koch et al. 1997). Chronic infection by *P. aeruginosa* is characterised by continuous growth of *P. aeruginosa* in airway secretions, development of antibodies against *P. aeruginosa* and a change in phenotype of the microorganism from non-mucoid to mucoid (Hoiby, Frederiksen et al. 2005). Once chronic infection by *P. aeruginosa* is established, frequent and extended antibiotic treatment is given (orally, intravenously and via inhalation, for example oral ciprofloxacin in combination with colistin inhalation, or intravenous tobramycin in combination with a ß-lactam antibiotic) to slow respiratory deterioration, however the infection is never completely eradicated. Additionally, the use of chest physiotherapy for bronchial drainage, anti-inflammatory agents (i.e. bronchodilators and corticosteroids), regular physical exercise and aerosolised human recombinant DNase (Welsh, Tsui et al. 1995) is utilised to halt respiratory deterioration.

Chronic airway infection with *P. aeruginosa* is normally preceded by a period of recurrent colonisation of the airways with the non-mucoid phenotype of the microorganism (Hoiby, Frederiksen et al. 2005). In Denmark, a retrospective study has shown that early aggressive treatment of non-mucoid *P. aeruginosa* infection in children, using
the Copenhagen Model (developed by Hansen, Pressler et al.) can protect up to 80% of patients from the development of chronic infection for up to 15 years (Hansen, Pressler et al. 2008). It is expected that this early aggressive therapy will contribute to the continuing improvement of CF patients (Hoiby, Frederiksen et al. 2005).

New regimes and formulations are continually being developed with the aim to reduce the prevalence of antibiotic resistance and improve antibiotic airway deposition. Additionally, new ways of preventing the formation of biofilms by inhibiting ‘bacterial communication’, or quorum sensing, and increasing the susceptibility of bacteria to the body’s immune system are being investigated (Bjarnsholt and Givskov 2007; Fulghesu, Giallorenzo et al. 2007).

c. Pseudomonas Vaccination

Vaccines against *P. aeruginosa* continue to be developed, assessed and improved with the aim of eliciting a long-term protective response, (Moser, Kjaergaard et al. 2000) (Johansen, Hougen et al. 1995). An extensive review of clinical trials of *P. aeruginosa* vaccines performed by Johansen and colleagues concluded that the vaccines tested were ineffective (Johansen, Hougen et al. 1995). Indeed, most published studies are pre-clinical, some describing phase I and II results, but only two vaccines have continued to phase III studies in CF patients (Lang, Rudeberg et al. 2004; Doring, Meisner et al. 2007). There is evidence that *P. aeruginosa* is able to undergo phenotypic variation in response to changing environmental conditions, such as in the airways of CF patients (Doring and Pier 2008) which makes the development of a successful vaccination more challenging. Effective vaccines have been developed against other bacteria, for example *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae*, thus there is clearly a need for further research to increase our understanding of the immune
response to *P. aeruginosa* in order to allow the development of a successful vaccine in the future.

**d. Organ Transplantation**

Lung transplants may be recommended as last resort treatment options for patients with lung failure diseases such as CF, but it is important to balance the risks of surgery with the health status of the patient. A recent study described survival figures of 82% survival at 1 year, 70% at 3 years, 62% at 5 years and 51% at 10 years post-transplant, but this study also showed a 41% rejection rate within the first month post-transplant (Meachery G 2008). Interestingly, this study showed that there was no significant difference between survival in CF and non-CF transplantation cohorts, although no explanation was elucidated. Clearly, further investigation into this is warranted.

The first successful heart-lung transplant took place in 1981, with the first successful single lung transplant occurring in 1983. It was not until 1986 the first successful double lung transplant took place. An even more recent development is the living lobar transplant, with the first successful transplant done in 1990 (Schum 2002). The main advantage of the living lobar lung transplant is that it doesn’t require a cadaveric donor as the procedure involves 2 donors, each donating a single lobe. This technique is often used with CF patients who are not expected to survive the waiting time until a cadaver transplant is available. Living lobular transplantation is also appropriate when the recipient is either a child or petite (as many malnourished CF patients are) and their chest area is not large enough for a double lung transplant. The survival rate for lung transplantation continues to rise with the introduction of new surgery techniques, drug therapies and physical therapy.
e. Mucolytics

Mucolytics reduce the viscosity of mucus to facilitate its clearance from the airways via MCC and coughing. Mucolytics, such as, DNase (Pulmozyme), hydrolyses the DNA, originating from dead cell debris, present in sputum/mucus of CF patients, thereby reducing mucus viscosity in the lungs, increasing clearance. Several studies have shown the effectiveness of DNase in adolescents and adults and DNase is now the most widely used mucoactive therapy in patients with CF (Suri 2005). Daily inhalation has been reported to slow lung function decline, reduce the frequency of exacerbations and decrease airway inflammation (Paul, Rietschel et al. 2004). While some patients respond extremely well to Pulmozyme treatment, others don't respond at all (Personal Communication, Dr Greg Smith, WCH).

There is a limited amount of information available concerning the usage of Pulmozyme in patients who are younger than 5 years however clinical trials are currently recruiting in the USA (http://clinicaltrials.gov) to investigate usage in 3-5 year olds. In South Australia, Pulmozyme can be used in children under 5 years, but only if they are able to complete a lung function test and are a stable out-patient. If Pulmozyme treatment is approved, the patient will then undergo a 1 month trial, during which there must be a minimum 10% improvement in lung function for Pulmozyme therapy to be continued (personal communication, Dr Greg Smith).

f. Airway Hydration

Increasing airway hydration has been investigated to restore MCC mechanisms and reduce mucous adhesion to the airway surface. It has been demonstrated in vitro that hypertonic saline added to cultures increases airway hydration and improves MCC
A reduction in pulmonary exacerbations and a marginal increase in lung function was reported after a 12 month trial of inhaled hypertonic saline (Elkins, Robinson et al. 2006). Interestingly, data from a long-term trial of patients on inhaled HS therapy showed exacerbations did not differ significantly between users and non-users of Pulmozyme (Bye and Elkins 2007), suggesting that HS and Pulmozyme may be equally effective at increasing mucous clearance as a result of increased airway hydration, leading to an overall reduction in exacerbations in CF patients (Elkins, Robinson et al. 2006).

g. Nutrition

The importance of nutritional care in CF is well recognised, with poor nutritional status negatively impacting on survival (Corey, McLaughlin et al. 1988; Beker, Russek-Cohen et al. 2001; Sharma, Florea et al. 2001; WHO, ICF(M)A et al. 2002), increasing exacerbations and reducing bone mineral density (Ionescu, Nixon et al. 2000; King, Toplis et al. 2005). Malnutrition in CF is caused by inadequate dietary intake, malabsorption and increased energy expenditure due to excessive coughing and breathing difficulties, lung infection and lung inflammation. The clinical manifestations of malnutrition in CF, such as growth retardation, weight deficits, muscle wasting, anaemia and immune dysfunction can all be attributed to a protein-energy deficit or malabsorption of essential nutrients (Wilson and Pencharz 1998).

Thus, it is recommended that people with CF consume between 120% - 150% of the daily amount of kilojoules recommended for a normal person of the same age and weight without CF (Dodge 1988). This is to ensure they increase their chance of absorbing the nutrients needed for normal and healthy growth (in children) and generally to sustain health and increase body weight in order to fight off infections, particularly in times of exacerbation of lung illness. Additionally, since people with CF
have difficulty absorbing vitamins, especially those that are fat-soluble; they often need to take supplements of vitamins A, D, E and K. While dietary management regimes have improved many of the problems associated with malnutrition in CF, it continues to be a major clinical problem, as evident in the retardation in weight gain and linear growth of most CF patient populations (Dodge 1988; Wilson and Pencharz 1998).

**b. Pancreatic Enzymes**

Approximately 85–90% of CF patients suffer from PI from birth, with symptoms including steatorrhea, diarrhea, abdominal pain, passage of excess bowel gas, deficiency of fat-soluble vitamins, and failure to thrive (Davis, Drumm *et al.* 1996). Pancreatic enzyme replacement therapies can assist in controlling these symptoms by delivering synthetic pancreatic enzymes to the small intestine to help digest fats, proteins and starches. Effective treatment with pancreatic enzyme replacement therapy should allow CF patients to consume a normal to high fat diet, control the symptoms of PI, correct malabsorption and achieve growth and a normal nutritional state. Although, while improved fat absorption has been achieved in a clinical trial setting, in practice a substantial number of CF patients do achieve entirely normal absorption (Littlewood, Wolfe *et al.* 2006).
1.3 The Airway Epithelium

1.3.1 A General Overview

Lungs are structurally complex organs whose primary function is the uptake of oxygen and the elimination of carbon dioxide. They are anatomically divided into two parts: the proximal conducting airways, comprising the trachea, bronchi and bronchioles; and the distal respiratory region, consisting of the alveolar ducts and alveolar air sacs (alveoli). This second region is where gas exchange occurs.

Due to the vast array of potentially pathogenic foreign particles that are drawn deep into the lung with each breath (indoor air typically has approximately $10^6$ bacteria per metre cubed (Feazela, Baumgartner et al. 2009)), an effective series of defense mechanisms has evolved to ensure a sterile environment is maintained (Figure 1.3). Firstly, mucociliary clearance (MCC) is a crucial primary defense mechanism that protects the lungs from deleterious effects of inhaled pollutants, allergens, and pathogens. Mucociliary dysfunction is a primary feature of CF airway diseases in humans. The MCC mechanism consists of three components, the cilia, a protective mucus layer, and an airway surface liquid (ASL) layer, which work in concert to remove inhaled particles from the lung, (Mall 2008). It is postulated that dysfunction of MCC results in chronic lung infection as pathogens, most commonly Staphlococcus aureus and Psudomonas aeruginosa, are not rapidly cleared from the airway surface. The continued presence of infectious particles leads to constant recruitment of neutrophils into the airway and significant inflammation of the airway. As the lungs of newborns with CF are sterile (Proesmans, Vermeulen et al. 2008), it is hypothesised that if MCC mechanisms can be restored sufficiently from an early age, then there is the possibility that chronic lung infection may be eradicated (Clunes and Boucher 2008).
Secondly, the glycocalyx is comprised of highly glycosylated tethered mucins located on the luminal surface of the airway cells. The glycocalyx is a sticky mesh layer that acts as a secondary barrier, trapping any particles that get through the mucus. Finally, epithelial cells are encircled by tight-junctions which act as a physical barrier to protect the deeper regions of the lung surface. Together these all help prevent any inhaled potential pathogens such as dust, allergens, bacteria or viruses, which may cause severe respiratory infections (Yeaman, Grindstaff et al. 1999).

### 1.3.2 Antimicrobial Properties of Airway Surface Liquid

A diverse range of proteins and peptides with broad-spectrum antimicrobial activity are found in the mucosal surfaces at many sites in the body, including the airway, (Diamond, Beckloff et al. 2008) and have been proposed to play an important role in the innate defence against pathogenic microbial colonization. While many different antimicrobial factors have been identified in the airway, by far the most abundant are lysozyme, lactoferrin and secretory leukoproteinase inhibitor, and thus is it speculated that these are the most critical to lung defence (Travis, Conway et al. 1999). Interestingly, the activity of antimicrobial factors is highly dependent on the ASL environment, for example many become ineffective in high salt concentrations (Travis, Conway et al. 1999). It is thought that the loss of CFTR alters the normal ASL environment, which reduces the potency of the antimicrobial agents with the ASL, thereby impairing the innate immune system and increasing the susceptibility of CF airways to infection.
1.3.3 Innate Immune System

In addition to the antimicrobial factors present within the ASL, roaming macrophages engulf and eliminate bacteria and other pathogens present in the airway. This in turn initiates the release of cytokines, recruiting neutrophils and other circulating immune cells (Travis, Conway et al. 1999). Interestingly, a study by Theo Moraes and colleagues showed that when bacteria were incubated with neutrophils in the presence of ASL from CF epithelia the antimicrobial effect was significantly less when compared with normal ASL (Moraes, Plumb et al. 2006). Subsequently, cytokine release would also be reduced, resulting in an inferior immune response (Moraes, Plumb et al. 2006).

Figure 1.2: Structure of Airway Epithelium. The ASL layer traps inhaled or introduced particles that are moved out of the airway via mucociliary clearance mechanisms. The glycocalyx, closer to the cell surface, also acts to trap particles and prevents particle entry via the apical surface. Finally, the tight junctions between the epithelia cells from another barrier to prevent foreign particles infiltrating the lungs by disallowing access to the basolateral surface of the epithelium and cilia.
1.3.4 Structure of the Conducting Airways

Cystic fibrosis lung disease begins in the conducting airways, so the target of gene therapy must include the epithelial cells that line them. The human conducting airways are lined with pseudostratified columnar superficial epithelium, which consists mainly of submucosal glands, ciliated epithelial cells, non-ciliated columnar epithelial cells and basal cells. The apical surface of each non-ciliated epithelial cell is covered with microvilli which appear as finger-like structures about 1 μm long (Mygind and Dahl 1996). The apical surface of each ciliated epithelial cell is covered in approximately 200 cilia, thin tail-like projections which extend 5-10 μm out from the cell body (Satir and Sleigh 1990). The coordinated wave-like movement of the cilia effectively clears foreign particles from the apical surface. Additionally, this surface is covered with ASL, the depth of which is dependent on the species, but is about 10 μm in humans (Widdicombe 2002). The ASL layer is further divided into a periciliary liquid (PCL) layer and a mucous layer (Boucher 1994). The PCL has a low viscosity which facilitates rapid ciliary beat while it also acts to protect the epithelium from the mucous layer (Knowles and Boucher 2002). The mucous layer which overlies the PCL is rich in mucins and acts to bind and trap most inhaled particles, which are then removed from the lung via MCC and airway reflexes such as coughing (Knowles and Boucher 2002). These transport mechanisms and the overall ability to maintain a sterile environment for the underlying epithelial cells are highly dependent on the depth and viscosity of the ASL layer.

1.3.5 Human Airway Epithelial Ion Transport

Cystic fibrosis lung disease presents as persistent bacterial infections of the large and small airways (Davies, Geddes et al. 2001), with the consensus being that the innate
defences of the normal airway are somehow degraded by the absence of functioning CFTR protein (Boucher 2007). However, as CFTR is a Cl⁻ channel, most research has focused on linking defective airway defence in CF to impaired ion transport (Boat and Cheng 1989; Grubb, Vick et al. 1994; Guggino 1999; Guggino 2001; Tarran, Grubb et al. 2001; Grubb, Chadburn et al. 2002; Boucher 2004; Tarran 2004; Boucher 2007) which subsequently leads to abnormal airway surface liquid (ASL). Initially, there were three hotly-debated hypotheses trying to link CFTR impairment with abnormal ASL and thus defective airway defence. One has emerged as dominant, but all are outlined in the following sections.

### a. The Low-Volume Hypothesis

The low-volume hypothesis was proposed by Richard Boucher and colleagues at the University of North Carolina (UNC). While CFTR is a Cl⁻ channel, it also functions to regulate the activity of other ion channels (i.e. the Na⁺ channel ENaC) (Boucher 1999). Thus, the function of the CFTR protein is to co-ordinate and control the relative rates of airway epithelial Cl⁻ secretion and Na⁺ absorption and, ultimately, the hydration level of the ASL. When CFTR is defective, the inhibition of the ENaC channel is lost, which leads to an increase in Na⁺ transport, which drives increased absorption of Cl⁻ and water via transcellular pathways. The dehydration of the airway surfaces allows the mucus on the epithelium to adhere to the airway surface, which in turn inhibits mucociliary clearance (MCC) (Figure 1.5). Effective MCC relies on a hydrated ASL to allow cilia to fully extend and perform an effective wave movement to clear pathogenic bacteria out of the airways. Therefore the reduced depth of the ASL in response to non-functioning CFTR impedes effective MCC mechanisms allowing pathogenic bacteria to reside for greater periods on the ASL layer, which leads to chronic infections.
The low-volume hypothesis is supported by experiments which studied cultured airway epithelial cells from both normal and CF airways. They found that the CF cultures had a significantly reduced ASL depth. By adding a 30 μm layer of fluid onto the cell cultures they found that the CF cultures reduced the ASL height at a significantly faster rate than the normal cultures, but no change in Cl− concentration was seen in either the CF or normal cultures. Additionally, these cultures secreted mucous and microscopic inspection revealed that in the CF cultures this mucus had collapsed onto the cilia and inhibited MCC after 24 hours. Additionally, when fluid was added back into the CF cultures, ASL height was restored, as were MCC and mucous movement.

Figure 1.3: The ASL in healthy (A) and CF (B) airways, Modified from (Boucher 2007). High ASL depth in normal airways (A) allows MCC mechanisms to clear foreign particles from the airway surface (the thinner mucous layer is not evident in this electron micrograph). In CF airways (B) however, a dehydrated ASL layer results in MCC impairment, note the bent-over cilia (lateral and cross-sectioned) that adhere to the large amount of mucous present in the airway.
b. The High Salt Hypothesis

The high salt hypothesis was proposed by Jeffery Smith and colleagues at the University of Iowa (Smith, Travis et al. 1996). They proposed that CFTR functions primarily as an anion channel and plays a vital role in the regulation of salt (NaCl) in the ASL which is thought to provide a suitable environment in which antibacterial factors can act against bacterial infection. In normal airways, the ASL contains several antibacterial factors including β-defensins and lysozymes (Smith, Travis et al. 1996; Zhao, Wang et al. 1996; Goldman, Anderson et al. 1997; McCray and Bentley 1997), but in CF ASL the activity of these factors is inhibited by high salt concentrations in the ASL, which then predisposes the CF airway to infection. In CF, non-functioning CFTR inhibits Cl⁻ absorption into the cell, while active Na⁺ absorption into the cell continues to occur via the ENaC channel, exiting via the basolateral membrane Na-K-ATPase (Welsh, Tsui et al. 1995). This leads to more Cl⁻ (which is negatively charged) in the ASL, which the cell then tries to counterbalance by secreting Na⁺ (positively charged) into the ASL. These molecules then combine, resulting in a higher than normal salt (NaCl) concentration in the ASL. While the epithelial layer is permeable to water, the Iowa group hypothesises that there is “some force” to counter the osmotic pressure generated by the ion concentration difference (Zabner, Smith et al. 1998). Zabner and colleagues suggest once osmotically driven water absorption lowers the ASL to the tops of the cilia, the tightly packed microvilli and cilia combined would produce a large surface tension, countering osmotic pressure and thus preventing further water absorption (Zabner, Smith et al. 1998).

The Iowa group proposed the high salt hypothesis after conducting several experiments in which they cultured airway epithelial cells from both normal and CF airways on filters. Unlike the UNC group, the Iowa group found no difference in the volume of the ASL, but they did discover significantly different salt concentrations in the
surface liquids from normal and CF airways, with almost double the amount of NaCl present in CF ASL compared with normal. When challenged with bacteria in the fluid covering the apical surface of the cells, the normal cell cultures readily destroyed the bacteria, the bacteria thrived in the CF cultured cells. When the bacteria was placed on the basolateral surface of the cultures, bacteria flourished in both normal and CF cultures airway cells, suggesting that the anti-bacterial properties of the normal cells were present only on the apical surface. Further, when the group manipulated the cultures by adding water to the CF cultures and diluting the salt concentration, the bacteria was destroyed, and by adding NaCl to the normal cultures, creating a high salt environment, bacteria was able to grow. It was thus concluded that while both normal and CF airway epithelial cells secrete antibacterial factors into the ASL, the high salt concentration of the CF cultures diminished their effectiveness.
Figure 1.4: Ion Transport across the Airway Epithelium: Low Volume and High Salt Hypotheses.

In this figure the UNC group’s low volume hypothesis is shown in A (normal) and B (defective CFTR), while the Iowa group’s high salt hypothesis is represented in C (normal) and D (defective CFTR).
c. The Serous Cell Malfunction Hypothesis

A less publicised, but equally relevant hypothesis was proposed after experiments by Walter Finkbeiner and colleagues at the University of California, San Francisco; Stephen Ballard and colleagues at the University of South Alabama; John Engelhardt and colleagues at the University of Pennsylvania; and Jeffery Wine and colleagues at the University of Stanford. These groups suggest that normal human submucosal glands are comprised of 60% serous cells and 40% mucous cells (Wine and Joo 2004) and that serous cells play a key role in airway defence by secreting a fluid rich in antibacterial and anti-inflammatory factors in response to irritants. Additionally, it has been shown that within human airways, CFTR is most predominandy expressed in those serous cells (Engelhardt, Yankaskas et al. 1992), so this hypothesis suggests serous cell secretions to be CFTR-dependent. When CFTR is defective, fluid secretion from these cells is greatly diminished, resulting in mucus that is thicker and deficient in antibacterial factors. This would then result in an exacerbation of the defects proposed in both the high salt and low volume hypotheses in that natural antibacterial agents would allow infection to infiltrate the airway and reduced hydration of the ASL would result from decreased serous cell secretion. There is clear evidence that submucosal gland epithelia is dysfunctional in CF, the specific regions of the airway that require CFTR delivery for the restoration of normal physiological function are not well established (Zhang, Button et al. 2009).

1.3.6 Evaluating The Hypotheses

The relationship between defective CFTR, defective ion transport, and chronic infection and inflammation in the lung continues to be controversial. Convincing evidence has been lacking that these or other mechanisms are responsible for airway
disease in CF and there is still debate. However, based on the data from *in vitro* studies, mouse studies and human clinical studies as outlined below, I believe the balance of evidence supports the low-volume hypothesis.

### a. In Vitro Studies

Many *in vitro* studies assessing ASL depth and composition have obtained samples using invasive techniques, such as filter paper and micropipettes, which require direct contact with the ASL. These methods therefore have the potential to produce inaccurate results by stimulating fluid secretion or *via* capillary action drawing interstitial fluid and intracellular fluid into the sample. A small number of research centres have used non-invasive sampling techniques, such as microdialysis (Tarran, Grubb *et al.* 2001), to measure ion concentration. Although these have shown no difference between normal and CF ASL ion composition, (so supporting the low-volume hypothesis overall), studies of ASL composition fail to consistently support one hypothesis.

Tarran and colleagues have also shown that normal airway cultures are capable of regulating ASL height, reducing and increasing in response to various experimental conditions, while CF airway cultures did not alter Na⁺ and Cl⁻ movement and subsequently ASL height remained static (Tarran, Button *et al.* 2005; Tarran, Trout *et al.* 2006). These experiments support the low-volume hypothesis.

### b. Mouse Studies

*In vivo* studies on normal and CF (*cftrtm1Unc*) mouse septum epithelial cells demonstrated a significant reduction in ASL height in the CF mouse compared to wild type (Tarran,
Grubb et al. 2001). The low-volume hypothesis was further tested when Mall and colleagues at the University of North Carolina generated a transgenic mouse model that over-expressed the β-subunit of the ENaC channel, in an attempt to shift the balance of salt and water movement away from Cl− secretion towards Na+ absorption, (the ENaC mouse model) (Mall, Grubb et al. 2004). These mice demonstrated increased Na+ absorption, but no alternation in Cl− secretion, resulting in a reduced ASL height (Mall, Grubb et al. 2004). The mortality rate of these mice was significantly higher than their littermates and morphological examination revealed they were dying from airway obstruction from adherent airway mucus (Mall, Grubb et al. 2004). Additionally, ASL reduction in the ENaC mouse model resulted in neutrophilic inflammation of the airways, goblet cell hyperplasia and difficulty clearing inhaled bacteria (Mall, Grubb et al. 2004), which are all characteristic of CF lung disease. These observations provide strong evidence that the balance between Na+ absorption and Cl− secretion produced ASL dehydration and support for the low-volume hypothesis.

c. Human Studies

Studies designed to measure the ion composition of the ASL in vivo have shown the concentrations of salts in the ASL of CF airways are no different from normal ASL salt concentrations (Knowles, Robinson et al. 1997; Kotaru, Hejal et al. 2003). However, similar problems as occurred in cell culture models (section 1.3.7a) with the sampling techniques resulted in inconsistent results, the result is that this data does not fully support one hypothesis over another.

More recently studies have been conducted in which hypertonic saline (HS) was administered to the airway. In the low-volume theory ASL salt concentration is always near isotonic. This theory predicts that the administration of HS, with a salt
concentration significantly higher than the ASL, would serve to osmotically draw water into the ASL. This increase in ASL liquid depth would allow the restoration of MCC (thus mucus movement), improve lung function and reduce the number of infections in the lung. In several studies it was found that the administration of inhaled HS produced an increase in basal MCC, an increase in lung function and a reduction in the number of lung infections recorded (Donaldson, Bennett et al. 2006; Elkins, Robinson et al. 2006; Tarran, Donaldson et al. 2007). If the high salt hypothesis was correct, the administration of HS would further increase the salt concentration of the ASL, thus worsening CF lung disease. However, the delivery of aerosolised HS (7%) to CF patients over a two week period was shown to increase the rates of MCC, improve pulmonary function tests and over a one-year interval, reduce exacerbations and improve quality of life (Donaldson, Bennett et al. 2006; Elkins and Bye 2006). Furthermore a pilot study conducted in young children (aged four months – seven years) using 3% and 7% HS showed a significant improvement in MCC in a dose-dependent manner (Dellon, Donaldson et al. 2008). If the administration of HS were to exacerbate CF lung disease, there would be no evidence of improvement after treatment, as seen in these studies.

An absolute resolution to this controversy is imperative to the overall understanding of the lung pathophysiology of CF and the development of effective treatments for CF airway disease.
1.3.7 Animal Models of Cystic Fibrosis Airway Disease

a. The Murine Model

The 1991 cloning of the murine CFTR gene allowed the development of CF mouse models by gene targeting (Tata, Stanier et al. 1991). Subsequently, several knock-out mouse models (with no CFTR) have since been developed (Snouwaert, Brigman et al. 1992; Ratcliff, Evans et al. 1993; Colledge, Abella et al. 1995; Hasty, O’Neal et al. 1995; Delaney, Alton et al. 1996; Rozmahel, Wilschanski et al. 1996), as well as mice homogenous for both the ΔF508 (Colledge, Abella et al. 1995; van Doorninck, French et al. 1995; Zeiher, Eichwald et al. 1995) and the G551D CF gene mutations (Delaney, Alton et al. 1996). In total there are now 11 CF mouse models available (Davidson and Rolfe 2001).

While a number of CF mouse models have been developed, none accurately mimics the ion transport defects and pathophysiology that characterises the human CF disease profile. Specifically, these mouse models don’t exhibit the spontaneous lung disease, the leading cause of death seen in CF patients. A number of factors could underlie the absence of pulmonary disease in the CF mouse models. Mice have far fewer mucus-producing submucosal glands and goblet cells lining the airway than humans and this could reduce much of the mucous needed for disease to establish. More importantly mice possess a second ion-transport system in their lungs (but not in the nose). It is thought that this additional ion-transport system may compensate, at least partially, for the loss of functional CFTR (Grubb and Boucher 1999) in lung airways.

While the CF mouse models don’t display lung disease, they do exhibit ion transport deficiencies in the nasal airways, so studies examining CFTR absence and treatment effects can be performed on nasal airway tissue, but must be interpreted with caution.
Additionally, the cell types (i.e. ciliated and non-ciliated epithelium) and mechanisms (i.e. MCC) present throughout the CF mouse nasal epithelium are comparable to the human lung, thus making it a suitable model tissue to assess the potential of CF gene therapy (Grubb and Boucher 1999).

More recently however, there have been questions raised as to the suitability of the mouse nasal airway as a test site for therapies designed to correct CFTR malfunction or absence. A study conducted by Griesenbach and colleagues concluded that when using *in vivo* CFTR functional endpoints, such as TPD measurements, the numbers of mice required to detect the small changes in mouse, which may be therapeutic in man, is prohibitive (Griesenbach, Sumner-Jones *et al.* 2009). Additionally, it has been questioned whether murine nasal epithelium is sufficiently representative of the human lung tissue in regards to gene transfer agents. Ostrowski and colleagues have also urged caution when using the mouse model to assess gene therapy success, as human and mouse CFTR share only 78% identity at the amino acid level and the interaction of CFTR with the ENaC channel may be species specific (Ostrowski, Yin *et al.* 2007). While there continues to be insights gained through the use of the mouse nasal epithelium (knock-out), which does exhibit ion transport defects that are nearly identical to those in human CF airways, the results gained from gene therapy experiments must be interpreted with caution.

### b. Development of Other Animal Models

The lack of an animal model that mimics the phenotype of human CF lung disease has been a major barrier to understanding CF pathogenesis and to developing therapeutic approaches for this disease. The similarities between human, ferret and pig in terms of lung anatomy, physiology and cell biology highlight them as potentially useful species...
to develop CF animal models. The ferret has been extensively used to study various lung diseases, such as influenza type A and B (Arroyo and Reed 1978; Piazza, Carson et al. 1991). Additionally, studies have shown the expression of CFTR, anatomy, airway cell types and distribution of submucosal glands within the ferret lung is very similar to human lungs, pointing to the potential of the ferret to be a good species on which to model CF lung disease in humans. Recently, the development of a ferret with a homogenous CFTR gene-disruption was developed (Sun, Yan et al. 2008) however, it is not yet known whether homogenous CFTR gene-disrupted ferrets will develop spontaneous lung disease similar to that in humans with CF (Sun, Yan et al. 2008). Regardless of this, the ferret will be a valuable animal model for studying CFTR lung biology.

The pig is another potentially useful species for generating a model of CF lung disease due to the many similarities to humans in terms of anatomy, physiology, size, life span and genetics (Rogers, Stoltz et al. 2008). Like the ferret, the pig has submucosal gland abundance and locations comparable to humans. In contrast, mouse submucosal glands are found only within the trachea (Innes and Dorin 2001). Additionally, when used as a model of chronic bronchitis the pig develops submucosal gland hypertrophy, also a feature of CF lung disease (Baskerville 1972). The electrophysiological properties of the pig epithelium have also been extensively studied and found to mimic that of humans (Ballard, Trout et al. 1999; Crews, Taylor et al. 2001; Ballard and Inglis 2004; Ballard, Trout et al. 2006).

Overall, the successful cloning of ferrets by somatic cell nuclear transfer (Sun, Yan et al. 2008) and of pigs using nuclear transfer with site-specific genetically modified somatic cells (Rogers, Stoltz et al. 2008) will provide new opportunities for dissecting the pathophysiology of CF and testing of new therapies not previously approachable in mouse models of this disease.
1.4  Therapies for Cystic Fibrosis Airway Disease

1.4.1 Gene Therapy for Cystic Fibrosis Airway Disease

The goal of gene therapy for CF is to deliver to the affected cells a functional CFTR gene, in sufficient quantity, for a duration that is therapeutically meaningful. Restoration of CFTR function would theoretically prevent the cycle of bacterial colonisation and recurrent infections by restoring the ion balance of the ASL. However, it is unknown how much CFTR is needed for ion transport restoration and the prevention of chronic and destructive lung disease. Reduction of CFTR function by 50% does not lead to any phenotypic display of CF lung disease as this is the carrier level of function. Additionally, people with another CFTR-related disorder – congenital bilateral absence of the vas deferens – display no lung disease despite having only 10-20% of normal CFTR function (Hantash, Milunsky et al. 2006). Patients with mild CF lung disease display as little as 5% CFTR function, thus to prevent CF lung disease it can be postulated that a 10% - 20% correction is required. However, in these natural models CFTR is expressed at lower than normal levels across all cells. Some vectors may have the potential to produce higher CFTR expression in some cells but, some cells may remain with zero expression. Accordingly, while 20% CFTR function may be achieved overall, this may not be physiologically relevant or sufficient. Zhang and colleagues used a recombinant parainfluenza viral vector to transduce ciliated airway epithelial cells. A range CFTR expression levels (from 3% to 65%) was assessed and it was found that once 25% of the ciliated epithelial cells were expressing CFTR, normal airway surface hydration and mucous transport rates appeared to be restored (Zhang, Button et al. 2009). While only assessed in *ex-vivo* and *in vitro* systems, this demonstration of efficient and functional CFTR delivery will act as a benchmark for future *in vivo* studies into CF gene therapy.
There are many different gene transfer vectors being used in CF gene therapy research, in both *in vitro* airway epithelial model systems and *in vivo*, in both animal models and human gene therapy clinical trials. These vector systems can be most easily divided into two categories, viral and non-viral-based gene transfer vectors, and their properties and usage are outlined in the following sections.

1.4.2 Non-Viral Gene Transfer Vectors

Non-viral vectors are generally less efficient than viral vectors at transfecting lung epithelium, however if free of non-human protein components, they are less likely to induce an immune response, thereby allowing for repeated administration (Romano, Pacilio *et al.* 1998; Griesenbach and Alton 2009). Consequently, non-viral vectors were developed to circumvent the safety concerns of viral vectors, and have since been employed for lung and CF gene transfer (Ziady, Davis *et al.* 2003). The most basic form of non-viral gene therapy is the use of naked DNA, which is ineffective in the lung (Zabner, Cheng *et al.* 1997; Glasspool-Malone, Steenland *et al.* 2002; Ziady, Davis *et al.* 2003; Ziady, Gedeon *et al.* 2003). To protect the naked DNA from endonuclease degradation, a primary defence mechanism of the airways, DNA was complexed with cationic lipids to produce positively charged lipoplexes (Ziady, Davis *et al.* 2003). Lipoplexes are very efficient at fusing with membranes and entering cells, making them efficient at transducing dividing cells, but in fully differentiated airway epithelia *in vivo* this technique has so far been unsuccessful (Ziady and Davis 2006).

Technologies to improve non-viral gene transfer are continually being developed. For example, ultrasound has been shown to increase gene transfer in several tissue types (including the mouse lung epithelium) by causing transient pore formation in the cell
membrane, however haemorrhaging was also noted, most likely due to micro-vessel rupture within the tissue (Xenariou, Griesenbach et al. 2007).

To move forward into clinical trials any vector must transfect airway epithelial cells, allow for repeat administration without provoking an immune response, have low toxicity and comply with GMP protocols for vector production. In February 2009, the UK Gene Therapy Consortium began a single-dose pilot study to investigate the safety of a single nebulised dose of their non-viral gene transfer agent. If successful, the consortium hopes to further investigate the use of non-viral gene therapy as a treatment for CF airway disease through a larger multi-dose clinical trial. However, to date, no non-viral vector system has been successful.

1.4.3 Viral Gene Transfer Vectors

Viral vectors have emerged as the vehicles of choice for CF gene transfer, but the various different categories of viral vectors all have limitations and risks, including complexity of production, limited packaging capacity, and unfavourable immunological features. These may restrict gene therapy applications and hold back the potential for preventive gene therapy. An outline of the benefits and drawbacks of different viral vectors is discussed in the following sections.

a. Adenovirus

Although initially widely used in CF gene transfer research, more recently the use of adenoviral vectors (AdV) has decreased. Adenoviruses are derived from common pathogens of the respiratory tract, so were rapidly identified and developed as vectors
for use in airway gene therapy. Recombinant adenoviruses (AdV) are able to efficiently transduce many cell types and produce 5-20% airway epithelial cell transduction \textit{in vitro} with the majority of expression in ciliated cells (Curiel, Pilewski \textit{et al.} 1996). AdV gene transfer \textit{in vivo} is much less efficient with between <1% (no pre-treatment) (Grubb, Pickles \textit{et al.} 1994) and 2.65% (pre-treated with polydocinol) (Parsons, Grubb \textit{et al.} 1998) airway epithelial cells expressing the transgene. Additionally, AdV preferentially transduce non-differentiated or damaged epithelial over healthy differentiated ciliated cells \textit{in vivo} (Grubb, Pickles \textit{et al.} 1994). Adenoviruses are able to be produced in high titres (up to $10^{13}$ particles/ml) which may help overcome this lack of efficiency \textit{in vivo}, but since AdV are non-integrating and non–replicating vectors, they produce only transient gene expression (Hemmi, Geertsen \textit{et al.} 1998). Gene therapy for the treatment of hereditary disorders such as CF requires extended transgene expression, or the ability of repeat administration to periodically re-establish therapeutic gene expression levels, to be effective. The inability of AdV to be re-administered remains a significant obstacle for its use in gene therapy for CF lung disease. Adenoviruses elicit an innate host immune response resulting in the inflammation of transduced tissues and the elimination of positively transfected cells (Muruve 2004). This innate response is followed by antigen-specific adaptive immunity, which provides the host with long-lasting immunity and memory, thus re-dosing is ineffective (Muruve 2004). Newer generations of AdV (helper-dependent AdV) have been developed which, by deleting all viral genes, have alleviated the host antiviral adaptive immunity (Kay, Glorioso \textit{et al.} 2001) and improved both the efficiency and duration of gene transfer \textit{in vivo} (Parks, Chen \textit{et al.} 1996; Muruve, Cotter \textit{et al.} 2004). Another approach has been to briefly suppress the immune response when the vector is administered, but given the many bacterial, fungal and viral infections associated with CF, this may be undesirable (Chirmule, Raper \textit{et al.} 2000).
Adenoviral vectors have many advantageous characteristics, particularly their ability to package large quantities of DNA, however, it is likely that AdV will be restricted to pre-clinical proof-of-principle studies for CF unless the problems of repeat administration and heightened immune response can be overcome.

b. Adenovirus Vectors: Progress in Gene Therapy Clinical Trials

In 1993 the first clinical trial for CF was conducted by Zabner and colleagues (Zabner, Couture et al. 1993). In this non-blinded study an AdVCFTR vector was instilled into the nasal cavity of three CF subjects. All three patients showed baseline TPD correction for up to ten days, however localised nasal inflammation was noted and may have affected the TPD measurements as it has been previously demonstrated that some inflammatory stimuli may change airway ion transport (Galietta, Folli et al. 2000).

A second clinical trial was conducted in 1994 by Crystal and colleagues (Crystal, McElvaney et al. 1994). A first generation E1/E3-deleted replication defective AdVCFTR vector was delivered to the nasal cavity and the bronchi of four CF patients with the ΔF508 mutation. Using RT-PCR analysis, CFTR mRNA was detected in bronchial scrapings of only one out of the four patients and a transient acute inflammatory response was observed one of the patients.

A double–blinded, dose–escalation (four logarithmically increasing doses) Phase I clinical trial using an E1–deleted AdV5CFTR vector was performed in 1995 by Knowles and colleagues (Knowles, Hohneker et al. 1995). Twelve CF subjects were intra-nasally given the AdVCFTR vector. Inflammatory responses were reported in two of the three patients who received the highest dose (1 x 10^10 pfu) of vector. While TPD showed no functional correction of ion transport in any of the twelve subjects,
RT-PCR analysis detected CFTRmRNA in nasal scrapings of five of the six subjects who received the two highest doses of AdVCFTR (1 x 10^{10} pfu and 1 x 10^{8} pfu).

In another dose-escalation phase I clinical trial (Zabner, Ramsey et al. 1996) a second–generation E1/E4–deleted AdVCFTR vector was delivered intra-nasally in six CF subjects approximately every six weeks. Using TPD measurements, partial correction of ion transport was reported in two subjects who received the highest dose of AdVCFTR. Humoral immune responses were noted in all six subjects.

In 1997 a phase I clinical trial Bellon and colleagues (Bellon, Michel-Calemard et al. 1997) delivered an aerosolised E1/E3-deleted AdVCFTR vector into the nasal and lung airway epithelium of six CF subjects. RT-PCR analysis detected very low levels of CFTR mRNA in nasal biopsies from all six patients, but in only one of the six bronchial biopsies was CFTR mRNA detected. No immune or inflammatory responses were noted.

In a similar dose-escalating Phase I clinical trial conducted in 1999 by Zuckerman and colleagues (Zuckerman, Robinson et al. 1999) a third generation E1/E4-deleted AdVCFTR vector was delivered to the conducting airways of eleven CF subjects. Four days after the instillation of the AdV vector low levels of CFTR mRNA were detected by RT-PCR analysis in bronchial scrapings from all four subjects. By the 43 day time point no evidence of gene transfer was found. Additionally, the majority of patients showed a T-cell specific response, and subjects who received the highest doses of AdVCFTR (2 x 10^{11} pfu) displayed influenza-like symptoms.

Harvey and colleagues conducted a Phase I clinical trial in 1999 were an AdVCFTR vector was aerosolised into the nasal cavity of fourteen CF subjects and gene transfer was assessed by RT–PCR analysis of CFTR mRNA (Zuckerman, Robinson et al. 1999).
High levels of transient gene expression (72 hours) were observed after the administration of the first dose of the highest titre of AdVCFTR. Following the second administration of AdVCFTR the levels of expression were reduced and after the third administration, no detectable level of gene expression was observed. Surprisingly, the decrease in gene expression was not associated with an increase in the levels of neutralising AdV antibodies. This clinical trial was among the first to demonstrate that while repeated doses of AdV were safe, it would not produce persistent gene expression (Zuckerman, Robinson et al. 1999).

In yet another dose-escalating clinical trial Joseph and colleagues delivered an AdV2CFTR vector to the lungs of thirty-four CF subjects as either an aerosol administration or via a bronchoscope (Joseph, O’Sullivan et al. 2001). The high doses produced a mild to moderate inflammatory response regardless of which instillation method was used. Additionally, gene transfer to the epithelial cells of the lung was inefficient using both methods of delivery (Perricone, Morris et al. 2001).

In summary, in early gene therapy trials for CF AdV were the most commonly used vector due to their natural tropism for the airway epithelium; their ability to infect non–dividing cells; and the ease with which high titres of AdV vector stocks can be produced. However the results gained through these clinical trials have highlighted some significant characteristics of AdV that, while potentially beneficial for other applications, are deleterious for CF gene therapy. AdV vectors can only confer transient gene expression and also evoke inflammatory and immune responses that limit the effectiveness of repeated AdV vector administration. Subsequently, other viral vectors have been investigated that can mediate stable transgene expression while reduced or no immune/inflammatory response.
c. Helper-Dependent Adenoviral Vectors

Also known as ‘gutless’ vectors, the helper-dependent adenoviral vector (HDAdV) was constructed from an AdV by removing all the viral genes and leaving only those sequences required for vector propagation and packaging (Mitani, Graham et al. 1995; Kochanek, Clemens et al. 1996; Parks, Chen et al. 1996). The remainder of the vector can contain either the desired transgene or is filled with non-coding ‘stuffer’ DNA sequences. This allows much larger transgene capacity (approximately 37kb) (Morsy and Caskey 1999) than the adeno-associated viral vector (discussed in section 1.4.3.d). This is especially important with regard to CF gene therapy, given the size of the CFTR coding sequence (about 4.5kb) it is too large to fit into many of the available gene therapy vectors. Additionally, the removal of these viral sequences reduces the host immune response because of the absence of the viral protein expression in the transduced cells that was present in the first-generation AdV. This reduced host cell immune response allows HDAdV mediated pulmonary gene expression to last more than 15 weeks (Toietta, Koehler et al. 2003). In addition, because the HDAd genome exists episomally in transduced cells, the risks of germline transmission and insertional mutagenesis are negligible (Palmer and Ng 2005).

As with the AdV vector, the cellular receptors for HDAds are located on the basolateral surface of the epithelial layer and transduction is therefore prevented by epithelial tight junctions, preventing access to these receptors. Highly efficient pulmonary gene transduction by a HDAdV was achieved however by Koehler et al., using a co-formulation of the vector with 0.1% lysophosphatidylcholine (LPC), an agent able to transiently modulate the tight junction permeability and permit access to the basolateral receptors.
While HDAdV-mediated transgene expression has been shown to last significantly longer than AdV-mediated expression in the lung, the potential duration of pulmonary transgene expression from HDAd in lung has not been determined. Stable HDAdV-mediated transgene expression in the liver has been demonstrated to last for more than 12 months in non-human primates (Brunetti-Pierri, Ng et al. 2006). However, the cellular turnover rate of the airway, particularly in the CF airway, is much greater than hepatocytes (Brunetti-Pierri and Ng 2008) so it is unlikely that gene expression would last as long in the airway as in the liver. Where expression is inadequate or is lost, HDAdV-mediated gene therapy protocols will require re-administration to effectively treat CF lung disease (Brunetti-Pierri and Ng 2008). Current studies have demonstrated that although the first round of HDAdV administration does not induce an immunological response, subsequent rounds of re-dosing result in decreased transgene expression and an increase in neutralising antibodies to the vector (Koehler, Martin et al. 2006). While the use of cell-specific promoters can limit this immune response (Kushwah, Cao et al. 2007), more research is required before these vectors can be effectively and safely re-administered in a clinical trial setting.

d. Adeno-Associated Viral Vectors

Initially discovered as a contaminant in an adenovirus preparation, the adeno-associated virus (AAV) is a human parvovirus that has not yet been associated with any human disease and is therefore considered safe for use as a gene therapy vector (Romano, Pacilio et al. 1998). Additionally, AAV has many properties desirable in a gene therapy vector, such as its ability to infect a wide range of cells, and the capacity to infect non-dividing cells. It can also establish a latent infection by integrating into the cell chromosome (Curiel, Pilewski et al. 1996; Sumner-Jones, Gill et al. 2007). Wild type AAV integrates specifically into the q arm of chromosome 19, but recombinant AAV gene vectors
integrate randomly, raising the possibility of insertional mutagenesis (Kay, Glorioso et al. 2001). A recent study reported that mice developed hepatocellular carcinomas containing AAV vector proviruses at a specific chromosomal locus. While the protocol used in this instance is significantly different to methods employed to target airways (in this study they delivered the vector via neonatal intravenous injection to neonatal mice) the presence of tumours (Davies, McLachlan et al. 2008), raise important questions about the safety of AAV gene therapy.

While appropriate for many gene therapy applications, the packaging capacity of AAV (about 5.0kb) (Kay, Glorioso et al. 2001) is a major limitation for its use in CF airway disease. The size of CFTR cDNA is about 4.5kb, which is close to the cloning capacity of AAV, thus complicating the addition of regulatory sequences needed to achieve adequate CFTR gene expression in vivo. An advantage of AAV for use in CF gene therapy is that as the vector genome lacks viral coding sequences, the vector itself has not been associated with any toxicity or inflammatory responses (except for the generation of neutralising antibodies that may limit re-administration). Additionally, AAV have been shown to infect non-dividing cells, an important feature for lung gene therapy (Miao, Nakai et al. 2000).

e. Adeno-Associated Viral Vectors: Progress in Gene Therapy Clinical Trials

In 1998 the first clinical trial for CF using AAV-based gene transfer vectors was conducted by Wagner and colleagues (Wagner, Moran et al. 1998; Wagner, Reynolds et al. 1998; Wagner, Messner et al. 1999). This study used a tgAAV2CFTR vector (a replication–defective AAV2–based vector that contains the hCFTR cDNA) which was instilled into the maxillary sinuses of ten CF subjects. The maxillary sinuses provide a good model of the human conducting airways as they are completely lined with ciliated respiratory epithelium. A non-blinded, randomised dose-escalating Phase I/
II clinical trial, Wagner and colleagues demonstrated transient correction of the ion channel (assessed via TPD) in subjects who received the highest doses (> 5 x 10^4 AAV replication units) for up to fourteen days post instillation. No significant immune or inflammatory responses were reported.

Another Phase I, single administration, dose-escalating clinical trial was conducted in 2001 by Aitken and colleagues who delivered a tgAAV2CF vector into the lungs of twelve CF subjects as an aerosol (Aitken, Moss et al. 2001). This study demonstrated that high doses of AAV vector administration resulted in the production of serum neutralising AAV antibodies even though gene transfer was inefficient.

Wagner and colleagues conducted another clinical trial in 2002 (Wagner, Nepomuceno et al. 2002). In this Phase II double-blind, randomised, placebo-controlled trial a tgAAV2CF vector was administered to one of the maxillary sinuses of twenty-three patients. While no evidence of gene transfer was reported, the administration of the vector was well-tolerated and no safety issues were noted.

In 2003, Flotte and colleagues (Flotte, Zeitlin et al. 2003) conducted a Phase I study in 25 CF patients with mild to moderate lung disease using a tgAAVCF vector administered to one side of the nose and to the superior segment of the lower lobe of the right lung. Gene transfer, measured by DNA-PCR, was only observed in the nasal and bronchial epithelia of subjects who received the highest dose of vector (1 x 10^9 RU). Several adverse events were noted prior to and/or after vector delivery, but it was reported that these were related to the endogenous CF lung disease or a result of the bronchoscopic procedures. Serum-neutralizing antibodies were detected after vector delivery to subjects in the highest dosage cohorts.
Moss and colleagues conducted a randomized, double-blind, placebo-controlled, phase II trial in 2004 where subjects were delivered aerosolized doses of tgAAVCF vector (1 x 10^{13} RU) every 30 days for a total of 3 doses (Moss, Rodman et al. 2004). Improvements in FEV(1) was observed at day 30 and vector shedding was observed in the sputum up to 90 days after the third dose of vector. It was reported that all subjects exhibited an increase (by at least four-fold) in serum AAV2-neutralizing antibodies and while gene transfer was detected, gene expression was not seen.

On the basis of the Moss study, Targeted Genetics Corporation initiated a large-scale repeat-administration study. While the study failed to meet its primary objective of statistically significant improvements in lung function, it confirmed that tgAAVCF doses to CF patients was safe and tolerable (Moss, Milla et al. 2007). It was postulated that the low gene transfer efficiency seen in these clinical trials appeared to be due to the poor binding efficiency of the AAV2 vector to the apical surface of the epithelial cells or the LTR promoter used to drive CFTR expression being too weak.

Studies involving using the AAV2 vector genome with different AAV capsids, such as AAV1, AAV5 and AAV6, have also been evaluated for CF gene therapy. These serotypes are thought to be more suitable to gene transfer to the airway epithelium, and have been shown to be more efficient at transducing airway epithelial cells than AAV2 (Sirninger, Muller et al. 2004; Virella-Lowell, Zusman et al. 2005). Molecular techniques have been employed by Gao and colleagues in an attempt to search for potent AAV vectors with enhanced performance profiles from a variety of human and non-human primate (NHP) tissues (Gao, Vandenberghe et al. 2005). While ten novel pro-viruses were identified, it is still unknown if these viruses are suitable for use in gene therapy.
Retroviruses are among the most efficient tools for gene transduction of mammalian cells and have been successfully used in early clinical trials for the treatment of inherited diseases (Anderson, Blaese et al. 1990) and cancer (Anderson, Blaese et al. 1990; Rosenberg, Aebersold et al. 1990; Osanto, Brouwenstyn et al. 1993; Mahvi, Sondel et al. 1997; Stopeck, Hersh et al. 1997). Retroviruses integrate their genome into the host chromosome (Kalpana 1999), which allows the introduced genetic information to be passed onto daughter cell populations (Kay, Glorioso et al. 2001). Retroviruses, however, integrate into the genome randomly which, in addition to raising the possibility of gene expression being subject to gene silencing (Bestor 2000), increases the risk of insertional mutagenesis, significantly hindering their potential for use in a clinical setting. Additionally, retroviral vectors are only able to transduce dividing cells, and thus have limited applications in gene therapy for the airway where only approximately 1% of the lung cell population is actively dividing at any given time (Leigh, Kylander et al. 1995). The most common retroviral vector is based on the Moloney murine leukaemia virus (MLV) (Romano, Pacilio et al. 1998) as this has been extensively studied and was well understood. This has allowed the development of vector systems with the genes essential for virus particle formation removed, allowing for up to 8kb of exogenous cDNA to be inserted (Kay, Glorioso et al. 2001). The developments in MLV vector design combined with helper-plasmid construction have greatly increased the number of recombination events required for a replication-competent retrovirus to be reconstituted, improving the safety of MLV vectors for clinical use.

The successful application of MLV vectors to CF lung gene therapy is limited by their ability to transduce only actively dividing cells (Kay, Glorioso et al. 2001). With such small levels of active cell turnover in normal airway epithelium the transduction efficiency of MLV in vivo is significantly hampered. However, the level of MLV-
mediated gene transfer can be improved by increasing cell proliferation \textit{via} the use of a mitogen, as demonstrated \textit{in vitro} by Wang and colleagues (Wang, Davidson \textit{et al.} 1998). A major limitation for MLV vectors is that while they do not elicit major host immune responses, they are rapidly inactivated by a component of the innate immune system known as human complement–mediated inactivation in both the airway and in other organs (Romano, Pacilio \textit{et al.} 1998).

The safety of retroviral vectors has been highlighted in recent years following the development of acute T-cell leukaemia in four of the eight patients, all of whom experienced very successful long-term correction of SCID-X1 after infusion of vector-modified bone marrow cells in a French-based trial (Baum 2007). In the first three patients, one of whom has since died, investigation revealed that the vector had inserted in proximity to the LMO2 oncogene promoter, leading to aberrant transcription and expression of LMO2, resulting in an uncontrolled exponential clonal proliferation of mature T cells and leukaemia (Hacein-Bey-Abina, Von Kalle \textit{et al.} 2003). The cause of leukaemia in the fourth patient is yet to be determined, but it is likely a similar insertional mutagenesis event will be observed. Recently obtained molecular profiles of vector insertion sites in this trial (Von Kalle 2006), and the long 60-month latency from tumour development in the latest case, compared to about 30 months latency in the three earlier cases, raise concerns for the remaining patients. A similar parallel study conducted in the United Kingdom using the same gene therapy protocol has reported one of the ten patients in their trial developed acute T-cell leukaemia. Further investigation found this patient also developed leukaemia as a complication of this gene therapy, with an activating insertion in the LMO2 locus (Howe, Mansour \textit{et al.} 2008). While many theorise as to the cause of such frequent insertional mutagenesis, the cause remains unknown.
g. Lentiviral Vectors

The successful development of gene therapy for CF airway disease requires a vector with specific properties; the ability to transduce both dividing and non-dividing cells, to provide stable and sustained gene expression, to not invoke significant host immune and inflammatory responses and with an adequate vector packaging limit for CFTR. In terms of these properties, lentiviral vectors are promising candidates for CF gene therapy.

Lentiviral-based vectors are able to transduce non-dividing cells because their pre-integration complexes undergo nuclear import independent of mitosis (Bukrinsky, Haggerty et al. 1993) and they encode an integrase enzyme that facilitates stable integration of their genome into the host chromosome. An added benefit of LV is that CFTR cDNA easily falls within the packaging limit of LV vectors and high titres are easily generated (Kumar, Keller et al. 2001; Koldej, Cmielewski et al. 2005).

Another advantage of LV is that it is able to be pseudotyped with envelope glycoproteins from a number of other viruses, allowing them to be targeted to the apical or basolateral surfaces of airway epithelial cells. The most common envelopes used for pseudotyping for airway gene transfer are vesicular stomatitis virus G (VSV-G), which targets receptors on the basolateral surface of the epithelium, baculovirus glycoprotein 64 (GP64), which targets receptors on the apical surface of the epithelium, and Filovirus envelopes which target both the apical and basolateral surfaces with equal efficiency (Sinn, Burnight et al. 2005). While VSV-G-pseudotyped LV vectors remain a popular choice for use in CF gene therapy as they produce high titres and have broad tropism, application in polarised airway epithelia in vivo requires transient disruption of the tight junctions to allow access to the basolaterally located receptors (Wang, Slepushkin et al. 1999; Johnson, Olsen et al. 2000; Wang, Zabner et al. 2000).
Different envelope glycoproteins suit different applications. In a recent study comparing transduction efficiency of a VSV-G-pseudotyped vector, in combination with a pretreatment with lysophosphatidylcholine (LPC), and the same vector pseudotyped with the apically-targeted baculovirus GP64 envelope (without any pretreatment), the GP64 vector was found to be significantly less efficient (Kremer, Dunning et al. 2007). Interestingly, if the airway was pre-treated with LPC, GP64 and VSV-G-pseudotyped vectors produced the same levels of gene expression. This suggests that the barrier effects of MCC and the glycocalyx may also be affected by LPC regardless of the location of the receptors used by the vector. In this study the VSV-G-pseudotyped vector resulted in persistent gene expression for six months; the levels of gene expression after infection with the GP64-pseudotyped vector declined to undetectable levels over six months. On the basis that airway cellular turnover occurs approximately every three months (Borthwick, Shahbazian et al. 2001), the persistence suggests that the use of the VSV-G-pseudotyped vector successfully targeted airway progenitor cells, which then produced transduced daughter cells during natural airway cell turnover, while the apically targeted GP64-pseudotyped vector did not. Interestingly, other studies have demonstrated long-lasting gene expression (>50 weeks) after infection with a GP64-pseudotyped vector formulated with a viscoelastic gel (Sinn, Williams et al. 2002; Sinn, Shah et al. 2005), which acts to inhibit MCC and increase residence time of the vector on the epithelial surface. However, the process by which an increased residence time allowed the transduction of airway progenitor cells, it as yet undetermined. One hypothesis was that a niche of airway progenitor cells is in the submucosal glands (Liu, Driskell et al. 2004), and so it may be these cells which are transduced with this method.

While the LV is arguably one of the few vectors with strong potential for CF airway gene therapy, there are still significant questions that must be answered before LV could be taken into a clinical setting. Such as whether a clinically relevant volume of vector
can be easily manufactured; if their advantages regarding long-term gene expression and progenitor cell transduction will translate into reality; and in light of the recent insertional mutagenesis seen in the SCID-X1 trial, whether they are safe.

1.4.4 The Women's and Children's Hospital Lentivirus

The lentivirus used in this project, an HIV-1-based self-inactivating (SIN) vector pseudotyped with the VSV-G protein, was designed and developed by Associate Professor D.S. Anson and colleagues at the Women's and Children's Hospital in South Australia. The Women's & Children's Hospital (WCH) LV contains none of the HIV accessory genes, but retains both the rev and tat regulatory genes, which play essential roles in viral replication. To increase the generation of high titres without increasing the risk of producing replication-competent viruses, all the sequences have been codon-optimised (to ensure optimal gene expression). Similarly to other third-generation lentiviral packaging cassettes, the WCH Lentivirus expresses all proteins from different constructs, however the 5' HIV-1 LTR U3 sequence is not replaced by a constitutive promoter, but retains a third packaging plasmid coding for tat (Fuller and Anson 2001). SIN vectors utilise a heterologous constitutive promoter in place of the HIV-1 U3 and are safer as they don’t contain the sequences required to reconstitute a functional HIV-1 LTR, (Miyoshi, Blömer et al. 1998). The WCH SIN vector is tat-dependant, thus functional, replication-competent virus can only be generated if both the LTR and the ability to express tat are restored (Koldej, Cmielewski et al. 2005). This is an important safety feature as it means the virus cannot exit the cell and indiscriminately infect other cells, as wild viruses are able to do. A schematic of the WCH Lentivirus is shown in Figure 1.7.
pHIV-1-SDm-SV-LacZ-ΔLTR

Figure 1.5: Women’s & Children’s Hospital Lentiviral Vector Expression Constructs, adapted from (Koldej, Cmielewski et al. 2005). In the vector pHIV-1-SDm-SV-LacZ-ΔLTR, RRE represents the Rev response element, cPPT is the central polypurine tract, SV40 is the simian virus type-40 early promoter and β-Gal is the β-Galactosidase protein coding sequence. For pcDNA3.1tat101ml, CMV represents the simian cytomegalovirus immediate early promoter. The tat101ml coding sequence is followed by the bovine growth hormone gene polyadenylation signal. In the pHCMV constructs, hCMV represents the human cytomegalovirus early promoter, the dashed lines represent splicing signals from the rabbit β-globin gene, whv is the woodchuck hepatitis virus post-transcriptional regulatory element followed by the rabbit β-globin polyadenylation signal and VSV-G is the vesicular stomatitis virus G-protein coding sequence.
1.4.5 Stem Cells & Cell Therapies for Cystic Fibrosis

Although in its infancy, the use of embryonic and adult stem cell therapies for CF lung disease is an exciting area of emerging study. *Ex-vivo* transduction of bone marrow-derived stem cells (BMSC) with integrating lentiviral vectors, which are then transplanted back into their irradiated host, is a well-established technique (Sueblinvong, Loi *et al.* 2008). Additionally, it is known that BMSC have the capacity to differentiate into airway epithelial cells. Thus some groups have speculated that BMSC may be useful in treating CF lung disease (Kotton, Ma *et al.* 2001; Krause, Theise *et al.* 2001; Sueblinvong, Loi *et al.* 2008). Unfortunately, others have not reproduced the results showing that BMSC were able to adopt respiratory and alveolar cell properties and the data has been questioned (Kotton, Fabian *et al.* 2005).

It has been well documented that specific cell types within the murine airway possess progenitor-like properties (Borthwick, Shahbazian *et al.* 2001; Schoch, Lori *et al.* 2004; Liu and Engelhardt 2008), although comparative data in humans has not yet been determined. In the future, lentiviral vectors may be designed to specifically target receptors on these resident progenitor-like cells to produce permanent correction of the CF defect, although it will be important to complete long-term studies and monitoring prior to any proof-of-principle trials in humans can be conducted.

1.4.6 Barriers to Viral Gene Transfer to the Airway Epithelium

When gene therapy to correct CF airway disease was demonstrated *in vitro* (Johnson, Olsen *et al.* 1992) and appeared relatively simple, it was assumed that the process would be easily replicated *in vivo*. However, the highly effective series of airway defences that have evolved to protect the mammalian airway from inhaled allergens, irritant, dust and
viral and microbial pathogens similarly defend the airway from gene transfer vectors (Bevins 1999). To improve gene transfer to the airway a greater understanding of the biological barriers protecting the airway, and thus limiting gene transfer, was needed, with the aim of devising methods to circumvent these barriers during gene delivery.

1.4.7 Physical Barriers Present in Normal Airway Epithelium

Inefficient delivery of various vectors has highlighted the complexity of the surface barriers which protect the underlying airway epithelium against foreign particles (Bevins 1999). Gene therapy vectors are foreign particles, and are thus subjected to the same surface barriers that protect the airways (Figure 1.2). The first barrier a gene therapy vector will encounter is the ASL mucous layer, which acts in conjunction with the cilia to continually trap inhaled particles for removal via MCM mechanisms. At the cell surface the glycocalyx, an intricate network of secreted glycoproteins, glycolipids and proteoglycans assembled into an organised structure attached to the cell surface (Wang, Williams et al. 2002), also prevents viral vectors from gaining access to receptors for binding and fusion. Finally, epithelial tight junctions (TJ) present yet another physical barrier to the delivery of gene transfer vectors to their receptors, predominantly located on the basolateral cell surface below the TJs.

1.4.8 Physical Barriers Present in Cystic Fibrosis Airway Epithelium

In addition to the physical barriers in the normal lung, the CF lung poses barriers for gene therapy vectors to overcome. The mucous layer in CF lungs is thought to be dehydrated, resulting in poor MCC and the inhibition of the normal clearance mechanisms within the lung (Boucher 2007; Boucher 2007). The lack of efficient
clearance mechanisms means that inhaled pathogens, such as P. aeruginosa, are able to
easily infect the airways. When the viscosity of the mucus was reduced after treatment
with DNase gene transfer the efficiency of AdV-mediated gene transfer was significantly
increased (Stern, Caplen et al. 1998). Additionally, the small fraction of CF mucous
which is aqueous and CF bronchoalveolar lavage fluid has been shown to inhibit gene
transfer in vitro, hypothesised to be due to the presence of neutralising antibodies to
the vector envelope (Perricone, Rees et al. 2000) or to the presence of viral infectivity
inhibitory factors (Bastian and Bewig 1999). Interestingly, it has also been shown that
in mice lungs infected with P. aeruginosa, a pathogenic organism found in most CF
patients, AdV-mediated gene transfer was significantly hampered compared to non-
infected mouse lungs (Parsons, Grubb et al. 1998; van Heeckeren, Ferkol et al. 1998).
This finding suggests that bronchorespiratory inflammation inhibits AdV-mediated
gene transfer, and so may play a similar inhibitory role when other systems are used.

1.4.9 Progenitor Cells of the Airway Epithelium

Cystic fibrosis is a lifelong disease, so any treatment must be effective for the lifetime
of the patient. One way of achieving this is via repeated treatments with the gene
transfer vector, which, as described in section 1.4.3, can lead to a myriad of undesirable
immune responses, such as neutrophil infiltration, macrophage recruitment and
the release of inflammatory cytokines and more immune cell recruitment. A more
appropriate method of achieving lifelong correction of the CFTR defect may be to
target gene therapy to airway progenitor cells. Successful transduction of the progenitor
cell population of the airway would result in the generation of an expanding progeny
of corrected daughter cells capable of re-populating the epithelium. There is much
scientific debate as to which cells of the airway constitute progenitor cell population
and several widely accepted methods have been developed to aid in the characterisation
of adult airway progenitor cells (Liu, Driskell et al. 2006). These include localization of
label-retaining cells (LRCs) (Borthwick, Shahbazian et al. 2001), retroviral tagging of
epithelial cells seeded into xenografts (Engelhardt, Schlossberg et al. 1995), air–liquid
interface cultures to track clonal proliferative potential (Schoch, Lori et al. 2004) and
multiple transgenic mouse models (Liu, Driskell et al. 2006). Advances in the study of
airway progenitor cells have not identified a single lung progenitor able to give rise to
multiple epithelial lineages in both the proximal and distal airways. There is a widely
accepted concept that local regional niches in the lung control both the phenotype
and expansion of progenitor cells required for their distinct regions of airway (Liu,
Driskell et al. 2006). Additionally, the potential of adult-derived bone marrow cells to
assume an epithelial phenotype has been demonstrated in vivo (Harris, Herzog et al.
2004; Macpherson, Keir et al. 2005), but these results have been subsequently refuted
(Kotton, Fabian et al. 2005), leading to much scientific debate. While the specific nature
and location of human airway progenitor cells remain controversial, basal cells and cells
that reside in submucosal glands have been shown to function as progenitor cells in the
conducting airway of mice (Borges, Linnoila et al. 1997; Borthwick, Shahbazian et al.
2001; Engelhardt 2001; Hong, Reynolds et al. 2004).

1.4.10 Mild Damage to the Epithelium Increases the Level of Gene Expression

Various studies have demonstrated gene transfer with several recombinant viruses,
including retrovirus (Wang, Davidson et al. 1998) AAV (Morgan, Nussbaum et al.
1993; Duan, Yue et al. 1998), and AdV (Pickles, McCarty et al. 1998; Walters, Grunst
et al. 1999) was very inefficient when the vectors were applied to the apical surface of
airway epithelia. Gene transfer efficiency increased greatly when the vectors were applied
to the basolateral surface, indicating that their receptors are restricted to the basolateral
membrane. Subsequently, a number of research groups have been investigating ways to allow the vectors to gain access to the basolateral surface of the epithelium.

Exposure of the epithelium to EDTA (Wang, Zabner et al. 2000), Ca\(^{2+}\) chelators (Wang, Zabner et al. 2000) polidoconol (an irritant that is used clinically at higher levels in the treatment of varicose veins) (Parsons, Grubb et al. 1998) or sulfur dioxide (SO\(_2\)) (Johnson, Olsen et al. 2000) influences the integrity of the airway and increase the permeability of airway epithelia to viral vectors. The result has been to greatly enhance airway gene expression.

### 1.4.11 Lysophosphatidylcholine Pre-Treatment to Improve Gene Transfer

Lysophosphatidylcholine (LPC) is a naturally occurring molecule within the airway and possesses detergent-like properties (see Figure 1.8). Detergents have been shown to improve viral mediated gene transfer in airway epithelium *in vivo* (Parsons, Grubb et al. 1998; Limberis, Anson et al. 2002). Detergents have been demonstrated to act on cellular membranes *in vitro* by disrupting the structure of cellular surfaces that are held together by hydrophobic interactions between the various lipid structures that comprise the membrane (Helenius and Simons 1975). When applied to intact airway epithelial tissue, LPC has been shown to permeabilise epithelial TJs (Parsons, Grubb et al. 1998), and improve vector particle deposition onto the epithelium (Parsons, Hopkins et al. 2000). Additionally, the mucolytic properties of LPC may act to solubilise airway mucus by reducing its viscosity and elasticity (Martin, Marriott et al. 1978) and reduce cilial beat, and thus reduce MCC (Merkus, Schipper et al. 1993; Limberis, Anson et al. 2002). These direct effects on airway surface epithelium may each contribute to enhanced gene transfer *in vivo* by improving vector delivery and retention, but there
are likely to be other biological effects that are yet to be characterised.

Figure 1.6: Molecular Structure of Lysophosphatidylcholine. LPC (1-acyl-sn-glyero-3-phosphocholine) is generated by the hydrolysis of an acyl group of phosphatidylcholine by phospholipase A2. R1 and R2 are fatty acids and in this form of LPC consist of palmitic acid (66%) and stearic acid (33%).
1.5 Research Aims

1.5.1 Overarching Aim

To study the ability of the HIV-1-based Women’s & Children’s Hospital lentiviral vector to produce long term, stable and functional gene expression in the murine airway epithelium.

1.5.2 Specific Aims

1. To establish an LPC pre-treatment protocol that will produce optimal conditions for gene transfer to the murine airway epithelium.

2. To achieve long term gene transfer in the murine airway epithelium after a single dose of the HIV-1-based Women’s & Children’s Hospital lentiviral vector containing the β-galactosidase marker gene.

3. To achieve long term functional correction of the CFTR defect in the cftrtm1unc knockout mouse model after a single dose of the HIV-1-based Women’s & Children’s Hospital Lentiviral Vector.
Chapter 2
Materials & Methods
2.1 Chemicals and Reagents

All chemicals and reagents were of analytical grade and obtained from Sigma Chemical Company or BDH Chemicals unless otherwise specified. All other suppliers are listed in Appendix One in alphabetical order. All buffers and solutions were made using MilliQ water (MQ H\textsubscript{2}O) (Millipore Corporation). The recipes for all buffers and solutions are described in Appendix Two, again these are in alphabetical order.

2.2 Virus Production

2.2.1 Cell Culture

All cell lines were cultured at 37 °C, in a humidified 5 % CO\textsubscript{2} atmosphere. NIH3T3 and 293T cells were maintained in DMEM / 10 % (v/v) FCS with regular subculturing in T75 flasks (NUNC, Nalgene Nunc International, NY, USA). A549 and 293T/5%FCS cells were maintained in DMEM / 5 % (v/v) FCS. Once cells had reached confluency, the media was aspirated, the cells rinsed with 4 mL PBS and incubated in trypsin / EDTA solution until the cells detached. For NIH3T3, 293T/5%FCS and 293T cells, a 1:10 (v/v) dilution of trypsin / EDTA solution in PBS was used. For A549 cells, undiluted trypsin / EDTA solution was used. Once the cells had detached, 8 mL of the appropriate growth medium was added to neutralise the trypsin / EDTA solution. The cells were pipetted to single cell suspension and subcultured as required (usually 1:2 to 1:10) into 10 - 15 mL of the appropriate growth medium in T75 flasks and incubated until confluency was reached once more. When culturing, harvesting or splitting 24, 12 or 6 well plates, volumes were adjusted such that 0.5 mL, 1 mL or 2 mL respectively was used in each well and after trypsinising the cells were added.
directly to fresh wells containing the appropriate growth media or to 1 % FCS in PBS as required to neutralise the trypsin.

2.2.2 DNA Plasmid Preparation

Plasmid DNA was prepared using the Qiagen plasmid midi–kit according to the manufacturer’s (Qiagen, Victoria, Australia) instructions.

2.2.3 DNA Construction and Virus Production

This study used a replication-deficient lentiviral (LV) vector system derived from HIV-1. The vector was produced by transient transfection of 293T cells using a five plasmid system as previously described (Koldej, Cmielewski et al. 2005). The LVLacZ vector contains from 5’ to 3’ the human immunodeficiency virus type 1 (HIV-1) YU-2 5’ viral long terminal repeat (LTR) and contiguous sequence extending 550 base pairs into the gag gene, the cPPT sequence, an extended RRE sequence, the SV40 early promoter, the LacZ gene sequence, the HIV-1 YU-2 polypurine tract and the 25 base pair sequence immediately 5’ of the PPT and the 3’ LTR with the rev response element (RRE) replacing sequences between the EcoRV and PvuII sites in the 3’ region (see Figure 2.1). This renders the vector self-inactivating because vital transcriptional elements in U3 have been replaced with the RRE sequence. Virus titre was determined on NIH3T3 cells (Limberis, Anson et al. 2002) to be 6x10⁸ tu/mL and was assessed to ensure the virus was replication competent virus-free as previously described (Limberis, Anson et al. 2002).
Figure 2.1: The Structure of the LacZ Lentivirus Vector Construct.

The LV/LacZ vector construct is shown. The long terminal repeat is represented as a white box comprised of the U3, R and U5 sequences. SD: major splice donor; ψ: packaging signal; RRE: Rev response element; cPPT: central polypurine tract; gag: gag gene sequence; SV40: simian virus 40 early promoter; LacZ: β-galactosidase gene coding sequence. Scale bar= 100 bp

2.3 Large Scale Recombinant Lentivirus Production & Processing

2.3.1 General

This protocol was designed for 30 x 245 mm square plates, which makes approximately 5 L of virus containing medium resulting in approximately 2 mL of concentrated virus

2.3.2 CaPO₄ Co-Precipitation & Transfection

293T cells were grown in T75 flasks until confluent and were subcultured according to the following schedule:

Day 1: Subculture 293T cells 1:5 into 5 x T75 flasks
Day 3: Subculture 293T cells 1:2 into 10 x T75 flasks
Day 4: Subculture 293T cells into 10 x 150mm dishes
Day 5: Subculture 293T cells into 25 x 150mm dishes
On the sixth day the cells were counted using a haemocytometer and plated into 30 x 245-mm square plates with 3.89 x 10^7 cells per plate in 105 mL of culture medium (DMEM / 5% FCS (v/v)). Plates were placed into an incubator at 37° C overnight.

Between 20 and 24 hours after the cells have been plated the medium was aspirated and the cells were fed with 10 ml of DMEM/ 10% (v/v) FCS. One hour later the cells were transfected with a mix of five different plasmids comprising of the LV vector plasmid, pcDNA3gagpolml, pcDNA3TAT101ml, pHCMV–G and pCMV–rev as previously described (Koldej, Cmielewski et al. 2005). The volumes of DNA required are outlined in Table 2.1.

Table 2.1: Virus was prepared using plasmids mixed in the volumes described.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Amount of DNA (for 31 plates)</th>
<th>Conc. of Plasmid Prep</th>
<th>Vol. of prep required (for 31 plates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHIV-MPSvlns-LacZ</td>
<td>4898 μg</td>
<td>2.323 μg/μL</td>
<td>2108 μL</td>
</tr>
<tr>
<td>pcDNA3Tat101ml</td>
<td>97.96 μg</td>
<td>1.189 μg/μL</td>
<td>82.4 μL</td>
</tr>
<tr>
<td>phCMVRevmlwhvpre</td>
<td>97.96 μg</td>
<td>2.80 μg/μL</td>
<td>35.0 μL</td>
</tr>
<tr>
<td>phCMVgagpolmlstwhv</td>
<td>489.8 μg</td>
<td>1.601 μg/μL</td>
<td>306 μL</td>
</tr>
<tr>
<td>phCMV-G</td>
<td>244.9 μg</td>
<td>1.265 μg/μL</td>
<td>194 μL</td>
</tr>
<tr>
<td>Combined Volume of Plasmids</td>
<td></td>
<td></td>
<td>2725 μL</td>
</tr>
</tbody>
</table>

The DNA was made up to a volume of 86.55 mL with sterile H2O before adding 9.92 mL of 2.5 M CaCl₂ (see Table 2.2). The DNA–CaCl₂ solution was added drop–wise to an equal volume of 2 x Hepes buffered saline (HeBS) buffer whilst vortexing. Once all the DNA–CaCl₂ solution was added, the tube was vortexed for an additional 20
seconds and the solution was allowed to stand for 90 seconds. Once 90 seconds has elapsed the DNA / CaCl₂ / HeBS mixture was added drop-wise (evenly distributed) onto the plate and gently swirled to mix. Plates were then placed into the post-transfection incubator at 37 °C overnight. Following the overnight incubation the transfection medium was removed and 170 mL of pre-warmed Opti-pro serum free media (Invitrogen, Victoria, Australia) supplemented with glutamine, penicillin and streptomycin was added to each plate prior to the plate being returned to the incubator for another 24 hours.

Table 2.2: Transfection Medium Composition.

<table>
<thead>
<tr>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5M CaCl₂</td>
</tr>
<tr>
<td>H₂O</td>
</tr>
<tr>
<td>Plasmid DNA mix</td>
</tr>
<tr>
<td>Total Volume</td>
</tr>
</tbody>
</table>

2.3.3 Filtration & Concentration Of The Lentivirus Vector

The supernatant from the plates was collected into a 5 L weighed container (supernatant volume is approximately 4.5 – 5 L). The weight of the container was measured once again after the supernatant was removed, so the volume of supernatant could be determined accurately. A 1 mL sample was taken and stored at 4 °C for use in the virus assay the following day.
The LV supernatant was filtered through an Amersham 0.45 μm hollow-fibre cartridge (GE Healthcare, NSW, Australia. Product Number CFP-4-E-4MA) at room temperature. Once the majority of the LV supernatant was filtered through, 50 – 100 mL of 0.1% BSA in PBS was put through the filtration system to ensure all the LV supernatant was retrieved.

The virus was then concentrated approximately 20-fold using an Amersham Quickstand system (GE Healthcare, NSW, Australia) (see Figure 2.2) with an UFP-750-E-4x2MA 750 kDa cut-off cartridge, again at room temperature. The virus was then passed through a 0.8 μm syringe filter unit (Millipore Corporation, Victoria, Australia) before ultracentrifugation at 50 000 g for 90 min (Beckman SW40) (Beckman Coulter, NSW, Australia) at 4°C. The resulting pellet was gently resuspended in 100 μL of PBS, incubated on ice for 1-2 hours and then pooled into a single tube. Resuspended concentrated virus was divided into 80 μL aliquots and stored in a -80°C freezer until required.

Figure 2.2: Filtration set-up for large scale Recombinant Lentivirus Production & Processing.
2.3.4 Real Time-Polymerase Chain Reaction (RT-PCR) To Determine Virus Titre

All PCRs were performed using the FastStart High Fidelity PCR System with the reactions containing 1 μL of each primer and 100 μL dNTPs. All primers were resuspended to a concentration of 100 μM in MQ H₂O and stored as stock concentrations at -20 °C. All DNA oligonucleotides were designed by R. Koldej and manufactured by Invitrogen Corporation (Invitrogen Corporation, CA, USA).

2.3.5 Sample Preparation

The desired cells (NIH3T3 or A549) were transfected with virus as described in Section 2.3, except the cells were split 1:4 into 24 well plates and incubated for 48 hours at 37 °C/5% CO₂. After the cells were harvested and spun down, the cells were washed twice in PBS and the cell pellet stored at -20 °C until required. The genomic DNA was isolated from the cells using the Wizard SV Genomic DNA isolation Kit and stored at -20 °C until required.

2.3.6 Virus Titre Assay

The gDNA was assayed for the presence of the desired gene (LacZ or CFTR) in either MicroAmpR Optical 96 well Reaction Plates with an ABI PRISM™ Optical Adhesive Cover or ABI PRISM™Optical tubes with ABI PRISM™ Optical Caps (All Applied Biosystems, CA, USA). Each reaction contained 2-5 μL gDNA, 1 x TaqMan Universal PCR Master Mix (Applied Biosystems, CA, USA), 0.9 μM of each primer (Invitrogen Corporation CA, USA) and 0.25 μM of probe in a total volume of 20μL. Each assay
also included standards and no template controls in triplicate for each gene analysed. The assays were run under the following conditions: 50 °C for two minutes, 95 °C for ten minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for one minute (Applied Biosystems 7300 Real Time PCR machine, Applied Biosystems, CA USA). The fluorescence of each reaction was read at the end of each cycle. The data was outputted as an amplification plot. Using the standards as a guide, the threshold bar was placed in the linear part of the plot and the cycle threshold value (Ct) for each sample determined. The following formulas were then used to determine the titre of the virus initially added to the cells.

\[
\text{Ct} = \text{cycle threshold and } K = \text{constant as detailed in Sequence Detection Systems Chemistry Guide (Applied Biosystems, California, USA).}
\]

\[
\text{Ct} = \text{Ct gag} - \text{Ct transferrin (control gene)}
\]

\[
\text{Ct} = \text{Ct of sample} - \text{average Ct of standard}
\]

Copy number per cell = \(\frac{1}{2 \text{ Ct}}\)

\[
\text{Titre} = \text{copy number per cell} \times \text{number of cells initially plated} \times 1000 \\
\text{volume assayed in } \mu\text{L}
\]

2.3.7 X-Gal Determination of LVLacZ Viral Titre

CHO-K1 cells were grown on a 12-well tissue culture plate and transduced with the LVLacZ vector as described in section 2.3. Four days later the cells were washed three times with 1mM MgCl\(_2\) in PBS for 15 minutes at room temperature and fixed with
4% PFA before being exposed to 1 ml of X-gal solution at 37 °C. Cells were exposed to X-gal solution until staining had reached sufficient intensity to allow easy numeration of LacZ positive cells (generally 4 - 6 hours). The X-gal solution was then aspirated and cells washed in PBS and then covered in 80% glycerol. Using an inverted microscope, the number of LacZ positive cells in three 0.25 cm² squares was determined and the titre of the lentivirus vector was determined by counting the number of blue cells, multiplying by the area of the well and then adjusting for the volume of virus assayed, to give the number of transducing units of virus per mL.

2.3.8 Assay for Detection of Helper Virus

To monitor for replication competent retrovirus (RCR) the p24 levels in the media of LV-transduced 293T cells was examined. 293T cells were plated in DMEM with 5% FCS for four hours, after which the media was changed and polybrene and gentamycin were added. Cells were transduced with a sample of concentrated lentivirus vector stock (LVLacZ or LVCFTR) as described in Section 2.3 and maintained in culture for 18 days. Once a week, a 450 μL sample of media was collected and stored at -20 °C until all samples were collected. The media from non-transduced cells was also collected to use as a negative control. The p24 assay was performed using the HIV-1 p24 ELISA kit (Perkin Elmer). Standards ranging from 6.25 pg / mL to 800 pg / mL were prepared in PBS / 1%FCS and samples were diluted in PBS / 1 % FCS as required. 20 μL of triton X-100 was added to each well followed by 200 μL of sample or standard. The plate was then sealed and incubated at 37 °C for two hours after which it was washed thoroughly six times with 300 μL of 1 x wash buffer and 100 μL of detector antibody added to all wells. The plate was sealed and incubated at 37 °C for 60 minutes. The plate was again washed six times, 100 μL of streptavidin-Horse Radish Peroxidase (HRP) concentrate diluted 1:100 in streptavidin-HRP diluent was
added and incubated at room temperature for 30 minutes. Just before required, 1 orthophenylenediamine-HCl (OPD) tablet was added to 11 mL of substrate diluent. The plate was washed as above, 100 μL of OPD solution was added to each well and incubated at room temperature for approximately 10 minutes for colour development. Once colour had developed sufficiently, 100 μL of stop solution was added to each well and OD490 determined.

2.4 Mice

2.4.1 General Information

Adult (8-24 week) C57/Blk6 females were used throughout these experiments, unless specified. All mice used were obtained through the Child, Youth and Women’s Health Service (CYWHS) Animal House Facility and were housed in PC1 laboratory conditions until vector dosing, after which they were moved into PC2 laboratory conditions. In PC1 and PC2 holding areas animals were exposed to a 12-hour light / 12-hour dark cycle with food and water ad libitum. All experiments were approved by the Child, Youth and Women’s Health Service Animal Ethics Committee (Ethics Number 557/12/07) and by the University of Adelaide Animal Ethics Committee (Ethics Number M074 - 2006).

2.4.2 In Vivo Instillation of Lentiviral Vector Into the Mouse Nasal Airway

Mice were weighed and identified by tail colour-coding with marker pen, or ear punches. Mice were anesthetised with a mixture of Xylazil (12 μg/g body weight)
(Lyppard Australia Limited, Adelaide, Australia) and Ketamine (40 μg/g body weight) (Lyppard Australia Limited, Adelaide, Australia) given intramuscularly in preparation for LPC delivery. Approximately 45 minutes after the initial anaesthetic, mice were re-anesthetised with one half of the initial anaesthetic dose, to maintain anaesthesia until the second delivery (of virus vector) that is given one hour after the LPC delivery. For instillation of the lysophosphatidylcholine (LPC) (Sigma Chemical Company, MO, USA Product Number L4129) pre-treatment and lentivirus, mice were anaesthetised and suspended by their dorsal incisors with their body supported from beneath. Then, 4 μL of the LPC pre-treatment (at various concentrations, or a PBS control) was delivered as a bolus droplet into the mouse right nasal airway via inhalation-driven instillation using a pipettor with a gel-loading pipette tip (Finnpippette, Thermo Fisher Scientific, MA, USA) as previously described (Kremer, Dunning et al. 2007). Delivery was achieved without the pipette tip contacting the nose. At various time points after the pre-treatment solution was instilled (as specified within each experiment), a 20 μL dose of the lentivirus was administered into the same nasal airway in two 10 μL aliquots, again using the inhalation-driven instillation method.

2.4.3 Processing of Mouse Heads for LacZ Gene Expression

Mice were sacrificed at specified time points via CO₂ asphyxiation, and cardiac puncture was performed to minimise blood contamination of the airway lumen during dissection as blood can interfere with X-Gal staining. The head was cut from the body at the neck and the fur and skin removed. The soft cartilage at the nose tip was cut off and the nasal cavities were immediately flushed via the cut trachea with 2% (w/v) paraformaldehyde / 0.5% (v/v) glutaraldehyde in PBS. Tissues were fixed, stained and decalcified as previously described (Limberis, Anson et al. 2002). The tongue and jaw were removed and heads were hand-sectioned according to specified landmarks equivalent to levels
6, 16 and 24 as described in Mery et al. (Mery, Gross et al. 1994). At this stage, each
*en face* section was examined and photographed under 10x – 40x magnification using
a Nikon stereomicroscope (Nikon Australia, NSW, Australia). These three gross tissue
sections were embedded in paraffin wax and 6-8 μm standard sections cut from the wax
blocks (i.e each slide had three cross-sections, one from each level). The sections were
counter-stained with Safranin-O (Saf-O) as described in Section 2.4.4 and numbers of
LV LacZ positive cells counted at 400x light magnification (Olympus BX5, Olympus
Australia, NSW, Australia). The total cell count was the total number of LacZ positive
cells in the three cross-sections.

### 2.4.4 Safranin-O Histological Staining

Safranin-O (Saf-O) non-specifically counterstains the whole tissue, with the red
contrast produced allowing for the number of LacZ positive cells to be easily identified
and counted. The wax tissue sections were hydrated in water and lightly stained in
Saf-O for 15 seconds, and washed with water. Stained sections were then dehydrated
through alcohols (35% for 1 minute, 70% for 1 minute and 100% for 1 minute) and
cover-slipped using DPX (APS Finechem, NSW, Australia).

### 2.4.5 Haemotoxylin/Eosin Histological Staining

Haemotoxylin stains nucleic acids while eosin stains both extracellular organelles and
the cytoplasm, so the combination of these stains allows for the identification of cell
types within the tissue. The wax tissue sections were lightly stained in haemotoxylin for
two minutes, washed in water and then exposed to Li₂CO₃ before being counterstained
with eosin for 30 seconds. The sections are again washed with water, dehydrated
through alcohols and cover-slipped using DPX (APS Finechem, NSW, Australia).
2.4.6 Transduced Cell Type Identification Within The Airway Epithelium

The cell types expressing LacZ (i.e. respiratory, transitional, squamous and olfactory – the four types of nasal epithelium) were determined in adjacent sections counter-stained with haematoxylin and eosin (H&E) (Parsons, Grubb et al. 1998). Cell type was identified at 1000x magnification using the haematoxylin and eosin stained sections. Cells were categorised as follows:

- Ciliated or Non-Ciliated Cells: These cells have a columnar appearance with or without visible apical cilia.
- Secretory Cells: These cells show a narrow base and expanding apical portion that sometimes projected into the lumen, with a nucleus in the bottom half of the epithelial layer.
- Basal Cells: These cells appear approximately triangular to cuboidal in shape, were closely adherent to the basal laminar and the nucleus was in the bottom third of the epithelial layer.
2.4.7 Identification Of Label-Retaining Cells (LRC)

a) Repeat Damage and Bromodeoxyuridine (BrdU) labelling study

Female C57Bl/6 mice (6-8 weeks old) received weekly damage to the nasal airway epithelium by instillation of 4 μL of 1% lysophosphatidylcholine. In addition, these mice (n = 8) received intraperitoneal injections of BrdU (2mg) (Abcam, Ma, USA) every 48 hours beginning two hours after the first injury and extending until 24 hours after the final (weekly) injury. One hour after the final injury 20 μL of LVLacZ vector was delivered as described in Section 2.3. Seven days later the mice were sacrificed and their nasal airways were examined for BrdU incorporation by immunohistochemistry. Controls included groups of mice (n=8) receiving BrdU but without nasal airway damage, and LPC-damaged mice (n=8) receiving no BrdU.

b) Bromodeoxyuridine (BrdU) Immunohistochemistry

BrdU incorporation was detected in sections of nasal airway epithelium using a staining procedure modified from previously reported methods (Borthwick, Shahbazian et al. 2001). Mice were sacrificed and heads were removed as previously described (Section 2.4.3). Mouse heads were fixed with Shandon Glyco-Fixx (Thermo Scientific, product number 6764262) and decalcified using Immunocal (United Biosciences, Queensland, Australia). Slides were baked for two hours at 60 °C and then dewaxed and dehydrated though xylene and ethanol. The tissue was quenched in 1.5% H₂O₂ in PBS for 30 minutes under gentle rocking, prior to being washed twice in water. The slides were placed into warm 1M HCL (in a water bath) for 12 minutes then washed twice in water and then again in PBS under gentle rocking. The slides was treated with Proteinase K in PBS for 15 minutes, washed in PBS three times and then
blocked with 1% normal goat serum in PBS. After rinsing in PBS for five minutes, a mouse anti-BrdU monoclonal IgG (Dako, Victoria, Australia) was applied at a 1:100 dilution, for two hours at room temperature, in the dark. Labelling was visualized with a rabbit anti-mouse biotinylated IgG and avidin / biotinylated horseradish peroxidase reagents (Dako, Victoria, Australia) (at room temperature for one hour) and with DAB substrate (Sigma-Aldrich, NSW, Australia) (1:400 dilution) for 15 minutes in the dark. A normal mouse monoclonal IgG (Dako, Victoria, Australia) was used as a negative control. The tissues were counterstained in haematoxylin for 30 seconds. The slides were then dehydrated via 70% and 100% ethanol and xylene and cover-slipped.

2.4.8 LacZ Immunohistochemistry

The slides were rehydrated via 70% and 100% ethanol and xylene and cover-slipped. Endogenous peroxidise was inactivated using hydrogen peroxide and slides were blocked with normal goat serum (1:30 dilution) for 15 minutes and 30 minutes respectively at room temperature. Rabbit anti-LacZ antibody (Clontech, California, USA) was applied, 1:100 in 10% FCS / PBS, for 1 hour at 37 °C followed by an overnight incubation at 4 °C. The primary antibody was washed off in PBS and the goat anti-rabbit antibody (Abcam, MA, USA) was applied (1:100 in 10% FCS / PBS) for one hour at 37 °C. The tissue was incubated with DAB substrate (Sigma-Aldrich, NSW, Australia) for 15 minutes in the dark. The slides were counterstained in eosin for 30 seconds and then dehydrated via 70% and 100% ethanol and xylene and cover-slipped.
2.4.9 Heat-Induced Epitope Retrieval

Sections were deparaffinised in two changes of xylene, for five minutes each before hydrating through 100%, 70% and 35% ethanol (for two minutes each) and rinsed in distilled water. A glass Coplin jar was filled with 2N HCl, in distilled water, the cover was placed on the jar, and the HCl was equilibrated to 40 °C in a water bath. The slides were then incubated in the HCl solution for 20 minutes. At the end of this incubation immunohistochemical processing was initiated with a PBS wash of the slides.

2.4.10 The Cftr<sup>tm1Unc</sup> Mouse Colony

The cftr<sup>tm1Unc</sup> knock-out mouse was originally generated at the University of North Carolina, Chapel Hill, in 1992 by Snouwaert and colleagues (Snouwaert, Brigman et al. 1992). The CF mouse colony at CYWHS was established in 1993 with three breeder pairs bought from The Jackson Laboratory and is now housed under PC2 conditions in the CYWHS Animal House Facility. Breeder cages are comprised of a CF homozygote male and a CF heterozygote female known to be fertile, resulting in a 75% probability of a homozygote CF mouse birth. New breeder pairs are set up when litter sizes have become too small (1 - 2 per litter) or there is an unexpectedly high newborn mortality rate (>60%). The typical success rate (at birth and weaning) for the generation of homozygote CF mice can be as low as 5%, or as high as 40% of the litter, but averages 25% to 30% of the litter (personal communication, L. Jenkins-White, CYWHS Animal House). The entire colony of Cystic Fibrosis knock-out mice are fed with standard mouse breeder cubes but drink 4.5% (w/v) ColonLytely (Dendy Pharmaceuticals, Victoria, Australia) in their feeding water to prevent the fatal obstructive gut disease associated with the CF homozygote phenotype. Homozygote
CF mice were positively identified by the distinctively white colour of the dorsal incisors 2 – 4 weeks after weaning (Wright, Kiefer et al. 1996), which has been shown in our lab and others to be as accurate as PCR analysis for the determination of CF phenotype in this colony.

2.4.11 Processing Of Mouse Heads For CFTR Gene Expression

Mouse heads were processed as in Section 2.4.3 with the following modifications. Tissues were fixed in Shandon Glyo-Fixx (Thermo Fisher Scientific, MA, USA) for 24 hours before decalcification in Immunocal (United Biosciences, QLD, Australia) for 24 hours. The three gross tissue sections (see Section 2.4.3) were embedded in OCT and 6 - 8 μm standard sections cut from the frozen blocks and used for CFTR Immunohistochemistry as described in Section 2.4.12.

2.4.12 CFTR Immunohistochemistry

Frozen section slides were allowed to thaw at room temperature in a humidified chamber, prior to being fixed at room temperature in 4% paraformaldehyde (PFA) and then dehydrated briefly in 100% ethanol and rinsed with phosphate-buffered saline (PBS) containing Ca²⁺ and Mg²⁺ (PBS+). Tissues were incubated in goat anti-mouse IgG Fab fragment (Jackson ImmunoResearch, PA, USA) to block endogenous binding sites and fixed briefly with 4% PFA, washed with PBS+ and blocked in 10% normal goat serum (NGS, Jackson ImmunoResearch, PA, USA). Sections were incubated at 41 °C overnight with anti-human CFTR antibody 596 (generously provided by Dr J Riodan, CF Center, University of North Carolina) diluted at 1:1000. Primary antibodies were diluted in 10% NGS. For immune detection of human CFTR, slides
were incubated with biotinylated goat anti-mouse (Jackson ImmunoResearch, PA, USA) diluted at 1:2000, followed by streptavidin alexa 568 (Sigma Aldrich, CA, USA) diluted at 1:500. All secondary and tertiary antibodies were diluted in washing solution (1% NGS). Slides were washed with PBS+/0.05% Tween and mounted using Vecta- Shield mounting medium containing 4,6-diamidino-2-phenylindole to label nuclei (Vector Laboratories, CA, USA) and sealed with nail polish.

2.4.13 Measurement Of Mouse Nasal Transepithelial Potential Difference

Mice were anesthetised with a mixture of Domitor (75 μg/g body weight) (Lyppard Australia Limited, Adelaide, Australia) and Ketamine (100 μg/g body weight), and suspended by their dorsal incisors on a loop of stiff wire (body supported). A subcutaneous agar-filled needle bridge was placed in the mouse abdomen as a reference electrode. A heat-drawn PE10 cannula marked at 2.5 mm and 5 mm was inserted in the treated nostril and connected to a perfusion/recording apparatus (consisting of a dual syringe pump (WPI SP2201Z Syringe Pump), a WPI Isomillivoltmeter and a laptop running DataStudio (Version 1.9.8r2, Pasco Scientific, CA, USA). The TPD set-up is shown in Chapter 5, Figure 5.1. The syringe pump was loaded with three 1 mL Hamilton glass syringes (Series 1000 gastight syringes, Hamilton Company, NV, USA) containing either the basal, basal plus amiloride or low Cl- plus amiloride (where the NaCl was replaced with Na gluconate) perfusion solutions and connected to the tubing system. The cannula was inserted to a depth of 2.5 mm to 3 mm to ensure the reading was taken from respiratory epithelial region of the nasal cavity. The basal solution was infused at a rate of 2.5 μL/min and the transepithelial potential difference (TPD) was recorded in DataStudio. The basal solution was infused until a stable TPD value was recorded (a plateau of at least one minute was required) before the solution
was switched to basal plus amiloride. Amiloride was included to block the function of the sodium channel so changes in airway TPD could be ascribed to the function of the chloride channel. Again, the basal plus amiloride solution was infused until a stable value was recorded (a minimum two minute plateau was required). The infusion was then switched to the low Cl\(^-\) plus amiloride solution until a new stable TPD value was obtained (a minimum two minute plateau was again required). At the end of each TPD recording, the reference electrode and the cannula were placed into 0.9% NaCl solution to assess any drift in electric potential of the recording system compared to the same assessment made prior to recording from an animal. If the drift value was greater than 2 mV the reading was discarded. The majority of drift values were less than 1 mV.

The mice were monitored for respiratory distress and excess fluid in the nostril was removed by wicking with fine twists of tissue. Wicking events were noted on the traces, as they could induce sudden changes in TPD that required notation and explanation. Once TPD readings were complete, mice were administered with Antisedan (0.2 μg/g body weight) (Lyppard Australia Limited, Adelaide, Australia) and allowed to recover in a 37 °C air chamber.

Individual ΔTPD values were blinded and assessed by experienced TPD technicians to remove the possibility of bias from printouts of the DataStudio. The ΔTPD values were calculated by subtracting the TPD value recorded under basal plus amiloride conditions from the TPD value recorded under low Cl\(^-\) plus amiloride conditions.

### 2.5 Statistical Analysis

Statistical analysis of the data was performed using SigmaStat 3.0 (SPSS), with advice provided by institutional biostatisticians. Data is presented as a mean ± standard
error (SEM). Student’s t-test was performed for two-group comparisons and multiple treatment groups were analysed by one-way analysis of variance (ANOVA) using post-test multiple comparisons to identify specific group differences. Where data did not satisfy normality assumptions, standard transformations or appropriate non-parametric methods were used.
Chapter 3
Airway Pre-Treatment Enhances Lentiviral Mediated Gene Expression in Living Mouse Airways
3.1 Introduction

The potential for gene therapy as an effective form of treatment for cystic fibrosis has been hampered by limited gene transfer efficiency. Indeed, issues of poor efficiency head the list of challenges for most gene vectors in most biological systems (Frecha, Szecsi et al. 2008). Parsons and colleagues have reported advances in the efficiency of gene expression in airway in vivo using a combination of a pre-treatment with the surface active agent lysophosphatidylcholine (LPC) and a lentiviral (LV) vector (Limberis, Anson et al. 2002). However, upon histological examination, the LPC dose used in that study (1%) produced short-term, though tolerable, epithelial damage.

A lentiviral vector was chosen for these experiments because it can stably integrate into the host chromosome, and it also has the ability to transduce both dividing and non-dividing cells, an important feature in this setting since airway epithelium has a low mitotic index (Leigh, Kylander et al. 1995; Naldini, Blomer et al. 1996; Wang, Slepushkin et al. 1999). Using a helper dependent adenovirus vector (HDAd), others have shown that LPC used at a lower dose (0.1%) in intact lung caused negligible side effects and yet it remained a potent enhancer of gene transfer in rabbit and baboon lung (Hiatt, Brunetti-Pierri et al. 2005; Koehler, Frndova et al. 2005).

Although there was some indication that different concentrations of LPC pre-treatment may alter the subsequent level of gene expression (Koehler, Frndova et al. 2005), there is little information on which to base LPC dose selection to achieve optimal gene expression. The consequences of varying LPC dose levels and pre-treatment timing were therefore examined to establish and to optimise the level of lentiviral gene expression, while minimising the generation of adverse effects on airway epithelium.

It was found that the effect of LPC use in mouse airway was dose-responsive and that
a dose setting could be identified that allowed enhanced airway gene transfer without producing histological evidence of epithelial damage.

3.2 Methods

3.2.1 LPC Pre-Treatment To Enhance Airway Gene Expression

Female C57Bl/6 mice were anaesthetised with an intramuscular injection of a mixture of Xylazil (12 μg/gram body weight) and Ketamine (40 μg/gram) before the delivery of 4 μL PBS control or LPC at one of the following concentrations: 0.03%, 0.1%, 0.3%, 1% or 2% w/v to the mouse nasal airway as described in section 2.3.2. One-hour post-instillation the LV LacZ vector (6 x 10^8 tu/ml) was similarly delivered into the same nostril in 2 x 10 μL aliquots. Seven days after LV vector delivery mice were sacrificed as described in section 2.3.3 and nasal airway LacZ gene expression was assessed after X-gal processing in safranin-O stained tissue as described in section 2.3.4.

3.2.2 Studies Of Altered Time Delay Between LPC And Lvlacz Delivery On Enhancement Of Airway Gene Expression

Female C57Bl/6 mice were anaesthetised as described above before the delivery of 4 μL PBS control or LPC (0.3% or 1% w/v) to the mouse nasal airway (see section 2.3.2). At various time points (5, 15, 30, 60 or 120 minutes) post-instillation the LV vector (6 x 10^8 tu/ml) was similarly delivered into the same nostril in 2 x 10 μL aliquots as described in section 2.3.2. Seven days after LV vector delivery mice were sacrificed and nasal airway LacZ gene expression was assessed as described above.
3.2.3 Histological Analysis Of The Effect Of LPC On The Airway Epithelium

Female C57Bl/6 mice were anaesthetised as described above before the delivery of 4 μL LPC (0.3% or 1% w/v). At varying time points (5, 15, 30, 60 or 120 minutes) post-instillation mice were sacrificed and the mouse heads were dissected and processed to wax blocks as described in section 2.3.3. Histological sections of nasal airway were cut and stained with AB/PAS to reveal mucosubstance (see section 2.3.5). The mean number and size of the mucosubstance-containing goblet cells along the epithelium exposed to LPC were assessed (blinded) and compared to the number and size of the goblet cells along the equivalent regions of epithelium in the non-treated nostril of the same animal.

3.3 Results

The dose-responsiveness of LPC in the mouse airway and the effect of different time delays between LPC and gene vector delivery, each affected the level of gene expression. The results showed that certain dose settings should be avoided to prevent damage of airway cells while ensuring satisfactory gene expression was retained.

In the gene transfer studies described in this chapter the treated mice showed no signs of behavioural distress (assessed using the CYWHS Animal House Animal Monitoring Sheet, see Appendix 3), nor was unusual weight change ever observed (weight was monitored daily for the duration of the experiment, see Figure 3.1). This suggests that immune or inflammatory responses were not evoked in response to the instillation of LPC, the LV vector or to the combination of both.
Figure 3.1 Effect of Anaesthesia and Exposure to LPC/LV Vector on Mouse Weight

The weight of mice (A - E) was measured prior to anaesthesia (day 1) and then monitored up to the termination of the experiment (day 7). Mice were exposed 1% LPC/LV LacZ. Mice F - J were only anaesthetised to monitor normal weight loss following anaesthesia.

### 3.3.1 LPC Enhancement Can Be Optimised For Airway Gene Transfer

The optimal level of enhancement of gene transfer that could be obtained with the standard 60 minute time interval between LPC pre-treatment and vector delivery was first assessed by varying the concentration of LPC, delivered one hour before vector delivery. The effects of these changes on gene transfer are shown in Figure 3.2. Except for the lowest concentration used (0.03%), all concentrations of LPC resulted in significantly increased gene transfer compared to the control (PBS), where no gene
transfer was apparent. Mice exposed to 0.3% or 1% LPC before LVlacZ instillation displayed significantly higher gene expression than at all other LPC concentrations. The levels of gene transfer obtained with 0.3 and 1% LPC, however, were not significantly different (ANOVA, p<0.05, Holm-Sidak post-hoc test). However, the level of gene transfer achieved with 0.3% LPC is clearly not inferior to that with 1% LPC, indicating that gene transfer efficiency is not compromised with the use of a lower concentration of LPC.
Figure 3.2: Airway LVLacZ gene expression in nasal airway after pre-treatment with increasing concentrations of LPC. LPC was administered 60 minutes before the instillation of LVLacZ gene vector and gene expression was measured 7 days after dosing. Gene transfer was evident after pre-treatment with all concentrations of LPC tested. Higher levels produced significant gene transfer compared to control (PBS) (*p<0.05, ANOVA, mean ± SEM, n= 8). Pre-treatment with either 0.3% or 1% LPC produced the highest levels of gene transfer**, and did not significantly differ from each other. (p<0.05, ANOVA, mean ± SEM, n= 8 except at the 1% LPC / 60 minutes delay combination where data from two independent experiments were pooled, n=15). It should be noted that the increased sample size at the 1% concentration did not reduce the variability, which suggests that large variability may be typical of this dosing regimen.
3.3.2 The Delay Between LPC And LV Gene Vector Delivery Affects The Level Of Airway Gene Expression

The optimal timing between LPC and LV delivery *in vivo* was determined in studies where the time delay was varied from 5 to 120 minutes, while using the two most effective concentrations of LPC determined above (0.3% and 1%). These studies demonstrated that a delay of 1 hour produced significantly greater levels of gene expression than all other time points tested (see Figure 3.3 (p<0.05, ANOVA, n = 7 – 15 per group). It is again interesting to note that the level of variability was not reduced at the 60 minute time point where the number of animals in the group is 15, suggesting that variability may be characteristic of this dosing regimen. Timing effect seems markedly more pronounced with 0.3% than 1% LPC, i.e. 3 - 4 fold higher than other time points, whereas 1% approximately 2-fold.
Figure 3.3: LVlacZ gene expression in nasal airway after different time-dosing regimes. LVlacZ was instilled after pre-treatment with either 0.3% (green bars) or 1% (blue bars) LPC. Although LVlacZ gene transduction was present at all times, use of 0.3% or 1% LPC 60 minutes before the LVlacZ vector produced significantly greater levels of gene expression (p<0.05, ANOVA, mean ± SEM, n = 7 – 8 per group except at the 1% LPC / 60 minutes delay combination where data from two independent experiments were pooled, n=15). Please note: The levels of LacZ gene expression seen in this figure are slightly different to the levels shown in Figure 3.2 because the two experiments used different groups of mice.
3.3.3 Distribution Of Gene Transfer

Gene transfer was unevenly distributed at all time points examined and did not extend into all areas of the nasal airway (see Figure 3.4). Analyses of the cell types expressing LVLacZ showed that predominantly ciliated cells in respiratory and transitional epithelium were transduced. Non-ciliated (non-secretory) and basal cells each comprised a smaller percentage of the total number of cells transduced (27.14% and 1.29%, respectively) while no transduced secretory cells were seen (see Figure 3.5). Transduction of olfactory epithelium was not observed after any of the time/dose combinations tested.
Figure 3.4: Enface view of LV LacZ gene expression in mouse nose. Gene expression was present only in the treated (right) nostril, shown here in the 3rd of 3 standard sections of nasal airway. In this example 0.3% LPC was administered 60 minutes before the instillation of LV LacZ gene vector. Left of the septum (S) is the untreated airway that shows no LacZ gene expression. Robust patchy transduction in ciliated respiratory & transitional epithelium is apparent (black arrows at septum, nasal turbinates (NT) and on the ventral lateral wall). The lighter, even staining seen at the top left is background artefactual staining which washes away during histological processing. Dorsal is at the top.
Figure 3.5: Transduced cell types after 0.3% LPC pre-treatment, 60 minutes before LVLacZ vector delivery. Pre-treatment with the chosen LPC concentration of 0.3% resulted in transduction of primarily ciliated cells in the respiratory and transitional epithelium. A smaller percentage of non-ciliated and a few basal cells were also transduced (mean ± SEM, n=8 across 3 standard sections).
3.3.4 Increasing LPC Concentration Increases The Histological Changes In The Epithelium

Significant mucosubstance release was present in the LPC-treated airway at concentrations between 0.1% and 2% (but not 0.03%) when compared to the mucosubstance levels present in the untreated contralateral airway (see Figure 3.6). Histological evidence of epithelial cell perturbation or exfoliation caused by these dosing protocols was also examined. Cell injury, as measured by mucosubstance release and cell exfoliation off the basal membrane, was noted only after treatment with the two highest concentrations of LPC, 1% and 2%. After exposure to 2% LPC, approximately 85% of the perimeter of the treated nasal airway in the 3 standard sections displayed significant mucosubstance release and/or cell exfoliation. After exposure to 1% LPC approximately 45% of the perimeter was similarly affected. No evidence of perturbation or injury was detected when LPC concentrations of 0.3% or below were used. Examples of the airway epithelium after exposure to different concentrations of LPC are shown in Figure 3.7.
Figure 3.6: Histological effects on airway epithelium. Goblet cells present as a percentage of the untreated side. Significant mucosubstance release* occurred after treatment with LPC concentrations 0.1% and greater compared to PBS (ANOVA on ranks, p<0.05 n=8 in each group).
Figure 3.7: Effect of LPC on the Airway Epithelium. The appearance of altered mucosubstance presence is apparent after LPC treatment (right hand side of the septum (S) in all photos). After exposure to 0.03% LPC (A) and 0.1% LPC (B) cilia remain intact and goblet cell number and content is equivalent to the untreated (left hand side) of the nostril. After exposure to 0.3% LPC (C), there was a reduction in the amount of cilia and lowered goblet cell contents on the treated side compared to the untreated side of the nostril. After exposure to 1% LPC (D) and 2% LPC (E) there was no cilia remaining on the treated side of the septum and the goblet cells were depleted. There was also obvious disruption to the integrity of the epithelia on the treated side. Scale bar = 25 μm
3.4 Discussion

The continued worldwide interest and research in producing an effective gene therapy for cystic fibrosis lung disease has not yet resulted in a clinical application. Poor efficiency of gene transfer, resulting in limited efficacy of the treatment, has been a central factor limiting the development of CF gene therapy (Rosenecker, Huth et al. 2006). This is undoubtedly a consequence of mammalian airway evolution where natural selection ensured that redundant and complementary protection mechanisms defended the host against inhaled foreign particles.

The first line of airway defense involves capture and binding of particles to respiratory surface mucous and to the glycocalyx which lines the airway epithelium apical surface structures. Foreign particles are readily trapped by these barriers and eventually transported out of the airways via mucociliary clearance mechanisms, described in Chapter 1. The epithelial cells that line the airways are interconnected by tight junctions, which act (in part) as a physical barrier preventing foreign particles from gaining access to the basolateral surface of the airway. The receptors required for virus vector binding and entry into the desired cells mostly lie on this basolateral surface.

The second line of defense that is closely associated with the cell surfaces is the airway epithelial cells, which synthesize substances such as complement, collectins, lysozymes and defensins (Kushwah, Cao et al. 2007). These can lead to localized destruction of foreign microorganisms. The third line of airway defense involves immune cells, such as airway macrophages and neutrophils which ingest and destroy foreign microorganisms. Finally, the airway epithelial cells can induce an inflammatory response, resulting in the production of pro-inflammatory cytokines and chemokines, which in turn leads to the recruitment of more leukocytes and neutrophils to defend the airway.

For a potentially-therapeutic gene vector delivery, all these barriers can result in the
bulk of any vector dose being blocked, removed or destroyed shortly after delivery into the airway. The resulting low efficiency of gene transfer encourages researchers to use the highest possible vector doses. It implies a reliance on the unstated philosophy that ‘more is better’. Perversely, higher doses can exacerbate the host inflammatory and immune responses initiated by vector delivery (Wu and Ertl 2009).

A key therapeutic question is therefore whether host responses can be made negligible, or tolerable, without affecting the gene expression levels and persistence required to achieve an effective therapy. One approach is to progressively reduce the titre that is able to produce effective gene transfer. An effective but lower titre may be utilised with the successful application of adjuvants and enhancers, which can allow smaller vector doses to continue producing adequate gene expression and duration. It has been recently shown that CFTR delivery to 25% of surface airway CF epithelial cells was sufficient to restore normal levels of airway hydration (airway surface liquid (ASL) height) and mucociliary clearance (MCC) mechanisms (Zhang, Button et al. 2009).

However there is a suggestion that less than 25% CFTR delivery may be sufficient to correct the defect and prevent CF lung disease based on sufferers of congenital bilateral absence of vas deferens (CBAVD) displaying no lung disease. The majority of men who suffer from CBAVD have a defect in both copies of the CFTR gene and therefore have a much reduced level of functional CFTR compared to normal (Patrizio and Zielenski 1996). However, the level of functional CFTR expression required to prevent CF lung disease is currently unknown, especially in an in vivo gene therapy setting, where an interplay between the percentage of cells expressing CFTR and the level of CFTR expression in each cell exists.

The use of the naturally occurring surfactant molecule LPC, previously shown to increase gene transfer, has been applied to use in the nasal airway prior to the delivery of the gene transfer vector (Limberis, Anson et al. 2002). LPC, when used as a gene
therapy enhancing agent, has shown little \textit{in vivo} toxicity at the doses that result in the strong enhancement of gene transfer, an effect achieved with both adenoviral (Koehler, Frndova \textit{et al.} 2005) and lentiviral vectors (Limberis, Anson \textit{et al.} 2002). Other types of gene transfer enhancing agents that have been studied include sulphur dioxide gas (Johnson, Olsen \textit{et al.} 2000), the synthetic detergent polidocanol (Parsons, Grubb \textit{et al.} 1998; Limberis, Anson \textit{et al.} 2002), sodium caprate-(C10) (Johnson, Vanhook \textit{et al.} 2003), and EGTA (Chu, St George \textit{et al.} 2001). Lysophosphatidylcholine was chosen over these agents for continued development because firstly, it is effective in boosting gene expression; secondly, it is naturally present in lung where it is a component of airway surfactant (Daniels and Orgeig 2003; Chen, Hyatt \textit{et al.} 2006); and finally, as also shown in here, LPC appears to have a relatively wide range of effective concentrations. The possibility of a degree of ‘automatic’ detoxification of the molecule, removing it as an ongoing source of potential airway irritation and damage, has been noted in some studies. Specifically, it may stem from the swift conversion of exogenously applied LPC to the innocuous and ubiquitous membrane lipid dipalmitoylphosphatidylcholine (Seidner, Jobe \textit{et al.} 1988; Lu, Huang \textit{et al.} 1999; Nakanishi, Shindou \textit{et al.} 2006).

When used at 0.1\%, LPC co-administered \textit{via} aerosol with a HDAd vector has produced high and widely-distributed airway gene transfer in rabbit and baboon models \textit{in vivo} (Hiatt, Brunetti-Pierri \textit{et al.} 2005; Koehler, Frndova \textit{et al.} 2005). These studies showed expression persisted for more than 90 days, and apart from a transient dose-dependent fever in some animals (Koehler, Frndova \textit{et al.} 2005) and minimal initial inflammation (Hiatt, Brunetti-Pierri \textit{et al.} 2005), the dosing regime was well tolerated. These findings support the continued development of LPC to significantly enhance gene transfer in airway epithelium with a variety of vectors.

Most researchers believe LPC acts primarily to open tight junctions, providing vectors access to the basolaterally-located receptors on the target ciliated epithelial and basal
cells, resulting in increased gene expression (Wang, Zabner et al. 2000). However, the ability of LPC to slow mucociliary clearance in the bronchial airway (Martin, Marriott et al. 1978) may be of importance by allowing a greater proportion of a vector dose to remain in the airway for an increased period and so reach the target cells.

3.4.1 LPC Concentration Can Be Optimised To Enhance Gene Transfer In Mouse Airway

The gene transfer produced by 0.3% LPC or 1% LPC was the highest achieved in these studies, and there was no significant difference between 1% and 0.3% LPC in the level of gene expression achieved. Although these two concentrations produced maximal gene transfer here, both a higher concentration (2%) and a lower (0.1%) concentration can also be regarded as effective in vivo enhancers of gene transfer if the noted side-effects (high dose) or the lower gene transfer (low dose) are considered acceptable or even desirable. For example, optimal therapeutic expression levels of a suicide gene to treat a specific cancer may require a lower level of gene transduction compared to treatment of disease. Airway gene therapies could include those that produce enzymes for controlling disease and very low levels may be adequate (Auricchio, O’Connor et al. 2002; Engelhardt 2002; Griesenbach, Cassady et al. 2002). Conversely, other airway diseases, such as chronic obstructive pulmonary disease (COPD) or asthma, may require over-expression of a protective gene, or genes, in order counteract the destructive mechanisms which underlie the disease (Kolb, Martin et al. 2006). There are specific barriers present in the CF lung, not seen in healthy lungs, which may require the use of a larger dose of LPC to penetrate. While 2% LPC produced a more pronounced effect on the airway epithelium, these experiments indicated that this was not deleterious to the animal (based on the behaviour and body weight health parameters monitored during the 7 days after LPC exposure, see Figure 4.1). Also,
there was no difference seen between the treated and non-treated sides of the nose 7 days after LPC delivery, so there were no obvious effects of LPC that lasted more than 7 days. Thus, if required, there is the potential for an increased concentration of LPC to be used with no obvious detrimental effects to the airway. Furthermore, compared to the zero gene transfer resulting when a control (PBS) pre-treatment was used, the improvement in gene expression resulting from even a sub-optimal LPC concentration remains substantial.

In all cases gene expression was found to be punctate and non-uniformly distributed through the nasal airway epithelium, consistent with most reports of the distribution of gene transfer in nasal airways (Parsons, Grubb et al. 1998; Limberis, Anson et al. 2002; Koldej, Cmielewski et al. 2005; Sinn, Burnight et al. 2005; Limberis and Wilson 2006). This distribution pattern is probably due to a combination of the complex physical topography of the rodent nasal airway (causing a non-homogenous distribution of instilled LPC and/or gene vector in nose) and the dose volume. The upper lung airway studies in rabbits and baboons, where the delivery of an aerosolised mixture of HAdV and LPC is into essentially tubular branching airways, showed a more homogenous pattern of expression (Hiatt, Brunetti-Pierri et al. 2005; Koehler, Frndova et al. 2005). Whether this homogeneity is due to the type of vector, the aerosolisation itself, or the comparatively simple topography of the upper lung compared to the nasal airway, is not clear. One factor that may be important is that in the single (combined) treatment used in the rabbit and baboon studies, all cells reached by the delivery aerosol particle will have received LPC and virus simultaneously. In these protocols airway epithelial cells exposed to the second (virus vector) instillation may not always have been exposed to the earlier, conditioning, LPC dose.
Other gene expression enhancer agents have demonstrated this patchy expression profile. Sinn and colleagues delivered a FIV vector suspended in a viscoelastic gel which acted to increase vector residence time at the cell surface leading to receptor-independent uptake in the nasal epithelium of mice. The published images clearly show patchy gene expression in both the respiratory and olfactory epithelia (Sinn, Shah et al. 2005). When polidocainol was used as a gene expression-enhancing agent by Parsons and colleagues, gene expression was evident throughout the nasal epithelium but again, irregular staining was noted (Parsons, Grubb et al. 1998). In contrast to this, several studies have demonstrated that gene expression in mouse trachea and lung is extensive and more uniform following treatment with a gene expression enhancing agent, such as EGTA (Chu, St George et al. 2001) or sodium caprate (Gregory, Harbottle et al. 2003). These studies suggest the uneven distribution of gene expression seen in the results presented in this chapter may be characteristic of gene expression in the nasal epithelium. Additionally, the ability of LPC to provide some degree of enhancement to in vivo airway gene transfer at most concentrations and delivery time intervals tested here, suggests a wide therapeutic index, at least in live mouse airways.

LPC dose and timing parameters can and should be tailored to suit the particular model employed. Recent studies of lung gene delivery using a HDAd vector (Hiatt, Brunetti-Pierri et al. 2005; Koehler, Frndova et al. 2005) revealed the widening scope for LPC to enhance gene expression in vivo. In those studies LPC concentration was set at 0.1%, slightly below the 0.3% chosen as the most suitable from these experiments. However, the target organ in the adenovirus studies was the lungs – in mice, rabbits and baboons – and any untoward effects in lung may have wider distribution and severity on organ function compared to the localised effects that would be produced in the nasal epithelium of a single dosed nostril. Nevertheless, it is notable that in baboon studies conducted by Hiatt and colleagues where an HDAdV formulated in 0.1% LPC was delivered directly into the airways of three baboons, there was an absence of host
toxicity, despite administration into the entire lung of over 12 mL of 0.1% LPC.

### 3.4.2 LPC-Enhanced Gene Transfer: One Hour Separation Between LPC And Vector Dosing Is Optimal

This data shows that a one-hour separation between LPC and lentivirus vector delivery provided the strongest gene transfer into mouse ciliated epithelium *in vivo*. An optimal timing relationship between LPC and lentivirus vector delivery has not been reported before, and these results support the earlier, and somewhat fortuitous, choice of a one-hour dosing separation used by CF Gene Therapy Research Group at the WCH where the approach was first developed.

Nevertheless, it is apparent that other timings can be effective when compared against the use of gene vector without LPC pre-treatment. Up to the peak at a one-hour separation (Figure 3.3), a general trend was found for gene transfer to increase as the delay interval increased, but a clear reduction in effectiveness accompanied a two-hour interval. It is not known why the one hour time interval is optimal. This may be a reflection of the time taken for LPC to be cleared from the airway coupled with the sensitivity of lentivirus to inactivation (at earlier times) by the detergent effect of residual LPC. Indeed, it has been demonstrated that LV viability was significantly reduced within only five minutes of co-incubation with LPC *in vitro* (Personal Communication, K. Kremer, CYWHS). Should residual LPC be present in the airway it may produce similar lentiviral vector inactivation. In contrast, the ability to use simultaneous LPC and vector doses does not appear to be important for adenoviral vectors, probably due to the robust capsids that surround adenovirus vectors.
3.4.3 Effects Of LPC On Vector And On Airway Epithelium

While LPC can significantly enhance airway gene transfer in vivo (Limberis, Anson et al. 2002; Hiatt, Brunetti-Pierri et al. 2005; Koehler, Frndova et al. 2005; Kremer, Dunning et al. 2007), this compound has inherent detergent-like activity that must be considered and minimised before use in a clinical setting could be envisaged. The study of rabbit airway gene transfer by Koehler and colleagues showed that immediately after aerosol delivery LPC-exposed animals developed a mild-to-moderate patchy pneumonia without edema together with a transient decrease in dynamic lung compliance; however these side effects had returned to baseline by day five of the experiment. The vector used in the current study was a Vesicular Stomatitis Virus G Glycoprotein (VSV-G)-pseudotyped LV which transduced the epithelium via receptors that are located on the basolateral surface of the airway epithelium. Thus, effective transduction requires transient disruption of the tight junctions to allow access to these basolateral receptors.

In the lungs, an important component of defense against bacterial infection is the combination of the ASL and MCC. In CF lungs MCC, and thus clearance of mucous and various inhaled infectious particles, is inhibited due to reduced ASL hydration (Donaldson, Corcoran et al. 2007), thus increasing the vulnerability of the CF airway to infection. It has also been proposed that modulating tight junctions in a CF lung, which already has impaired MCC mechanisms, may lead to an increased risk of severe lung infections (Wark and McDonald 2009). Conversely, without the use of a tight junction modulator, such as LPC, the level of gene expression is significantly reduced, even when an apically-targeted vector is used (Kremer, Dunning et al. 2007). To maximise gene expression in the face of lost efficiency, using higher vector doses may initially achieve similar levels of gene transfer. It will, however, inevitably be accompanied by increased host inflammation and immune responses; these then can destroy some or all of the desired gene expression. A careful cost-benefit appraisal of the different methodologies available, to balance any pre-treatment side-effects against decreases in the ensuing
airway gene expression, will be of vital importance when optimising the LPC pre-
treatment protocols in future animal and human studies.

There are multiple mechanisms by which LPC could affect gene transfer. Both the
virion particle and the target epithelial cells may be affected by LPC. After gross delivery
into the airway, lentivirus-mediated gene transfer begins with the attachment of the
virion to a specific cell-membrane receptor, a process mediated by specific interactions
between the virion envelope glycoprotein (VSV-G in this study) and one or more
surface receptor molecules on the target cell (the receptors involved in transduction by
VSV-G-pseudotyped LV vectors are not well known (Coil and Miller 2004)). Residual
LPC left in the airway after dosing may inhibit virion-cell fusion because LPC can
affect the viral-host protein binding process (Ohki, Baker et al. 2006). The effect of
residual LPC on the viral-host-binding process should be reduced by increasing the
time delay between LPC and vector delivery, thus allowing the LPC time to clear
before the introduction of lentivirus vector. It may also help explain why a one-hour
delay between LPC and vector exposures was found to be optimal. Since LPC is a
molecule with detergent-like properties, mixing with vector prior to delivery probably
disrupts the lipid membranes of fragile vectors, such as lentivirus, causing the rapid
decrease in vector viability.

In summary, these findings provide further support for the use of LPC as an in vivo
enhancing agent in developing gene transfer in airways with LVs. The effective
concentration of LPC used can be optimised via simple time-course and dose
concentration studies in an animal model. Furthermore the LPC dose can be tailored
to enhance gene transfer without invoking significant injury to airway surfaces. Based
on previous experience with LPC-enhanced lentivirus protocols (Limberis, Anson et al.
2002) and the protocols of other groups discussed in this chapter, a suggested starting
LPC concentration for other animal models is 0.1 to 0.3%, with a maximal dose of
the chosen vector following (as a first trial) 1 hour later. Although the full range of mechanisms by which LPC-enhanced gene expression are still to be determined, the minimal and tolerable disturbance produced with these optimised doses in mouse airway supports the contention that LPC has a role in the further development of effective and safe CFTR gene transfer into live CF airways.
Chapter 4
A Lifetime of Airway Gene Expression
After a Single Dose of Lentivirus Vector
4.1 Introduction

Successful gene therapy for the treatment of CF lung disease will require the CFTR gene to be expressed, at a therapeutic level, in a sufficient numbers of cells, for an extended period of time. This profile of expression will be necessary to negate the lack of endogenous CFTR gene expression, and in turn, theoretically prevent the initiation and progression of airway disease. Lentiviral vectors stably integrate the gene into the host chromosome, so in the right circumstances the gene transduction protocol used in this chapter could also produce gene expression lasting for the life of the transduced cell. The use of specific agents to transiently modulate epithelial tight junctions allows the vector to access populations of cells below the apical surface, including those within the basal-cell compartment which are thought to act as progenitor cells in vivo (Engelhardt 2001; Emura 2002; Otto 2002). If these progenitor cells within the basal cell compartment are transduced, theoretically, this may lead to lifelong expression of the transgene. A molecule commonly found in lung surfactant (and also used in some artificial lung surfactants), lysophosphatidylcholine (LPC), was used to enable this access and was delivered to the airways prior to LV dosing. Unless injured, mature human airways have low turnover rates, and any gene expression introduced solely in terminally differentiated cells will only be maintained until the epithelium has been renewed. Although gene expression will be maintained as long as the transduced cell lives, gene expression is eventually lost from terminally-differentiated cells (i.e. those located on the apical surface and basal cells that are not part of the progenitor cell population) during the course of natural cell renewal. Unless progenitor cells have been transduced and can provide daughter cells already expressing the desired gene, re-dosing would be necessary to ensure continued or renewed expression.

An important difference in CF airways compared to normal airways is that epithelial cell proliferation rates can be much higher as a result of chronic inflammation (Leigh,
Kylander et al. 1995), thus the need for re-dosing may be significantly increased in CF. However, re-dosing poses the threat of an increased level of host immune response against the vector. Vector presence (a modified virus in this instance) in the airway causes the epithelial cells to synthesise various products i.e. lysozymes, complement and defensins (Kushwah, Cao et al. 2007), that attract other immune cells into the airway. Recruited immune cells such as macrophages will capture and destroy the vector (Kushwah, Cao et al. 2007) and B-cells and T-cells, which provide long-lasting immunity to the host (Kindt, Osborne et al. 2006). Subsequent exposure of the airway to the same vector, such as from re-dosing, triggers a heightened immune response and a cascade of immune events resulting in cytotoxic T-cells destroying any cells which harbor the virus (Kindt, Osborne et al. 2006). This leads to further recruitment of inflammatory cells into an already chronically-inflamed CF lung.

In contrast, by using a stably integrating lentiviral vector able to transduce respiratory epithelial cells and also airway epithelium progenitor cells, only a single dose may be required, negating the complex immune response produced by re-dosing, and long term gene expression may be achieved.

The following studies were designed to determine if a single-dose of LV LacZ could produce extended gene expression in the airway epithelium, potentially avoiding the immune system responses associated with repeated doses of vector. The types of cells expression LV LacZ were also examined in order to investigate whether the proportions of transduced cell types differed over time. This may provide some indication if progenitor-like cells may have been transduced.
4.2 Method

4.2.1 Instillation Of A Single Dose Of LVLacZ & Transduced Cell Type Identification Within The Airway Epithelium

Female C57Bl/6 mice were anaesthetised as described in section 2.4.2. One hour post-instillation the VSV-G HIV1-derived LVLacZ vector was similarly delivered into the same nostril (section 2.4.2). Mice were then sacrificed at 1, 3, 6, 9, 12, 18 or 24 months post instillation. Using the anatomical study of Mery and colleagues as a guide, the mouse heads were sectioned at levels approximately corresponding to levels 6, 16 and 24 (Mery, Gross et al. 1994) and these gross sections of tissue were viewed under a dissecting microscope for observation of LacZ gene expression and distribution, prior to embedding in wax.

Gene expression was assessed in greater detail in Safo stained sections (5 - 10 μm thick) as described in section 2.4.4. The cell types expressing LacZ were classified as respiratory, transitional, squamous or olfactory in sections counter-stained with Haematoxylin and Eosin (H&E) at x1000 magnification as described in section 2.4.7.
4.3  Results

4.3.1 24 Months Of Gene Expression Is Produced After A Single Dose Of LVLacZ

En face examination of the grossly sectioned blocks showed LacZ gene expression at all time points (Figure 4.2), with the highest levels seen in sections approximately corresponding to sections 6 and 16 (Figure 4.1), and sometimes extending into the section corresponding to section 24 (Figure 4.1) in Mery et al (Mery, Gross et al. 1994).

When examined at 400x magnification, distribution of LacZ positive cells at each time point was seen to remain ipsilateral in the treated nostril, and no LacZ gene expression observed in the untreated nostril. High numbers of LacZ positive cells were located as far posterior as the nasopharyngeal meatus. The single nasopharyngeal airway is formed posteriorly (at approximately level 24, see Figure 4.1) where the two anterior nasal airways coalesce into one airway as it runs below the brain towards the pharynx. LacZ positive cells were not seen in the olfactory or squamous regions of the epithelium.
Three cross-sections were taken corresponding to Level 6 in Mery et al.: immediately posterior to the dorsal incisor, level 16: where the two nasal airways coalesce into the nasopharyngeal duct and Level 24: at the 4th ridge seen along the palate. The black bold lines indicate where respiratory epithelium is present (only the left side is marked in the figure) and where gene expression was observed. N: nasopharynx. Adapted from Mery et al., 1994.

To measure gene expression the number of LacZ-positive cells was counted in Safranin O counterstained sections (the 4 sections within each mouse were totalled). The number of cells showing LacZ expression was maintained with no significant change up to 18 months after a single dose of LV-mediated gene transfer. Between the 18 and 24 month time points the number of cells expressing LacZ dropped significantly (Figure 4.3).
Figure 4.2: Gene Transfer at Different Time Points after a Single Dose of the LV LacZ vector into the Mouse Nasal Airway

Representative gross tissue sections from the 1 month (A), 3 month (B), 6 month (C), 9 month (D), 12 month (E) and 18 month (F) treatment groups, where mice were pre-treated with 0.3% LPC 1 hour prior to the instillation of the LV LacZ vector are shown. These sections correspond to section level 16 in Figure 4.1 (above). The untreated, left nostril shows no LacZ positive cells while the treated, right nostril shows large areas of LacZ positive cells in the nasal turbinates, and along the septum (S). The diffuse blue/green visible in the left nostril (a) is artefactual staining not associated with a cellular distribution. It is commonly seen in X-gal processed nasal tissues prior to sectioning; however, the staining is not cell-associated and no evidence of this diffuse stain remains when the same areas are viewed in counter-stained cross-sections.
500 μm
After a single dose of LPC / LVLacZ, the level of gene expression remained stable and not significantly different to the 1 week time point up to 18 months. However, expression significantly reduced at the 24 month time point (ANOVA, *p<0.05).

The black squares represent individual mice within each group, the blue bars and error bars are mean (SE).

*NB* some black squares may represent multiple mice with close cell counts. Cell counts were the total number of LacZ positive cells in the 3 standard sections (corresponding to levels 6, 16, and 24 in figure 4.1). The table shows the numbers of mice treated at each time point as well as the number of mice at each time point where no gene expression was seen.
4.3.2 A Single Dose Of LVlacZ Produces Extended Gene Expression In Ciliated Respiratory Cells

LacZ gene expression was patchy and restricted to the anterior regions of transitional and respiratory epithelium within the dosed nasal airway (Figure 4.2) affecting primarily ciliated and non-ciliated cells epithelial cells, but also low numbers of secretory and basal cells (Figure 4.4). Within the target respiratory and transitional ciliated epithelium, the number of transduced cells represented between 5.8% (at 1 month) and 2.8% (at 18 months) (Figure 4.5) of the total cells lining the three sampled regions of nasal epithelium (based on the total number of perimeter cells taken as ~ 4000, as previously determined (Parsons, Grubb et al. 1998). Although 0.3% of the ciliated columnar target cells were transduced at the 24 month time point, this level was significantly reduced compared to the initial 1-week level of transduction.

Lentivirus-mediated LVlacZ transduction into ciliated epithelium was maintained at approximately 54% of total transduction (i.e. of the total number of cells expressing LacZ) for the length of the experiment. Similarly, non-ciliated cell transduction also remained relatively constant at approximately 42% of total transduction. Additionally, from the 3 month time point onwards, there was at least one mouse in each group that had no evident LacZ gene expression in the nasal sections examined (see the table included in Figure 4.3).
Figure 4.4: Transduced Cell Types after a Single Dose of LVlacZ Vector. The ciliated cell population (Blue) at 24 months was significantly different to the 1-week value (*p < 0.01, ANOVA). However, the Non-Ciliated groups (Red) show no differences (p = 0.21) and, although analysis of both Secretory (Yellow) and Basal (Green) groups indicates a significant difference is present within the study (p = 0.046 and p = 0.017, respectively), the group multiple comparison procedures could not identify significantly different groups (ANOVA on Ranks, Dunn's multiple comparison). Black squares represent cell counts for individual mice within each group.
The data from Figure 4.4 is expressed here as the percentage of LacZ positive cells within the treated nostril. The total percentage of LacZ positive cells (all cell types) over 24 months is shown in A. Gene expression was maintained for 18 months after a single dose of the LPC / LVLacZ treatment, but was significantly reduced by the 24 month time point. The percentages of transduced ciliated and non-ciliated cells (individually plotted in B) remained stable over the length of the experiment. Similarly, the percentages of LacZ-positive secretory and basal cells (individually plotted in C) was also maintained over the course of the experiment, however, this may be due to such small numbers. Mean levels of less than one cell are due to averaging across animals.
4.4 Discussion

4.4.1 24 Months Of In Vivo Gene Expression After A Single LVlacZ Vector Dose

Lentiviral vectors appear to comprise extremely attractive vehicles for CF gene therapy because they can mediate long-term gene expression in the airways of experimental animals. However, gene delivery must be targeted to the respiratory epithelium to be effective in treating cystic fibrosis, and results in the literature suggest the type of vector (i.e. non-viral, viral; as well as the particular viral pseudotype used) and the mode of delivery may alter the cell types targeted. For example, the LPC pre-treatment system results in delivery to ciliated and non-ciliated cells in the respiratory and transitional epithelium in murine nasal airway. Furthermore, no transduction was observed in olfactory or squamous epithelium in any of the LV-LacZ gene transfer studies at any time. By contrast, other LV, such as GP64-pseudotyped FIV (Sinn, Burnight et al. 2005; Sinn, Arias et al. 2008) and non-LV vectors, such as AAV serotype 5 (Sumner-Jones, Davies et al. 2006) reported substantial proportions of olfactory epithelium transduction (according to histological analysis using a LacZ reporter gene). The studies by Sinn and colleagues utilised a relatively new reporter gene, firefly luciferase; and a new assessment method, real time bioluminescence imaging. The use of luciferase is beneficial in reducing the numbers of animals required for each experiment as gene expression can be easily assessed in live animals and thus repeat measures within the same animal is possible. An example of luciferase expression detected using bioluminescence imaging is shown in Figure 4.6. The monitoring of luciferase gene expression, while beneficial in many ways, reveals a particular advantage of the LacZ reporter gene system, as it allows the specific identification of the cell types transduced at each time point. However, the luciferase reporter system, although permitting invaluable non-invasive repeated measures over long time frames and in the same animals, can only provide
a global measure of gene transduction. In that regard the LacZ studies reported here provided clear identification of gene expression in the relevant target cells.

Figure 4.6 – An example of real time bioluminescence imaging in a mouse after delivery of 0.3% LPC one hour prior to delivery of an HIV-1 derived LV vector containing the EF1a luciferase gene. Using this type of gene expression assessment, the general area of gene expression can be seen, however detail, such as the cell types expressing the luciferase gene, cannot be identified. Image courtesy of K. Kremer, CYWHS.
The targeting capacity of lentivirus vectors can be expanded or altered by pseudotyping the vector with envelope glycoproteins derived from another virus. The pseudotyped vector then possesses the natural tropism of the virus from which the envelope glycoproteins were derived as implied from the nature of the native virus. In airway gene therapy studies several different pseudotyped have been utilised, including GP64 (broad tropism) (Cronin, Zhang et al. 2005), filovirus (tropism for the respiratory epithelium) (Kobinger, Weiner et al. 2001) and various strains of the influenza virus (tropism for the respiratory epithelium) (McKay, Patel et al. 2006). The findings of the present study suggest that the VSV-G pseudotype (broad tropism), in conjunction with LPC pre-treatment, may be well-suited for in vivo targeting of the ciliated airway epithelium that is needed for successful CF airway gene therapy.

After delivery of the VSV-G pseudotyped lentivirus (with LPC pretreatment) there appeared to be a trend for higher gene transduction levels at some of the earlier time points, however analysis showed that gene expression did not significantly alter from the 1-week level for at least 18 months. There was LacZ gene expression present in a small number of mice for up to 24 months (the planned termination of the study). However, for any gene expression to be present so long after the initial single transduction event is in itself notable. These results shown in Chapter 3 also show that LPC is effective in a dose-responsive manner. The lower expression levels achieved in the present study using 0.3% LPC suggests the potential use of LPC at lower doses in settings where too much gene transduction (Farmen, Karp et al. 2005) may be problematic. In airways, it has been speculated that over-expression of CFTR would result in over-correction of Cl-transport generating excessive fluid secretion, however a recent study has demonstrated that transgenically over-expressed CFTR does not over-correct ASL regulation or cell toxicity in air liquid interface cultures of respiratory epithelium (Zhang, Button et al. 2009). Gene expression levels that closely mimic the natural expression profile will be preferable to greatly over-expressed genes as long as the level of phenotypic correction
remains therapeutic. Whether lower initial levels of gene expression could also support lifetime gene expression was not tested in the current study, and this remains a potentially fruitful area of investigation since lower doses that remain effective will, in general, help to reduce the inflammation and immune responses that may arise from introduction of the gene vectors into airways.

Pre-treatment with LPC is expected to provide access of vector to normally protected resident adult progenitor cells located near the basement membrane of the epithelium (Limberis, Anson et al. 2002; Koehler, Frndova et al. 2005). These cells act as the mother cells of airway epithelium. Once initially transduced, these cells can generate already-transduced daughter airway cells and lineages at a rate dependent on cell turnover time. By this mechanism, progenitor cells may extend the desired gene expression for the life of the animal. Although this notion is consistent with an approximate 3-month cell turnover time originally established for murine tracheal airway (Borthwick, Shahbazian et al. 2001), recent data suggest longer turnover times in deeper regions of the mouse lung (Rawlins and Hogan 2008). Unfortunately, no assessment of the turnover time of murine nasal airway epithelium has been reported to enable its consideration as a factor here. Nevertheless, the enduring LacZ gene expression observed in the majority of the LVLacZ treated mice in this study is likely to be derived at some point, in part, from daughter cells of transduced airway progenitor cells.

It is noteworthy that from the three month time point onwards there was at least one mouse in each time point where no LacZ gene expression was evident (see table in Figure 4.3). While the reason for the absence of a response is not known, it may be that no progenitor-like cell transduction occurred in these particular mice. Subsequently, once the terminally differentiated LacZ-positive epithelial cells turned over, only non-transduced progenitor cells within the airway were present to re-populate the epithelium, and gene expression was therefore lost when terminally-differentiated
cells died out and were normally replaced. Though speculative, this can provide an explanation why some mice appeared to have no LacZ gene expression, while other mice in the same cohort had considerable levels of LacZ gene expression.

An immune response to the transgene in some of the mice may be contributing to the reduction of LacZ gene expression in some mice. As LacZ is a marker gene not naturally expressed in the mouse, the cells expressing LacZ would be identified by T-cells as “non-self” and an immune response may be elicited (Bessis, GarciaCozar et al. 2004). Maintained expression of the transgene would normally boost the immune system, resulting in destruction of all introduced transgene expressing cells and limiting the persistence of the introduced gene (Bessis, GarciaCozar et al. 2004). However, this immune response may not be the only contributing factor to the decline in LacZ gene expression as many mice within this study did have extensive gene expression at the 18 month time point. It may be that an immune response is mounted more rapidly in some mice compared to others, resulting in most mice having a significant decrease in gene expression by 24 months, while others had decreased gene expression earlier in the study. If the introduced gene was CFTR, which is naturally expressed, the immune system may not mount an immune response and gene expression may not be compromised. However, in CF airways, some mutations result in a complete lack of CFTR, and as such cells expressing CFTR may in fact be considered foreign and induce an immune response (Limberis, Bell et al. 2010). As there is no definitive answer regarding the immune response to LacZ or the LV in this study, it should be considered a possibility and assessed in any future studies.

At present, the significant reduction in nasal airway transgene expression noted at 24 months cannot be explained, although it is noted that this is close to the expected lifespan of laboratory mice. It may be that this decrease in expression has resulted from age-related changes of airway stem cell function. Aging is a complex process which is
not well characterised in any species and in particular, there is no information on the effects of aging on airway progenitor cell processes in such aged mice.

Nevertheless, there are some considerations surrounding cellular aging processes worth exploring. Some studies have demonstrated a number of physical and metabolic changes do occur with age and are related to an increase in reactive oxidative species (ROS) (Beccafico, Puglielli et al. 2007). Ito and colleagues have demonstrated that elevated ROS inhibits the re-populating capacity of HSC and inhibits the maintenance of HSC quiescence, resulting in defects of stem cell function in vivo (Ito, Hirao et al. 2006). In this instance, the age of the animal directly affects the re-populating capacity of the progenitor cell population and indicates that age may also be affecting the cellular renewal processes in the airways. The findings regarding haematopoietic stem cells (HSC) may provide clues about potential mechanisms. Although HSC's are not a static tissue like airway epithelium, self-renewal in haematopoietic stem cells produces differentiated cells in all haematopoietic lineages throughout the lifetime of the organism, analogous to the processes that occur in airway epithelium. Ito and colleagues have demonstrated that elevated ROS inhibits the re-populating capacity of HSC and inhibits the maintenance of HSC quiescence, resulting in defects of stem cell function in vivo (Ito, Hirao et al. 2006).

There are other studies that clearly show a change in the function of HSC with age. Rossi and colleagues reported differences in gene expression between young and old mice in purified HSC isolated from blood (Rossi, Bryder et al. 2005). Beccafico and colleagues have reported that quiescent cells -as airway stem cells are majority of the time - are resistant to this oxidative stress-related cell death (Beccafico, Puglielli et al. 2007). Although, the regulation of stem cell function specifically within the airway has not been described, it may be that stem cell regulation, and age-related dysfunction, results from the local microenvironment of the stem cell niche and the surrounding...
tissue. Thus, changes within the niche, such as changes in the membrane proteins and lipids in cells that make direct contact with the stem cells, changes in soluble paracrine and endocrine factors, and alternations in the amount and composition of the extracellular matrix, would ultimately affect the function of the stem cells located within it (Rando 2006). Although these effects could hamper the ability of resident airway stem cells to maintain LV LacZ expression within the epithelium, the important question is what exactly drives these changes. Ultimately, the role of stem cells in the biology of different organs and tissues is so diverse and incompletely understood, that the extent to which aging might affect stem cell function, specifically tissue/organ function in airways is an area demanding continued study.

In the current study, the percentage of secretory cells remained stable for the first 12 months of the experiment at approximately 0.3% of total transduction and appeared to increase (although not reaching statistical significance), to almost 5% by the 18 month time point. While there is a lack of consensus within the field about the location of stem cells within the airway, it has been hypothesised that the secretory cells within the murine airway may contain a niche of progenitor-like cells (Engelhardt 2001), and may assist in the maintenance of the longevity of gene expression seen in this study.

A subset of basal cells in the airway are also thought to have progenitor-like qualities, and can give rise to a fully differentiated epithelium \textit{in vivo} (Hong, Reynolds et al. 2004). While not a significant change, the percentage of transduced basal cells fell to 0.3%, 1% and 0.5% at 3, 6 and 9 months respectively after the 1 month time point of 2%, but returned to initial levels by the 12 month time point. Further studies are required to confirm the validity of this trend, which may have been a result of natural cell turnover in the lung, followed by repopulation at 12 months by LacZ gene-expressing progenitor-like basal cells. However, the number of cells detected was very small and variable; some mice had none, while others had several transduced basal
cells in the sections studied. It is also important to note that these cell counts are only samples taken from within the nose and there are inevitably stained cells outside the plane of the section, so the numbers and presence of gene expression basal cells may be underestimated. It is therefore difficult to draw firm conclusions about the meaning of transduction variability with time. A study that identified the location and cell type of stem cells within the mouse nasal airway at different ages would provide a valuable insight into the results discussed in this chapter.

This experiment demonstrated the maintenance of gene expression in mouse airway for 18 months in most mice, and 24 months for a small number of mice. If translated into a human system, the question arises whether gene expression would again last 24 months, or would expression last the equivalent proportion of the organism lifespan - approximately 60 years in humans? If the decline in expression noted at the 24 month time point in mice is related to a decrease in general mammalian stem cell function the potential longevity of gene expression in a human lung may be relatively short if it follows the same time course. Nevertheless, effective treatments of defective CFTR function in CF airways would be a valuable advance, particularly if the problems of redosing can be overcome.

Another possible explanation is the cell turnover time within the mouse nasal airway may be longer than hypothesised. While studies have demonstrated turnover times in other areas of the mouse respiratory system, such as the trachea, bronchioles, and terminal bronchioles (Borthwick, Shahbazian et al. 2001; Rawlins and Hogan 2008), no study has looked specifically at the turnover time within the nasal epithelium. The cell types of the nasal epithelium are similar to the cell types found in the trachea which may suggest cellular turnover times may also be similar (approximately 3 months), it is possible that the turnover time is much longer and may account for the extended gene expression seen in the current experiments. In order to speculate further, specific
investigations into the turnover time within the nasal epithelium of the mouse must be performed.

4.4.2 Appropriate Cell Types Transduced For Cystic Fibrosis Gene Therapy

Successful gene therapy for CF airway disease requires that the therapeutic gene be expressed at a sufficient level to correct function in the proper cell types. Wild-type CFTR functions at the apical membrane of ciliated airway epithelial cells (Harris, Mendes et al. 2004) thus these cells are one of primary target cells for any gene therapy protocol for CF.

Empirically, the LV vector used here has epithelial transduction targeting characteristics that make it well suited to the needs of airway gene transfer for CF situations. Olfactory and squamous epithelial cell regions cover at least 50% of the surface of the mouse nasal cavity (Parsons, Hopkins et al. 2000; Grubb, Rogers et al. 2009). Past studies using an LPC pre-treatment followed by an adenoviral vector transduced the olfactory, respiratory and transitional epithelium with equal efficiency (personal communication, Dr D. Parsons, CYWHS). In contrast, the LV vector used here exclusively transduced ciliated regions of respiratory and transitional epithelium. This apparent targeting was not expected since VSV-G pseudotyped vectors have previously been shown to transduce a broad range of cell types, albeit with varying efficiency (Wang, Slepushkin et al. 1999). It is possible that this apparent targeting is a result, in part, of the distribution pattern of LPC prior to vector delivery. The volume of LPC delivered is only 4 μL, so if this volume was not sufficient to penetrated deeper into the mouse nostril and coat the olfactory regions of the airway, thus providing basolateral access to the vector, it is unlikely that olfactory transduction would occur. However,
considering the large number of mice that have undergone the LPC followed by VSV-G-pseudotyped lentiviral vector protocol, it is surprising that not a single transduced olfactory cell has been noted in the studies presented in this thesis, and of others in the group, over the last decade. The specific targeting seen here may be regarded as an important advantage of this system, since CFTR expression in cells which do not naturally express the protein may have detrimental effects. However, a recent report has demonstrated the presence of CFTR mRNA in murine olfactory cells isolated from the dorsal meatus (Grubb, Rogers et al. 2007), which suggests that CFTR gene expression within olfactory regions may be a physiological requirement for that tissue, although if and how this might influence lung disease severity and progression is unknown.

In summary, the results obtained in the present study are the first to demonstrate such extended in vivo expression of LacZ (to 24 months in the desired target-cell epithelium) in adult mice after any gene therapy protocol. Importantly, this study has demonstrated proof-of-principle for a single-dose protocol, using an HIV-derived lentivirus vector. The data suggest that single-dose lifetime gene expression in the airway epithelium is a feasible methodology because it was shown that one brief lentiviral vector dose, when combined with LPC treatment, is sufficient by itself to produce enduring nasal airway transgene expression, extending for the lifetime of a mouse. Moreover, because re-dosing may not be needed, this method could entirely avoid the inherent immune-activation barriers that thwart repeated-dosing gene therapy protocols (Sumner-Jones, Gill et al. 2007).
Chapter 5
Correction of CFTR Function *In Vivo*
5.1 Introduction

Cystic fibrosis (CF) is caused by a defective cystic fibrosis transmembrane conductance regulator (CFTR) ion-channel (Riordan 2008), which can result in a debilitating and incurable airway disease. The goal of gene therapy for CF lung disease is the development of a safe and effective strategy to deliver a functional CFTR gene to the airway, which produces long-term gene expression. Despite the long recognised potential of gene therapy, encouraging data from animal models, and a number of early phase clinical trials, gene therapy for CF airway disease has not yet eventuated (Griesenbach, Geddes et al. 2006). A significant hurdle still to be overcome is the inability to achieve sustained corrective gene expression in ciliated airway epithelium (Griesenbach, Geddes et al. 2004).

One study has shown that transgene expression could extend for 12 months after a non-integrating adeno-associated (AAV) vector was delivered to mouse nasal airways (Sumner-Jones, Davies et al. 2006). However, in that study, only olfactory epithelium and alveolar type II cells were transduced, with neither of these being the desired targets for CF airway gene therapy. Continued gene expression for up to eleven months in mouse nasal airways has previously been demonstrated using a FIV-based lentivirus (Sinn, Burnight et al. 2005). However this was only achieved with a marker gene, such as LacZ or EGFP, with no therapeutic benefit, and the source of the longevity was shown to be in primarily non-respiratory tissues of the mouse nose.

The purpose of the studies in this thesis was to determine the longevity of in vivo airway gene expression through the LacZ studies described in Chapter 4. This chapter will deal with results in testing the persistence of function of the CFTR gene in vivo in a mouse model relevant to the therapeutic need – the CF knock-out mouse.
The assessment of CFTR function depends on measurement of the electrophysiology of the airway epithelial cells. Epithelial cells absorb Na⁺ from, and secrete Cl⁻ onto, the apical surface of the cells, and this active transport of charged sodium and chloride ions creates a voltage difference across this epithelium. The bioelectrical properties are measured using the transepithelial potential difference (TPD) assay and reflect the function of the CFTR ion channel. Because this method can have several sources of experimental error (as discussed in Appendix 4), the presence of CFTR protein was also sought using immunohistochemistry.

5.2 Methods

5.2.1 Instillation Of A Single Dose Of LVCFTR Into The Mouse Nasal Airway Epithelium

Mice were anaesthetized with an intramuscular injection of xylazil : ketamine prior to the delivery of a single dose of 4 μl of LPC to the mouse nasal airway via inhalation-driven instillation, as previously described (see section 2.4.2). After 60 minutes, the CFTR gene vector dose was delivered into the same nostril. The LVCFTR virus titre was 2.45 × 10⁸ tu/ml as determined via a real-time polymerase chain reaction assay (see section 2.3.4).

5.2.2 Transepithelial Potential Difference Assessment Of Functional CFTR Within Mouse Airway Epithelium

To assess levels of transduced functional CFTR, mice were first anaesthetized with a mixture of domitor and ketamine. Transepithelial potential difference (TPD) assessments were performed as described in section 2.4.13.
As part of the experimental design, TPD measurements were normally performed on each mouse only once. This design was chosen because of the unknown potential for sickness or death of CF mice from repeat anaesthesia, or other unknown causes, that might be present if repeat studies were conducted on individual mice. In cases where traces were rejected and additional TPD assessments were necessary (due to blind assessment of the technical quality of the traces determined they were not acceptable) they were performed two weeks after the initial TPD experiment as this time interval permits full regeneration of epithelium that may be damaged by TPD cannula insertion (personal communication, Dr D.W. Parsons, CYWHS).

5.2.3 Immunological Detection Of CFTR Within Mouse Airway Epithelium

Female and male \textit{cftr\textsuperscript{tm1Unc}} knockout mice (and heterozygote controls) were euthanized by CO\textsubscript{2} asphyxiation and selected tissues were surgically removed as described in section 2.4.3. The isolated tissues were rapidly frozen in Optimal Cutting Temperature compound (OCT; Sakura Finetek Co., Torrance, CA, USA) using dry ice. Frozen sections were prepared for immunostaining and stored at -80 °C. Immunohistochemistry was performed as described in section 2.4.11 with an anti-human CFTR antibody 596 (generously provided by Dr J Riordan, University Nth Carolina at Chapel Hill).
Figure 5.1: TPD Measurement set-up. The multi-syringe pump controls the flow of each of the TPD solutions into the cannula in the mouse nose. One electrode is placed in the mouse nostril and one in the KCL reservoir and the TPD was recorded on the laptop (not seen in photograph). The micromanipulator controls the placement of the cannula in the mouse nose and is placed in the nostril with the assistance of the microscope. A schematic of the TPD set-up is also shown.
5.3 Results

5.3.1 Restoration Of CFTR Function Following A Single-Dose Of LVCFTR

Heterozygote mice from the $\text{cfr}^{\text{tm1Unc}}$ mouse colony exhibited a $\Delta$TPD value of $-7.13 \pm 1.19 \text{ mV}$ ($n=8$), while untreated $\text{cfr}^{\text{tm1Unc}}$ mice exhibited a $\Delta$TPD value of $+5.47 \pm 0.99 \text{ mV}$ ($n=16$) (Figure 5.2). Across the entire 24 month study period ANOVA analysis identified that there were significant changes present in the dataset ($P < 0.001$). The groups responsible for the improvement in $\Delta$TPD post-treatment were determined using a Holm-Sidak Post Hoc comparison, as follows.

\textit{a} One month Post-Treatment

The exposure of the LPC-conditioned $\text{cfr}^{\text{tm1Unc}}$ mouse nasal airway epithelium to the LVCFTR vector resulted in functional expression of CFTR. Specifically, at one month the mean PD values ($-0.6 \pm 1.5 \text{ Mv}, n=5$) were significantly more negative compared to those of untreated $\text{cfr}^{\text{tm1Unc}}$ mice (values noted above). This change represented a 48.16\% functional correction towards the mean $\Delta$TPD value of the heterozygote CF colony mice ($\text{data } -7.13 \pm 1.19 \text{ mV}, n=8$).

\textit{b} Three months Post-Treatment

The TPD values at three months post-treatment ($\Delta$TPD = $2.14 \pm 2.42 \text{ mV}, n=7$) showed functional change in CFTR activity of 26.43\% toward the mean heterozygote $\Delta$TPD value. However, the post-hoc multiple comparison assessment indicated this was not a statistically-significant correction.
c Twelve months Post-Treatment

At the twelve month time point the mean ΔTPD value (1.10 ± 1.40 mV, (n=5) represented a statistically-significant 34.68% functional recovery of CFTR activity compared to the mean heterozygote ΔTPD value of heterozygote mice (noted above).

d Greater than Eighteen months Post-Treatment

By the last two time points examined (18 and 24 months) there were not enough mice left in each group to include as individual time points. This was due to the reduced overall survival of the \textit{cftr}^{mut/loc} mice and the exclusion of a number of TPD traces based on the assessment and acceptance criteria detailed in methods section 2.4.13 and Appendix 4. Therefore these two groups were combined; the group is designated as “>18” in Figure 5.2.

The ΔTPD values of the combined >18 post-treatment group (TPD = 3.13 ± 1.66 mV, n=4) showed a change in CFTR activity that was 18.6% towards the mean heterozygote ΔTPD value. This was not a statistically-significant change.
Figure 5.2: TPD Measurements of CF Mouse Treatment Groups. A single-dose LVCFTR gene transfer (20 μl, titre = $2.45 \times 10^8$ tu/ml) delivery produced significant partial functional correction of CFTR expression at 1 month and 12 months ($p < 0.05$, ANOVA, Holm–Sidak multiple comparisons against untreated CF). Each time-point examined a different group of transgenic CF animals, dosed once; with the subsequent TPD measures performed at the stated time-point.

This study began with larger CF mouse group sizes than shown in Figure 5.2, but in the final analysis the sample size was smaller due to an increased mortality rate within the CF mouse population compared to the normal mouse population, (Griesenbach, Smith et al. 2008), together with the challenging nature of TPD measurement that resulted in ATPD trace rejections, based on quality-control acceptance criteria (see methods section 2.4.13 and Appendix 4) applied throughout the study, see Figure 5.3.
5.3.2 Immunohistochemistry

In 2005, Alice Stocker (Doctoral Candidate) was trained in specific CFTR immunohistochemistry methods at The Cystic Fibrosis Pulmonary Research and Treatment Center at the University of North Carolina (UNC), under the guidance of the researchers in the research groups of Dr. S. Randell and Dr. L. Ostrowski, who routinely employ CFTR immunohistochemistry in their research. Nasal airway tissue slides produced in the current study were treated using these same CFTR IHC protocols, with the addition of a decalcification step prior to embedding.
No CFTR-specific labelling was detected in any of the samples compared to controls in the initial studies. In an attempt to increase the labelling, the dilution of primary antibody was increased (see Table 5.1), however this had no effect on the outcomes. The secondary antibody concentration was also increased and tested (see Table 5.1), again with no increase in CFTR–specific labelling detected.

Table 5.1: CFTR Immunohistochemistry Antibody Dilutions. There was no specific staining seen using any of the concentration combinations. Each experiment was conducted using tissue from mice with a previous TPD recording that showed significant correction compared to untreated cftr<sup>tm1UNC</sup> mice, in duplicate. N=3 animals (6 sections) for each experiment.

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5.4 Discussion

This is the first study of CFTR gene transfer in CF mice to successfully induce significant functional CFTR gene correction that persisted for a significant portion of the lifetime of these transgenic CF mice.

Importantly, only a single-dose event was required to produce functional CFTR correction that was still present after 12 months, and this finding substantially extends the persistence of functional CFTR gene transfer over that previously reported (Limberis, Anson et al. 2002), despite employing a lower pre-treatment dose of LPC (0.3% LPC) than in that earlier study (1% LPC).

Finding no significant correction of CFTR at an intermediate point (3 months) was unexpected. In examining the reasons for this anomaly it was first noted that in both the normal mice and the CF mice there was an absence of gene transfer in some animals. At or after 3 months LacZ gene expression was absent in some LV-LacZ treated mice (Figure 4.3). In these instances transduction (whether LacZ, or CFTR) may simply not have occurred. Another possibility is that the 3-level nasal airway sampling protocol (see section 2.4.3) may have missed detecting transduced cells in some mice because transduction is both regional and patchy. Indeed, when the wax histology blocks were reviewed there was evidence of patchy LacZ staining in respiratory and transitional epithelium regions below the cut face of the block for some mice. Accordingly, this sampling method will have underestimated the proportion of treated mice considered to show successful transduction of nasal airway.

Currently, there is no way to determine which specific cells or cell-types were responsible for the corrections of CFTR measured by the TPD methodology. However, the conditions of the study described in Chapter 5 mirrored the conditions of the LacZ
study described in Chapter 4. The vector used was identical other than the gene of interest (LacZ or CFTR), the manufacture of the vector was conducted using the same protocol, reagents and equipment and the dosing parameters were identical. Because of these similarities of vector, reagents, manufacture and dosing it is likely that the distribution of CFTR gene expression would mirror the distribution patterns of LacZ gene expression seen in the Chapter 4 studies.

It has been reported that mouse nasal olfactory epithelium can greatly influence measurements of nasal TPD, partly as a result of the large olfactory surface area in mice. The electrical potential signals from the olfactory epithelium may swamp measurements taken from respiratory epithelium and most gene vectors appear to transduce olfactory as well as respiratory epithelium (Sinn, Shah et al. 2005; Ostrowski, Yin et al. 2007). The VSV-G pseudotyped LV-LacZ vector used in the present studies was found to target only the ciliated respiratory and transitional epithelium. Because the distribution of CFTR expression from the LVCFTR vector is likely to be similar to that produced by the LV-LacZ vector, olfactory epithelium was not likely to have been transduced with CFTR. Any effect that olfactory epithelium may have had on TPD levels will therefore have been the same in treated and untreated animals. Thus, TPD recordings in this study will probably have been spared the confounding influence of olfactory epithelium on TPD measures.

At each time point in the LVCFTR study it was noted that there were some TPD values in treated CF mice that were not different to those in untreated CF mice (Figure 5.2). It is likely that unpredictable sampling of transduced regions by the TPD recording electrode tip may also have occurred (Parsons, Hopkins et al. 2000). For each TPD assessment conducted, the cannula tip is unlikely to have sampled from precisely the same area of airway epithelium. The mouse nasal airway epithelium is anatomically and morphologically complex, and depth-related differences in TPD values have been
previously reported (Parsons, Hopkins et al. 2000) This study showed that in 6 to 12-week-old mice cannula placement 2.5 mm from the nose tip should maximize the ability to record from nasal airway respiratory epithelium, however, the authors noted that the size of the animal could influence the eventual location the cannula tip in the mouse nose. Their finding is important in relation to the current study as the size of the mice increases with age, thus the placement of the cannula tip to a certain depth within the nose may affect the location of the tip and the TPD values recorded. There is a difference in basal TPD recorded from a cannula depth of 2.5 mm and 5.0 mm in the mouse nasal cavity; these depths tend to record from respiratory epithelium or olfactory epithelium, respectively (Parsons, Hopkins et al. 2000). In order to most accurately place the cannula in the nasal cavity of the experimental mice in the experiments described in this chapter, a combination of correct depth placement and appropriate basal TPD readings were utilised in order to obtain TPD readings recorded from respiratory airway epithelia. However, some TPD readings may still have been obtained from nasal olfactory epithelium or areas influenced by olfactory epithelium, resulting in measurements of TPD that do not accurately represent LVCFTTR mediated changes in respiratory epithelium.

If the CFTR gene expression mirrors the expression distribution seen in the LacZ studies (Chapter 4), another potential explanation for some TPD values in treated CF mice that were not different to those in untreated CF mice is the patchiness of gene expression. If the recording cannula is placed in an area with no LVCFTR gene expression, this lack of expression may be reflected in the TPD value recorded. By comparison, if the cannula was placed in a different location within the same mouse, where LVCFTR gene expression was abundant, the TPD value may reflect a strong correction in ion transport due to successful local CFTR expression.

A disadvantage of the original CFTR gene-transfer study design used in this experiment
is that it contributed to the reduction in the eventual power of the study. The study began with CF mouse group sizes that were expected to be adequate for the time periods (1 month n=7; 3 month n=15; 6 month n=8; 12 month n=9; 18 month n=15; 24 month n=14), but the final sample size in the analysis of some groups (see Figure 5.3) was smaller. This was due to unpredictable increased mortality (compared to C57Bl6 mice) with advancing age in transgenic CF mice, and the challenging nature of TPD measurement (Griesenbach, Smith et al. 2008; Parsons, Morgan et al. 2008; Siu, Morgan et al. 2008) which can cause technical rejection of the TPD measurements. Both factors resulted in exclusion of data points in the final analyses, and together with the high variability present a reduced statistical power was evident at the 3 and >18 month time-points this study.

Despite the measurement limitations noted here, it is important to note that both these latter time-points include individuals with moderate to high levels of apparent correction of their TPD values.

Attempts to localise CFTR presence in nasal airway epithelial cells using immunohistochemistry were not successful. This may have been due to a number of factors. Firstly, the decalcification process used in this experiment may have contributed to the inaccessibility of the epitopes, resulting in a lack of specific staining in the tissue samples. Secondly, the abundance of CFTR protein in the tissues may have been low. If the data gained in the LacZ experiments can be used as an indicator of CFTR distribution in the airways, it is likely that the levels of CFTR within the treated tissue may have been too low to detect with the CFTR antibody. Indeed, it has been reported that while CFTR is detectable via IHC in hyper-expressing cells, but has been more difficult to detect in tissues with endogenously expressed CFTR (Claass, Sommer et al. 2000). Thirdly, the sensitivity of the antibody to CFTR may not have been sufficient to detect the small amount of protein present within the treated tissue.
Finally, the epitopes recognized by the CFTR antibody may have become inaccessible after tissue preservation; although this is unlikely as the same preservation protocol was successfully used during IHC training at UNC. While the lack of CFTR staining seen in this experiment, may be attributed to one of these potential factors, it is of interest to note that in other *in vitro* studies in undertaken by other members of the Adelaide Cystic Fibrosis Gene Therapy Group *in vitro* also failed to detect specific staining for CFTR. This may indicate that there may, in fact, have been a problem with the antibody itself, rather than with the protocol (e.g. due to poor temperature control during shipping from the USA).

In summary, the results obtained in this study of single dose airway CFTR gene transfer are the first known to demonstrate such extended *in vivo* CFTR airway gene expression (to greater than 12 months) in adult mice after any type of gene transfer protocol. These encouraging findings support the potential of combining a mild preconditioning of the airway surface using LPC with a single brief LV vector exposure to produce extended therapeutic gene expression in live airway. Such persisting *in vivo* CFTR gene transfer offers hope that the theoretical promise of lentiviral gene therapy can be translated into a safe and effective treatment of CF lung disease.
6.1 Introduction

In Chapters Four and Five, extended gene expression was demonstrated following the implementation of a single gene vector dose. Eighteen months of expression were noted after delivery of LVlacZ, but importantly some mice displayed expression that lasted for 24 months. Using the LVCFTR gene vector, correction of the lack of CFTR function \((\text{via TPD measurement})\) emerged at 12 months; again, some individual mice appeared to display correction for more than 18 months. Total epithelial turnover time in the mouse trachea has been estimated at between 2 and 267 days (Blenkinsopp 1967; Bowden 1983; Rawlins and Hogan 2008). Therefore, achieving expression for the longest reported cell turnover time in the trachea (approximately 8.5 months) suggested that resident progenitor-like cells may have been involved if the nasal airway epithelium is similar to the tracheal epithelium. However, the average lifetime of nasal airway epithelial cell types has not been directly assessed.

It has been suggested that progenitor cells segregate their chromosomes asymmetrically during the S-phase of cell division. The immortal DNA is preferentially retained in the progenitor cell while the newly synthesised DNA strands segregate into the daughter cells earmarked for differentiation (Potten, Hume \textit{et al.} 1978; Smith 2005). Thus, Bromodeoxyuridine (BrdU), or any other DNA label that becomes incorporated into the DNA during the S-phase of cell division should be preferentially retained in the progenitor cell, while in the differentiating daughter cells, the BrdU signal is sequentially reduced with each cell division, and eventually is lost completely when cell death occurs. Therefore, BrdU labeling provides an effective means to determine the progenitor cell population in any tissue, as, after a given period cell division and death in the differentiating cell pool will mean the only cells that retain the BrdU label can be considered to be progenitor cells in that tissue. Additionally, in the case of the nasal airway, by injuring the epithelium, the rate of cell division can be increased and the
dilution of BrdU (until it is present only within a progenitor cell population) occurs more rapidly.

In order to determine if progenitor-like cells were involved in maintaining gene expression for up to 18 months in mouse nasal airways, an experimental approach based on assessing the co-localisation of vector mediated LacZ expression and BrdU staining after repeated epithelial injury was conducted. This was developed from the repeat injury / BrdU labeling studies first described by Borthwick et al. (2001) in combination with the LPC / LV dosing protocol developed by the Adelaide Cystic Fibrosis Gene Therapy Research Group.

### 6.2 Methods

#### 6.2.1 Repeat Damage by Lysophosphatidylcholine & BrdU Labelling

Female C57Bl/6 mice (6-8 weeks old) received weekly damage to the nasal airway epithelium by instillation of 4 μl of 1% lysophosphatidylcholine (see methods section 2.4.2). In addition, the mice received intraperitoneal injections of BrdU (2mg) every 48h beginning 2h after the first injury and extending until 24h after the final injury. One hour after the final injury 20 μl of LVLacZ was delivered as described in methods section 2.4.2. Seven days later mice were killed and their nasal airways were examined for both BrdU incorporation and LacZ expression using immunohistochemistry. Controls included groups of mice receiving BrdU but without nasal airway damage, as well as mice with nasal airway damage but no BrdU.
6.2.2 BrdU Immunohistochemistry

Mice were killed and heads were removed as previously described (section 2.4.3). Mouse heads were fixed, decalcified and cut and mounted onto slides. Slides were baked for 2 hours at 60°C and then dewaxed and dehydrated through xylene and a graded series of ethanols. The tissue was quenched in 1.5% H₂O₂ prior to being placed into warm 1M HCL. The tissue was treated with Proteinase K and blocked with 1% normal goat serum. After rinsing, a mouse anti-BrdU was applied. Labelling was visualized with a rabbit anti-mouse biotinylated IgG and avidin/biotinylated horseradish peroxidase reagents and with DAB substrate (Sigma-Aldrich, NSW, Australia). A normal mouse monoclonal IgG (Dako, Victoria, Australia) was used as a negative control. The tissues were counterstained and slides dehydrated through graded ethanols and xylene and finally cover-slipped.

6.2.3 LacZ Immunohistochemistry

The slides were rehydrated through xylene and ethanol. Slides were blocked with normal goat serum. Rabbit anti-LacZ antibody was applied and left overnight at 4°C. After washing in PBS the goat anti-rabbit antibody was applied for 1 hour at 37°C followed by incubation with DAB substrate. The slides were counterstained, dehydrated through graded ethanols and xylene and finally cover-slipped.

6.2.4 Heat-Induced Epitope Retrieval

To improve the detection of the LacZ, epitope retrieval was attempted. Sections were deparaffinised before being rehydrated through ethanol, then rinsed in distilled water. Slides were incubated in a 2N HCL solution for 20 minutes. At the end of this incubation IHC was performed as described in Section 6.2.2 and 6.2.3.
6.3 Results

6.3.1 BrdU Immunohistochemistry

No specific labelling was detected in any of the samples following staining using the recommended BrdU IHC conditions (Abcam Inc.). In an attempt to increase the labelling, the dilution of primary antibody was increased (see Table 6.1). Again, however, no specific labelling was detected. Nonetheless, with increased dilution, background staining increased. The secondary antibody concentration was also increased and tested (see Table 6.1) with no increase in labelling detected.

Table 6.1: BrdU Immunohistochemistry Antibody Dilutions. No specific staining was seen using any of the combinations noted here, and background staining was increased as the antibody became more concentrated.

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6.3.2 LacZ Immunohistochemistry

No specific labelling of LacZ was detected in any of the treated tissue slides after staining using the recommended IHC protocol (Clontech Inc.). However, a high level of background staining was evident. Different dilutions of the primary and secondary antibodies were tested in an attempt to achieve specific staining and reduce background staining (see Table 6.2). However, no specific labelling was detected when any of the dilutions were tested.

Table 6.2: LacZ Immunohistochemistry Antibody Dilutions. No specific staining was seen using any of the combinations noted here, and background staining was increased as the antibody was more concentrated.

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In an attempt to identify specific labeling, antigen retrieval was done. Antigen retrieval is used to unmask epitopes which may have become inaccessible to the antibody due to prolonged fixation (Hayat 2002). It can also act to denature proteins to reveal previously masked epitopes, and unmask epitopes by removing calcium ions (Hayat 2002). Following antigen retrieval, IHC was repeated using the methods described as above. However, no increase in specific labelling was detected.

6.4 Discussion

The airway epithelium is a dynamic tissue, which is constantly renewing, however, the average lifetime of different airway epithelial cell types has not been assessed in the mouse nose. Because the basal rate of mitosis in healthy airways was unknown, in this study the epithelium was injured to induce an increased cell turnover rate. As discussed in Chapter 3, the instillation of 1% LPC into the mouse nasal airway produced widespread epithelial exfoliation one-hour after exposure. The LPC-treated airway was indistinguishable from non-damaged airways 7 days post-exposure, indicating the recruitment of progenitor-like cells to repopulate and repair the airway cellular structure.

Differing methodologies, diurnal and seasonal variations in proliferation rates, and animal age, nutritional status, strain and overall health status have led to hugely variable estimations in cellular turnover timeframes in mouse airways (Kauffman 1980; Rawlins and Hogan 2008). Rawlins and colleagues have reported the longest estimated turnover time of 17 months in the bronchioles and 6 months in the trachea (Rawlins and Hogan 2008). They also found that epithelial turnover time was slower in the more distal lung airway regions than in the proximal, suggesting that while turnover in the bronchioles was approximately 17 months, turnover in the nasal airways may be
much more rapid. Turnover time in the ciliated nasal airway epithelium of mice has not been reported. Results from this thesis have achieved gene expression for greater than 18 months, strongly suggesting that progenitor-like cells may have been transduced and that these cells are providing the source of the maintained levels of gene expression.

The intention of this chapter study was to show co-localisation of LVLacZ and BrdU label retention within a subset of basal cells, thus indicating transduced cells that had potentially progenitor-like qualities. Successful double labeling would have been an important finding since, if a significant proportion of resident progenitor-like cells could be transduced, then repeat delivery of gene therapy might be unnecessary because gene expression would be continually transferred from the progenitor cells to daughter cells, down the cell-type lineages. Unfortunately, even after multiple attempts at modifying protocols and a limited amount of time, this result was not achieved.

A limitation of this study was that it did not include a positive control for BrdU. Because there was no BrdU-specific staining seen, one explanation is that there were no progenitor-like cells present within the sampled airways 3 months after the final dose of BrdU. If a positive control for BrdU was included (for example gut or hair follicles) in future studies when staining for BrdU was performed then a lack of BrdU staining in the sample tissues would show a lack of BrdU incorporation. If no specific BrdU staining emerged in the positive control, it would be appropriate to repeat the studies until the control-positive BrdU signal could be detected. This might involve utilizing different commercially available LacZ and BrdU antibodies, and different antigen retrieval techniques (such as proteolytic induced antigen retrieval or room temperature antigen retrieval using hydrochloric or formic acid). A different fixation method could also be trialed. The present data is incomplete and is not adequate to state whether BrdU labeled LacZ-stained cells may or may not have been present. Thus the hypothesis that LV-mediated transduction of (BrdU-incorporated) progenitor cells
in the mouse airway contributed to the extended maintenance of LacZ and CFTR gene expression reported in Chapters Four and Five cannot be supported or denied.

When the airway epithelium sustains damage, progenitor-like cells distributed throughout the airway epithelium are the source for renewal of lost epithelial cells, and have the capability to divide into all the cell types present within the epithelium (Kim, Jackson et al. 2005; Rawlins and Hogan 2006). Importantly, it has been demonstrated in rat airways, that the type of airway injury is an important determinant of the type of progenitor cell activation that will provide cellular renewal and repair. For example, Evans, Johnson et al. (1976) have shown that after nitrogen dioxide or ozone-induced injury, secretory cells within the airway can function as the progenitor-like cells (see also Evans, Shami et al. 1986), while naphthalene-induced injury resulted in basal cells acting as airway progenitor cells (Hong, Reynolds et al. 2004). In mice, several studies have reported that different regions of the respiratory system (the trachea, large airways, distal bronchioles and alveoli) use different populations of progenitor cells to initiate repair and re-population after damage (Borthwick, Shahbazian et al. 2001; Engelhardt 2001; Giangreco, Reynolds et al. 2002; Schoch, Lori et al. 2004; Rock, Onaitis et al. 2009). While progenitor-like cell populations have been identified in other airway tissues, the progenitor cell population responsible for maintaining the nasal airway region has not been described. The cell types present in the nasal airway are very similar to those present in the trachea, and the mouse nasal airway is indeed used as a surrogate for lung airways in many CF mouse research studies. For this reason, a similar process of cell renewal may be involved, relying on a subset of basal cells to act as progenitor-like cells. This theory was also based on results that showed if no LPC pre-treatment was used, expression levels were transient (regardless of the location of the vector receptor on the target cell, whether the apical or basolateral surfaces). However, if access was gained to the basolateral compartment of the airway, extended expression was seen (Kremer, Dunning et al. 2007).
While the majority of cells segregate their chromosomes randomly (Potten, Hume et al. 1978; Armakolas and Klar 2006), one mechanism to avoid accumulating mutations which may arise during cell replication is that progenitor-like cells do not duplicate their chromosomes in this way (Cairns 1975). Recently, however, it has been determined that not all progenitor cells behave and divide by segregating their chromosomes randomly. After chronic exposure to BrdU, Kiel and colleagues determined that less than 0.5% of all BrdU retaining cells in the mouse haematopoietic system were actually progenitor cells. This finding may be complicated by the fact that BrdU has poor specificity and poor sensitivity as a progenitor cell marker (Kiel, He et al. 2007). While this study by Kiel and colleagues specifically investigated haematopoietic cells, if BrdU label retention and asymmetric chromosome segregation are general properties of adult progenitor cells, these characteristics should be also evident in the airway’s progenitor cells. Studies are now required to establish the sensitivity and specificity of BrdU before assuming it marks progenitor cells definitively. Additionally, in other systems, normally quiescent differentiated cells are able to act as progenitor cells following an injury. For example, in the liver after a hepatectomy (the removal of part of the liver), turnover and regeneration involves the division of differentiated hepatocytes. Furthermore, if this hepatocyte proliferation is inhibited, the interlobular bile duct cells are able to replenish the hepatocyte cell population (Alison, Vig et al. 2004). Thus, cells recruited for repair and re-population after injury may be different to those that re-populate as part of normal cellular renewal in normal, uninjured epithelium.

When designing this long-term BrdU-labelling experiment, the repeat-damage methodology (see methods section 2.4.7) was considered the most appropriate way to show that the LPC-pretreatment / LV gene transfer method in airways was delivering the target gene into a (BrdU-labelled) population of slowly dividing cells with progenitor-like qualities. Applying this method was intended to identify a mechanism for the
greatly extended gene expression in live airways. However, a recent report indicated that BrdU has poor specificity and poor sensitivity as a progenitor cell marker (Kiel, He et al. 2007), highlighting that protocols used in these thesis studies may not be able clearly identify progenitor-like cells within the nasal airway.

The underlying question behind this study remains an important one in trying to understand the mechanism of extended gene expression that produces the findings described in Chapters Four and Five. However, the role of epithelial progenitor cells in the repair and maintenance of the airway is unclear and the identification of these progenitor cells remains contentious, and as can be seen in this chapter quite difficult, due to the lack to definitive progenitor markers (McQualter, Yuen et al.).

Investigations to answer the questions surrounding the mechanisms of extended gene expression, as well as airway repair, should continue as the development of new assays to accurately assess the progenitor cell potential in the airway appear.
7.1 Overview Of The Work Described In This Thesis

The two key findings of this study were: firstly, the persistence of LV-mediated LacZ gene expression for at least 24 months in some mice, and 18 months in most mice, after the single LV vector instillation; and secondly, that a single dose of LVCFTR vector could produce significant electrophysiological recovery in CFTR function for 12 months. In addition, these studies demonstrated the ability to optimise the pre-conditioning effects of LPC that enhanced the efficiency of gene transduction. At the same time it reduced damaging effects on the airway epithelium at higher doses. These significant in vivo findings should facilitate further development towards clinical application of safe and effective airway gene therapy for CF lung disease.

The initial experiments in this thesis were designed to optimise the LPC pre-treatment dosing regimen, previously developed by Dr Maria Limberis (Limberis, Anson et al. 2002) and colleagues at the CYWHS, Adelaide. Dr. M. Limberis, who described significant CFTR-mediated electrophysiological correction approximately 3 months after a single delivery of LVCFTR (n=2) (Limberis, Anson et al. 2002), achieved the longest-lasting CFTR gene delivery and expression reported until now. In her studies, 1% LPC was delivered as an enhancer agent to the nasal airway 1 hour prior to delivering the LV vector. However, the effects of LPC on airway epithelium were unknown. The experiments here were designed to determine the concentration of LPC that could produce the greatest level of gene transfer while minimising the amount of damage to the airway. I found that the level of cell perturbation increased as the concentration of LPC delivered to the airway increased. Additionally, increasing concentrations of LPC caused mucosubstance release (a measure of airway irritation) to increase in a dose-response fashion. The level of gene transfer peaked prior to reaching the highest LPC dose, indicating there was a maximum benefit at the 0.3% and 1%
LPC concentrations. We chose an LPC concentration of 0.3% to achieve high levels of gene expression, while inducing minimal disruption to airway epithelium.

I then wanted to establish the optimal time delay between the pre-treatment delivery and LV delivery. My studies showed that a one-hour delay produced the greatest level of gene transfer in these studies. Together, these experiments demonstrated that the optimal dosing protocol for gene transfer into live mouse nasal airways to be 0.3% LPC delivered one hour prior to the LV vector delivery via the same route. This combination was then utilised as the standard treatment to assess gene transfer over an extended period.

Using the marker gene LacZ, C57Bl/6 mice were instilled with 0.3% LPC followed one hour later with the LVLacZ gene therapy vector, with assessment of gene expression at several time points up to 24 months post-transduction – the usual lifetime of laboratory mice. The results provided the first in vivo demonstration of LV-mediated LacZ gene expression lasting for a lifetime in mice, after only a single LVLacZ vector instillation.

Interestingly, while mean LacZ gene expression remained stable over the initial 18 months post-instillation, by the 24 month time point expression had significantly declined. It is unknown why this reduction occurred, although an age-related effect is one obvious possibility. There are many examples of progenitor cell function changing with age (Ito, Liu et al. 2005; Ito, Hirao et al. 2006; Dykstra and de Haan 2008) and this may have an effect on the level of gene expression seen at this late time point. In haemopoietic stem cell populations for example, as aging occurs their response to changes in the levels of reactive oxygen species is altered and the repopulating capacity of the cells is diminished (Ito, Liu et al. 2005; Ito, Hirao et al. 2006).
The repeated airway epithelial injury combined with BrdU treatment study was conducted to uncover airway progenitor cells that may have been responsible for the extended gene expression seen in Chapters Four and Five. Cells that were double-labelled with LacZ and BrdU would suggest the gene expression longevity was caused by transduced progenitor cells re-populating the airway with transduced daughter cells. This is because the presence of BrdU staining would indicate the cell is a progenitor-like cell and LacZ staining would demonstrate that our standard protocol 0.3% LPC / LVLacZ transduced both differentiated airway epithelial cells and progenitor-like cells of the airway. Although it was hoped that the presence of LacZ / BrdU double-labelled cells might reveal clues about the involvement of airway progenitor cells in the extended LacZ and CFTR gene expression achieved in this thesis, these studies were not successful as there was no LacZ or BrdU labelling seen. This result does not rule out the possibility of progenitor cell involvement in gene expression longevity. The lack of staining may be attributed to: (a) the small number of basal cells within the airway compared to ciliated epithelium and thus even smaller number of progenitor-like cells present, as not all basal cells can act as progenitor cells; and (b) the patchy nature of LacZ staining that was seen throughout the experiments in this thesis. Future studies would benefit with the use of positive as well as negative controls, larger sample numbers per group (including both larger numbers of mice and a greater number of sections taken per mouse) and optimisation with different antibodies.

The study described in this thesis demonstrates extended LV-mediated gene expression after a single exposure to the gene therapy vector, and suggests the possibility of progenitor cell transduction. It may be possible to assess progenitor cell transduction after delivering a gene using our LPC / LV protocol dose by utilising a protocol similar to that used by Mitomo and colleagues (Mitomo, Griesenbach et al. 2008). In their study, Mitomo and colleagues instilled a sendai virus-pseudotyped SIV vector carrying the GFP marker gene. At 7 and 28 days post-vector delivery, artificially induced
cell division was induced by the instillation of 2% polidocanol to the mouse nasal epithelium in order to strip the cells of the airway epithelium while retaining the basal cell layer. GFP gene expression was analysed in the mouse nasal airway 4 weeks after the final detergent treatment, and clustered GFP-expressing cells (ciliated, non-ciliated and basal cell types) were present (Mitomo, Griesenbach et al. 2008). When progenitor cells divide via mitosis the resulting daughter cells created initially appear clustered around the originating progenitor cell before migrating throughout the airway. Therefore the presence of GFP-expressing cell clusters is indicates derivation from a common progenitor cell (Mitomo, Griesenbach et al. 2008). In replicating the Mitomo study to assess progenitor cell transduction in the LPC / LV system, we could strip the airway epithelium using a higher concentration of LPC at 7 and 28 days, and assess gene expression four weeks after the final LPC assault. If similar transduction patterns to the Mitomo study are seen these could support a role for progenitor cell transduction. In order to determine if the airway epithelial cells would regrow in a similar cluster-type fashion at later time points, it would interesting to conduct the epithelial stripping and regrowth study several months after the initial LV vector exposure.

The persistence of LV-mediated LacZ gene expression for at least 24 months in some mice, and 18 months in most mice, after the single LV vector instillation was encouraging and suggested that similar gene transfer would occur after LV-mediated CFTR gene transfer in cftrtm1Unc mice. The LVCFT vector used in these studies was essentially the same as the LVLacZ vector, but with the CFTR cDNA sequence replacing the LacZ marker gene sequence.

The reasons for the difference in gene expression persistence produced by the LVCFT and LVLacZ vectors are unknown. Designing gene transfer vectors that target specific cells (for example ciliated epithelial cells or progenitor-like cells) may be one way to begin to understand the difference in expression. Potentially, this could be done by
incorporating a target cell surface receptor antibody and a fusogenic molecule (to increase transduction efficiency) into the LV (Lei, Joo et al. 2010). By targeting only those cells that require CFTR, more effective and efficient airway gene transfer can be achieved when delivering the same amount of vector because it will only be expressed in the targeted cell types. Additionally, by delivering a GFP-tagged CFTR-containing vector (similar to the vectors used by Oceandy and colleagues (Oceandy, McMorran et al. 2003) and Ban and colleagues (Ban, Inoue et al. 2007)), the optimal level of CFTR expression per cell and the percentages of cells expressing vector-delivered CFTR may be identified. This would provide key information to help direct the development of how vectors can be targeted to achieve the most effective gene expression patterns to correct CF lung disease.

7.2 Future Directions

The findings outlined in this thesis can form a useful contribution to the field of CF airway gene therapy. It is important that the work in this thesis continues to be developed in future studies if the encouraging findings described within are to advance to a point where they can be considered for use in a clinical setting.

7.2.1 Immune Response To Gene Therapy

The immune system has evolved to eliminate foreign material, and this restricts the efficacy of gene therapy vectors. Adverse events caused by the activity of the adaptive or innate immune system were reported after phase I or II clinical gene therapy trials (Marshall 1999; Manno, Pierce et al. 2006; Bainbridge, Smith et al. 2008; Maguire, Simonelli et al. 2008). While the study described in this thesis focuses on producing
extended gene expression after a single exposure to the LV, there was no obvious immune
response seen (no changes to the nasal architecture, airway cavities clear of inflammation
and debris and no unusual cellular (i.e. neutrophil) infiltration into the nasal tissues).
However, if the level of expression waned (as it did at the 24 month timepoint in the
LVLacZ study) additional doses LV may be required and an immune response may
be elicited. It is therefore important for both the safety of the patient and the efficacy
of the therapy, that any potential immune response is controlled. A study by Koehler
and colleagues (Koehler, Martin et al. 2006) showed that although the first round of
vector administration does not induce an immune response, subsequent rounds of
re-administration result in decreased transgene expression and more circulating anti-
vector antibodies. Additionally, a study by Limberis and colleagues (Limberis, Bell et
al. 2010) showed that the decrease in gene expression was a direct result of activation
of transgene-specific T cells rather than as result of normal turnover of airway cells.

It is clear that unless clinically relevant levels of gene expression after a single dose of LV
can be achieved, vector re-dosing and methods to overcome this immune response will
be required. While several groups have investigated inducing tolerance to gene therapy
vectors, it has so far has proved unsuccessful using conventional immunosuppressive
agents (Kushwah, Cao et al. 2007; Wu and Ertl 2009). Additionally, immune suppression
may not be appropriate in CF lung disease as CF lungs exhibit an exaggerated response
to proinflammatory stimuli and are highly susceptible to infection (Kushwah, Cao et
al. 2007; Wu and Ertl 2009). The success of gene therapy and avoidance of deleterious
immune responses will require close collaboration between with immunologists and
virologists. Researchers may benefit in taking guidance from the numerous mechanisms
employed by the viruses themselves to avoid the destructive forces of the human
immune system.
7.2.2 Gene Delivery To The Lung

Cystic fibrosis affects the conducting airways, so the ultimate goal of CF gene therapy is the conducting airway of the lungs. There are two obvious methods for LPC and the vector administration via the airway, which could be employed in a clinical setting: instillation via a bronchoscope (as a bolus instillation or a course spray), or nebulisation. The Adelaide CF Gene Therapy Research Group has examined gene transduction in sheep lung using a bronchoscope to deliver a bolus of LPC, followed an hour later by a bolus of vector (personal communication, Dr. D.W. Parsons, manuscript submitted 2010). Studies have shown successful LacZ gene transfer, albeit at low levels. Since the two instillations occur an hour apart, care was taken to ensure the LPC and vector were delivered to the same region, by video recording the location of the first delivery site and then using this video to confirm the location when the vector was being delivered. However, it is possible that the second bolus delivery may not reach or affect the same cells that received the (first) LPC pre-treatment, which may reduce the number of transduced cells. Additionally, sheep airways possess much greater surface mucus levels than do mice, and this may provide a more extensive physical barrier to influencing transduction success.

Differences in the method of gene vector delivery in airways can affect the success of gene transfer. Administration of agents via bronchoscopy concentrates the material to a relatively small segment of the lung and can also force airway debris into the alveolar space (Joseph, O’Sullivan et al. 2001) thus causing alveolar contamination and subsequently, increase the likelihood of generating an inflammatory response (Joseph, O’Sullivan et al. 2001). Non-invasive delivery methods which may reduce the potential of an inflammatory response are clearly preferable, particularly if repeated administration of the vector will be required in a clinical setting. Using the relatively simple technique of nebulisation may be more convenient for future use in a clinical
setting. However, nebulisation cannot be targeted to a specific site within the lungs and the mouth, oesophagus, nasal airways, sinuses, and pharynx could be considered as off-target sites that would be exposed to both LPC and the gene vector. Additionally, the success of this technique relies heavily on the individual’s breathing pattern (Garcia-Contreras and Hickey 2002).

### 7.2.3 Animal Models To Assess The Efficiency Of Lentivirus Gene Therapy To The Airway Epithelium

Mouse models of human disease have inherent advantages in biomedical research of low cost, simple maintenance, and a rapid reproduction rate. Laboratory mice have contributed significantly to a better understanding of many aspects of human genetic diseases. However, CF is a complex human disease that affects many organs, and no mouse model accurately mimics the entire CF human disease state.

The mouse nasal airway was used to assess gene expression in these studies as it displays the CF bioelectric defect and most closely resembles the human conducting airway epithelium with respect to the types of cells present. The $\text{cfr}^{\text{TM1Unc}}$ mouse used in this thesis was produced by J.N. Snouwaert and colleagues in 1992 (Snouwaert, Brigman et al. 1992) and the primary pathology is intestinal obstruction due to thickened mucus. Although originally the hallmark of CF disease, intestinal obstruction is not seen in most CF patients today due to early detection of CF via neonatal screening programmes, and the use of pancreatic enzymes and vitamin supplements to prevent obstructions. Furthermore, while CF mice show abnormal chloride transport in nasal epithelial cells (Davidson and Dorin 2001) and some increase in the number of tracheal cells that produce mucus (Collins and Wilson 1992), these mice do not display the pancreatic or lung respiratory pathologies seen in CF (Clarke, Grubb et al. 1992). These limitations
as a model for CF lung disease have supported efforts to develop and use other animal models with a lung structure, size and function are more similar to humans.

Until recently, there were no large animal models for CF, but gene transfer studies (using reporter genes) had been conducted in sheep, pigs and non-human primates (McDonald, Lukason et al. 1997; Cunningham, Meng et al. 2002; McLachlan, Baker et al. 2007). The developments of nuclear transfer and gene targeting technology have recently permitted the successful targeting of the pig CFTR locus to produce transgenic CF piglets (Welsh, Rogers et al. 2009) which display a similar phenotype to newborn humans with CF prior to effective treatments. These CF pigs die from intestinal obstruction soon after birth, and though there is no evidence of morphological lung abnormalities, there is a lack of functional airway CFTR Cl− channel activity (as determined via TPD) indicating the basis for future CF lung disease development.

The normal pig lung is already an established model for normal human lung, in both assessment of disease state and in therapeutics research. It should therefore serve as an excellent research tool for studying the pathogenesis of CF lung disease and subsequently, the development of new treatments and therapies.

Recently, a ferret model of CF airway disease has also been developed (Sun, Yan et al. 2008). The ferret is an attractive animal model for human airway disease as the ferret and human lung architecture (Wang, Zhang et al. 2001), as well as their airway CFTR gene expression patterns (Engelhardt, Yankaskas et al. 1992; Sehgal, Presente et al. 1996), are extremely similar. The bioelectric properties of the CFTR chloride channel in the airway epithelia of the ferret is also analogous to the human airway epithelia (Liu, Luo et al. 2007). A recent study comparing ΔF508 forms of ferret CFTR and human CFTR found that the ΔF508 ferret CFTR contains the same processing defect seen in its human ortholog (Fisher, Liu et al. 2009). Another study by the same group has also reported that the bioelectric properties of the CF ferret airway is characteristic
of the human CF airway (Fisher, Zhang et al. 2009), suggesting that the CF ferret will also develop CF lung disease comparable to humans. The CF ferret model will undoubtedly be a valuable tool in CFTR biology research, as mice have been over the past 20 years.

### 7.2.4 Improved Assessment Of CF Gene Therapy Success

In the studies outlined in Chapter Five, both TPD assessment and CFTR immunohistochemistry were utilised to accurately assess the success of LVCFTR transduction. However, there was no staining seen in these studies. In order to obtain a more accurate and valid assessment of CFTR gene transfer, assays which together ensure full accurate and reliable assessment of the physiological and electrophysiological correction must be performed. This requires several assays to be done.

Several groups evaluate CFTR transfection efficiency by assessing transgene-specific mRNA using real time PCR (Gerard, Dell’Aringa et al. 2003; Holder, Stevenson et al. 2010). This assay is used to quantify expression in tissue homogenates and is therefore unable to distinguish which specific cell types within the homogenate are expressing the transgene. Consequently, mRNA detection does not necessarily indicate respiratory airway gene transduction. Recently, three methods have been developed by Holder and colleagues to isolate airway respiratory cells in order to assess murine cftr mRNA in the target cell population (Holder, Stevenson et al. 2010). After comparing the three different isolation methods, Holder and colleagues reported that laser microdissection of respiratory epithelial cells was successful, albeit too time-consuming to use in a high-throughput environment, while pronase digestion of the septum only achieved low yields of respiratory cells. Nasal brushing, however, was reported to achieve high yields of respiratory epithelial cells and cftr mRNA was detectable in both normal and
FABP-CF mice. As a new method to identify the specific cell types that support gene expression, laser microdissection could now be used to examine whether basal cells in LVCFTR-treated mice contain CFTR mRNA. Those findings could help decide whether the basal cell transduction was involved in the long-term LVCFTR gene expression described in Figure 5.2, Chapter 5). If so, the morphological and functional similarities across these two studies would lend further support to the stem / progenitor roles that have been ascribed to some groups of basal cells in the airway epithelium.

CFTR gene transfer to the respiratory airway cells in the mouse nose can be assessed by immunohistochemistry (IHC). As I experienced, however, this technique can be difficult to perform and optimise. The CFTR protein analysis based on detection of its presence, is critically dependent on using robust antibodies (Mendes, Farinha et al. 2004). While many papers report successful CFTR detection via IHC, the literature suggests that reliable detection of CFTR in tissue and primary cell cultures remains challenging task (Davidson, Wilson et al. 2009) and findings remain inconsistent across CF research groups. Furthermore, the epitopes recognised by some of these antibodies frequently become inaccessible or damaged after routine tissue preservation (Hayat 2002). An additional barrier in my studies was the relative harshness of the decalcification procedures needed to process nasal airway tissue within the bony nostril region.

The ability to identify the cell types that are expressing CFTR is an important aspect of CF gene therapy. The expression profile of recombinant CFTR should mimic endogenous CFTR activity as much as possible to avoid any complications inadvertently caused by erroneous CFTR expression. It is equally important that experimental design includes the appropriate positive or negative controls and that the results gained are interpreted with caution, since there is a real possibility for false positives. Interpretation of the images can be highly subjective.
In addition to CFTR presence, localisation and function assessment, the phenotypic features of the CF airway can also be examined to assess the efficiency of CFTR gene transfer. Once the basic defect is corrected, the ASL depth should increase and the goblet cell hyperplasia should decline. While airway cellular remodelling may take some time due to the airway cell turnover rates, the ASL depth should return to normal height as soon as ion channel function is restored.

The TPD assay remains a valuable method to assess successful correction of the electrophysiological defect in CF airways after CFTR gene transfer. However, results from this assay may be limited due to the small assessment area and the technical difficulties associated with TPD measurements (see Chapter 5 and Appendix 4). If used in conjunction with another assay, such as Real Time PCR on isolated respiratory epithelial cells, and immunohistochemistry, a clearer picture of the CFTR profile in the airway epithelium should emerge.

7.3 How Much CFTR Transduction Is Required In Order For A Clinical Benefit To Emerge?

A fundamental question in CF gene therapy studies is how much CFTR is required to restore ion channel function, ASL depth and MCC mechanisms to normal. Previous studies have demonstrated that the level of CFTR expression in any tissue can have significant consequences on organ physiology, development as well as susceptibility to disease. For example, mice heterozygote for wild-type CFTR, are thought to be resistant to cholera, while homozygote mice are not (Gabriel, Brigman et al. 1994), although this has recently been disputed by Högenauer and colleagues (Högenauer, Santa Ana et al. 2000). Additionally, a specific level of CFTR function is required for
the proper development of the vas deferens (De Braekeleer and C. 1996). Conversely, a study by Mohammad-Panah and colleagues reported that over-expression of CFTR may detrimental, demonstrating that in vitro, hyper-expression of recombinant CFTR proteins can lead to the loss of alternate ion channel regulation (Mohammad-Panah, Demolombe et al. 1998).

More recently Zhang and colleagues (Zhang, Button et al. 2009) reported that above-normal levels of CFTR expression were not detrimental to the function of the airway. This study showed as predicted that as the amount of CFTR increased, forskolin-stimulated Cl− secretion and Na+ absorption increased. Interestingly, they also found that when cells significantly over-expressed CFTR, ion movement remained identical to ion movement seen in normal (non-CF) cells. Additionally, ASL depth and mucus transport increased proportionally to increased CFTR expression, and again, a plateau was reached at normal, non-CF levels. Once 40% of cells were positive for CFTR, no further increase in ion transport was seen. That study concluded that CFTR delivered to 25% of the surface epithelial cells was sufficient to restore normal levels of ion transport, ASL depth and mucous transport. An important difference between the Mohammad-Panah study and the Zhang et al. study is the systems in which they were conducted. The Mohammad-Panah study examined CFTR expression levels in three different cell lines (COS-7 cells, derived from the African Green Monkey kidney, CFPAC-1 cells derived from a pancreas duct carcinoma from a patient suffering from AF508 CF and A549 derived from carcinomic human alveolar epithelial cells). The Zhang et al. study, however, utilised an air-liquid interface model of ciliated human CF airway epithelium and so the findings are more relevant to the normal airway environment.

The Zhang et al. study was important because it has provided a benchmark of CFTR delivery for the field. However, the work was conducted in vitro and because of the
often poor predictive value of \textit{in vitro} findings to \textit{in vivo} situations, the next step will be to examine these effects \textit{in vivo} if possible. Potentially, this could be achieved by combining several experimental protocols. Firstly, by delivering a GFP-tagged CFTR-containing vector (similar to the vectors used by Oceandy and colleagues (Oceandy, McMorran \textit{et al.} 2003) or Ban and colleagues (Ban, Inoue \textit{et al.} 2007)) to the mouse nasal airway and measuring CFTR function using TPD measurement, ion-channel restoration could be assessed \textit{in vivo}. By subsequently utilising immunohistochemistry to detect GFP-tagged CFTR, the specific cell types that are expressing CFTR could potentially be identified.

In order to accurately assess the percentage of respiratory epithelial cells expressing CFTR, the nasal brushing mRNA quantification method described by Holder and colleagues could be included (Holder, Stevenson \textit{et al.} 2010). As the nasal brushing technique and the IHC would require the same cells, these experiments would need to be done in different animals groups, however the TPD assessment could be performed on both groups of animals. Finally, the ASL depth could be assessed by OsO$_4$ and PFC fixation as previously described by Tarran and colleagues (Tarran, Grubb \textit{et al.} 2001). However, as recently described by Griesenbach and colleagues (Griesenbach, Sumner-Jones \textit{et al.} 2009) in order to see a significant improvement in ASL depth a large cohort of mice may be needed. This is due to the difference in ASL depth between normal and CF knock-out mice being so small. This ASL study may therefore be more suited to a larger animal model, where the normal depth and the difference in ASL depth is likely to be larger, and so smaller animal numbers may be required in the study groups. A successful study combining all these analytical methods would contribute significantly to advancing the field of CF gene therapy and potentially move us further towards answering the unanswered question: “how much CFTR is enough?”
7.4 The Ethical Considerations Of Gene Therapy

The developments in clinical genetics over the past 20 years, and in particular the increased knowledge of the specific genes responsible for a number of inherited diseases have resulted in steady, albeit slow, advances in research. This continual advancement provides hope that gene therapy treatments can be developed in order to cure many genetic diseases, including CF. However, this progress also raises important ethical issues about the potential application, misapplication and consequences of gene therapy.

The potential to cause harm using gene therapy has been readily apparent for many years and must not be forgotten amongst the excitement of the future success that any gene therapy protocols achieve. In 1999, a gene therapy phase I clinical trial patient, Jesse Gelsinger, died 4 days after being injected with a high dose of adenovirus vector containing a gene to correct deficiency in ornithine transcarbamylase enzyme (Batshaw, Wilson et al. 1999). This gene therapy trial was designed to develop a cure for a severe deficiency that impairs the urea cycle, resulting in a build up of ammonia in the bloodstream and early death (reference – Gordon 2003 OTD a urea cycle defect (Gordon 2003?) ). When Jesse volunteered for this trial, his disorder was non-life threatening and largely under control. However, upon receiving his dose of viral vector his body elicited an acute immune response to the virus and he died of systemic inflammation resulting in multi-organ failure (Wilson 2009).

Shortly after this incident, the first curative gene therapy success was reported. A clinical trial to treat the severe immune deficiency SCID-X1 was conducted at the Hôpital Necker Enfants Malades in Paris (ten patients) and at Great Ormond Street Hospital in London (four patients). This trial proved successful with all 14 patients developing a fully functioning immune system, and being effectively cured, after previously been classed as incurable (Hacein-Bey-Abina, Von Kalle et al. 2003). Disappointingly,
30 months after this successful treatment, one of the patients developed leukaemia. Since then, five of the fourteen patients have gone on to develop leukaemia (De Ravin and Malech 2009), due to the viral vector inserting in close proximity to the LMO2 promoter, a known proto-oncogene associated with T cell leukaemia (Rabbitts, Bucher et al. 1999). Without the gene therapy treatment, these patients would almost certainly no longer be alive, but they are now susceptible to leukaemia.

The case of Jesse Gelsinger and the SCID-X1 trial outcomes highlighted the potential dangers regarding vector doses and the possibility that some age groups may be at higher risk of developing complications (the patients who developed leukaemia were the youngest who participated in the trial). The important ethical question regarding gene therapy clinical trials thus becomes, when, and under what conditions, does it become morally and scientifically justifiable to commence a clinical trial?

7.4.1 Treatment Versus Enhancement

Genetic medicine will have a fundamental impact on both medicine and the ethics of medicine. However, the speed at which the gene therapy research is progressing is far greater than society’s ability to consider and deal with the resulting ethical consequences in a timely way.

Gene therapy can be utilised to cure diseases but what classifies a diseased state? Currently, the most significant ethical issue is the establishment of solid criteria to judge disease, disability and normality. Many believe that abnormality equates with disease, for example, blood pressure readings which fall outside the normal range, are considered indicative of disease. Another approach is that aligned with Nordenfelt’s
The Theory of Disability which states “one is disabled if one is unable to pursue goals one both wishes to pursue and has the opportunity to pursue” (Edwards 1998). The understanding that our society has around the concepts of health, disease and normality will play a key role in shaping the application of the emerging genetic medicines in which gene therapy plays a major role. Unfortunately, the distinction between therapy and enhancement is not always clear.

An inherent human desire is to improve appearance, mental capacity and physical performance (Miller 2005) and as a reflection on this, society places high values on certain physical and mental traits and shuns others (Wenz 2005). It is not then surprising that there are a variety of accepted techniques which aim to improve these desired traits. Examples of these techniques include cosmetic surgery to reverse the appearance of aging or remove unwanted body fat and hair transplantation. While these enhancing strategies are ethically acceptable in society, other enhancement procedures are not. For example administration of growth hormones is used to treat short stature when the underlying reason is growth hormone deficiency or Turner’s syndrome, but is not acceptable to use it to become taller for social or athletic reasons (Allen 2006).

The use of gene therapy for enhancement is widely debated in the ethics literature, with many scientists agreeing that gene therapy should solely be used to cure severe disease. Some, however, argue that genetic enhancement is not dissimilar to enhancement by way of plastic surgery, hair transplantation and growth hormone injections and should be permitted (Miller 1994; Fuchs 2006). The challenge is how to define the difference between disease treatment and enhancement, an immensely difficult issue to resolve.

Without an adequate regulation system, the potential for abuse of gene therapy is always present. It is important, as with any medical procedure, that gene therapy research and treatment developments continue to be critically reviewed at research
institutions and within government health systems around the world, to ensure that its use as a treatment option is compatible with acceptable ethical standards.

7.4.2 Somatic And Germline Gene Therapy

There are two types of gene therapy possible; somatic and germline. Somatic gene therapy requires only the affected organs to be transduced. It is designed to only affect the genetic make-up of that one individual and not be passed onto any subsequent offspring. Germline gene therapy involves insertion of the altered gene into the germ cells (sperm or egg) of the treated individual. Its effects ultimately involve not only the treated individual, but also all future generations in that lineage.

In medical terms it can be argued that somatic gene therapy is merely an extension of current medicine, which has evolved over the past 60 years to incorporate previously unimaginable procedures, such as organ transplants and in vitro fertilisation. In this sense, it could be regarded as raising no ethical problems; rather it is just a new medicine that is treating patients, but at a genetic level. However, as straightforward as this notion seems, issues such as safety, unpredictable consequences and informed consent would assume greater importance simply due to the uncertainty surrounding such an emerging field of science. Significantly, 28 policy statements from 28 government authorities, groups of experts consulted by state authorities, churches and medical associations published between 1980 and 1993 agreed that somatic gene therapy for treating serious diseases is ethically acceptable in principle (Walters and Palmer 1997).

In contrast, germline gene therapy presents a major ethical dilemma because the germ cell contains not just the information needed for one cell type to function, but the entire human DNA code. This becomes important when it is realised that currently
gene therapy protocols involve delivering a gene into the chromosome randomly. If this were to occur in a germ cell, it could create an entirely new genetic disorder which has not previously occurred naturally, which may cause death or lifelong disease in the offspring, with the ability to pass on the disease to subsequent generations.

Some scientists and ethicists argue that germline gene therapy is medically necessary to prevent certain classes of disease and that it should not only be allowable, but is advisable, as it fits with their duty to remove harm (Fiddler and Pergament 1996). It is argued that the possibility of developing applied and refined germline gene therapy is justified and should be considered imperative because it will:

- Provide a true cure for many genetic disorders
- Prevent the transmission of disease causing mutations
- Prevent the need for repeated somatic gene therapy generation after generation.

However, many others have significant ethical and social concerns against germline gene therapy, arguing that germline gene therapy must not be considered while alternative procedures offering theologically, socially and ethically acceptable technologies are being developed to treat genetic diseases (Frankel and Chapman 2000; Resnik and Langer 2001). It is argued that there is not sufficient evidence concerning the technology’s long- and short-term safety and efficacy profiles (Frankel and Chapman 2000; Resnik and Langer 2001).

The potential for a therapy that would affect future generations raises three important ethical arguments in opposition to its practice:

- Risk and Uncertainty
- “Slippery slope” notions, specifically in regard to genetic enhancement
- Lack of consent by future generations
Risk and uncertainty are associated with human germline gene therapy; however they are not unique to it. The introduction of many other medical interventions, such as organ transplantation and antibiotic drugs faced similar risk and uncertainty. With stringent experimental assessment of the potential risks, however, the technology can become mainstream lifesaving practice in medicine today. It may be possible that by the time gene therapy trials that could affect future generations through alteration of the germline are approved, the scientific community will have accumulated a significant level of experimental data on its impact in model systems to ensure the associated risks and uncertainty are minimal. It is, however, also possible that we are unable to minimise the associated risks at the time they become apparent, and may do irreversible harm to future generations.

The so-called ‘slippery slope’ argument in reference to genetic enhancement is an interesting one. On one hand, why should genetic enhancement be strictly prohibited while enhancements such as plastic surgery, hair transplantation and growth hormone injections are commonplace. Alternatively, caution is reasonable considering that governments and dictators could potentially impose a use that is not in the interest of society as a whole. There is the notorious example of the eugenic goals of Nazi Germany, which aimed to eliminate handicapped, diseased and “lower” classes of people (Guvercin and Arda 2008). Currently, concern about gene therapy being applied to asocial behaviour, deviant personalities and enhanced human development (i.e. skills in music, mathematics or athletics) appears to be ill conceived and unnecessarily inflammatory to the debate at hand. Considering that presently the ability to apply gene therapy to improve more undefined characteristics, such as personality, this seems implausible.

The question of consent by future generations to alterations in genetic inheritance is a complex and highly debated one. The position of some scientists is that any genetic
changes would permit the future generation to survive when they otherwise would not have, and believe that it is realistic for them to be grateful for this intervention (Fiddler and Pergament 1996). However, this assumes that germline gene therapy will produce no ill effects. A more sensible approach would be using a germline gene therapy protocol, which incorporated certain safety factors to reduce the risk of harm to following generations. To reduce the potentially negative impact on future generations a reversible germline modification has been developed, which after the first generation, is eliminated (Capecchi 2000). Similarly, Stock and Campbell proposed developing a transgene which will only be expressed when activated by the appropriate transcription factors (Stock and Campbell 2000). Both these concepts could provide important safeguards to reduce the risk to future generations; however, it must be remembered that these safety factors may not function according to their design specifications. Much more knowledge about the efficacy and reliability of these safety factors is required before reversible germline gene therapy becomes a possibility in a clinical setting. Furthermore, it is thought that these types of genetic interventions are not likely to have a significant effect on the human gene pool as long as there is a limited medical use (Resnik, Steinkraus et al. 1999). Extreme caution is necessary, regardless of the initial good intentions of germline gene therapy advocates. In 20 years time, safe and successful germline gene therapy may be a reality; and the question then will remain: can we afford to assume that powerful governments, leaders and indeed, scientists, will continue to practise a “limited medical use” or will the temptation to push the boundaries of science continue?

With regards to risks and benefits of germline gene therapy, it is important to remember that it is still at an early stage of development, and at present, has yet to be tested. It is also important to realise that some of the risks associated with genetic interventions are still unknown.
At this stage, I do not believe that we, as a society or as scientists, are currently equipped with sufficient knowledge to make moral decisions involving the use of germline gene therapy, especially considering the lack of globally agreed upon distinctions between treatment, prevention and enhancement. As research into this field continues, we will continue to gain a greater understanding of the potential benefits and risks, as well as more knowledge surrounding the safety of this technology. Subsequently, a more thorough assessment of the important bioethical, social and regulatory issues can be undertaken before germline gene therapy may become a reality.

Unless scientists proceed with both conviction and great caution, the potential to eradicate human suffering, while a lofty and perhaps unattainable goal, is lost. I believe that prior to making decisions on germline gene therapy, somatic gene therapy must be successfully achieved for multiple disorders and followed over a significant period of time in order to ascertain a long term safety profile of this genetic manipulation. Once a sound knowledge of the long-term outcomes of somatic gene therapy, in light of the potential to do harm to future generations has been fully elucidated, only then should consideration be given to germline gene therapy. Germline gene therapy offers to take us one step closer to a world where the burden of illness and disease is lifted, however the health of future generations cannot be jeopardised until we can fully understand the consequences of our interference. Worldwide, the debate on germline gene therapy will continue.

7.5.3 In Utero Gene Therapy

Advances in human prenatal medicine and genetics have allowed the diagnosis of many genetic diseases early in gestation, and as adult gene therapy is progressing several obstacles to its application as a treatment option might be overcome by *in utero* gene
therapy treatment. Compared to adult gene therapy, \textit{in utero} gene therapy offers the potential to correct genetic defects before the onset of disease, before any pathological changes have occurred. This would be particularly valuable in treating diseases such as Tay-Sachs disease, type 2 Gaucher disease and Krabbe disease (lysosomal storage diseases), each of which cause irreversible damage to the brain prior to birth (Shen, Meng \textit{et al.} 2004). As babies with CF have normal lungs at birth (Bush 2008) there is an opportunity to prevent lung disease using gene therapy without needing to perform \textit{in utero} gene therapy. While meconium ileus, an intestinal obstruction, occurs in approximately 20\% of newborns with CF (Waddington, Kramer \textit{et al.} 2005), the current treatment at birth is to use medication to liquefy and release the meconium, or in more serious cases, surgery may be required. There is no clear advantage of \textit{in utero} gene therapy over post-natal gene therapy for the treatment of CF, thus I believe that if a gene therapy treatment for CF is realised, \textit{in utero} gene therapy will not be required for CF.

\textit{In utero} gene therapy presents a complex ethical issue as there are two participants in the procedure and one of them (the foetus) is unable to give consent. As with any \textit{in utero} procedure, there is a risk of infection and in the induction of pre-term labour and prior to contemplating any trials in humans, a thorough evaluation or safety and efficacy in animal models must first be completed. The majority of concerns surrounding the ethics of \textit{in utero} gene therapy, however, have tended to focus on its potential to lead to the uptake and expression of genetic material in non-target cells, specifically the inadvertent transduction of germline cells. To date, no transduction of the germline after \textit{in utero} gene therapy has been reported in studies that specifically looked at germline transmission of gene therapy vectors into non-gonadal tissues (Porada, Tran \textit{et al.} 1998; Themis, Schneider \textit{et al.} 1999; Coutelle and Rodeck 2002). While these results provide some confidence the issue must continue to be investigated and \textit{in utero} animal trials should always incorporate an assessment of the risk to the germline.
Overall, many questions remain regarding the unseen and long term consequences of foetal gene therapy, specifically in relation to the potential advantages, safety and risks, which can only be answered by further \textit{in vivo} animal studies. Further discussion and evaluation of the methods for assessing the safety of applying \textit{in utero} gene therapy must be conducted before human trials are considered.

7.6 Concluding Remarks

Since the discovery of the CFTR gene in 1989, scientists have endeavoured to find a cure for CF airway disease. While a cure has proven elusive, continued progress in the field of gene therapy provides an optimistic outlook for the future. The demonstration in my studies of extended \textit{in vivo} gene expression (up to 12 months for CFTR) offers additional options and improves the promise that gene therapy can, eventually, be translated into a safe and effective treatment of CF lung disease. Additionally, the simple transduction protocol described in this thesis, utilising brief single exposures to LPC and the LV vector, may avoid re-dosing and thereby avoid the inherent immune activation barriers that thwart repeat-dosing gene therapy protocols. The findings described here provide evidence that a single-dose LV gene transfer methods could offer a novel \textit{in vivo} therapeutic paradigm in the pursuit of a cure for CF airway disease.
Appendices
## Appendix 1: Company Addresses

<table>
<thead>
<tr>
<th>Company</th>
<th>Location</th>
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<tbody>
<tr>
<td>Abcam</td>
<td>Massachusetts, United State of America</td>
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<tr>
<td>Amersham International</td>
<td>Amersham, United Kingdom</td>
</tr>
<tr>
<td>Applied Biosystems</td>
<td>California, United States of America</td>
</tr>
<tr>
<td>APS Finechem</td>
<td>New South Wales, Australia</td>
</tr>
<tr>
<td>Beckman Coulter</td>
<td>New South Wales, Australia</td>
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<tr>
<td>BDH Chemicals</td>
<td>Dorset, United Kingdom</td>
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<tr>
<td>Clontech</td>
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<td>Dako</td>
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<tr>
<td>Decal Chemical Corporation</td>
<td>New York, United States of America</td>
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<tr>
<td>Dendy Pharmaceuticals</td>
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<tr>
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<td>Geneworks</td>
<td>South Australia, Australia</td>
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<tr>
<td>Hamilton Company</td>
<td>Nevada, United States of America</td>
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<tr>
<td>Invitrogen Corporation</td>
<td>California, United States of America</td>
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<td>Jackson ImmunoResearch</td>
<td>Pennsylvania, United States of America</td>
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<tr>
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<td>NUNC</td>
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<td>Olympus Australia</td>
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<tr>
<td>Pasco Scientific</td>
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<td>Perkin Elmer</td>
<td>Victoria, Australia</td>
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<tr>
<td>Qiagen</td>
<td>Victoria, Australia</td>
</tr>
<tr>
<td>Sigma-Aldrich</td>
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</tr>
<tr>
<td>Sigma Chemical Company</td>
<td>Montana, United States of America</td>
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SPSS, Inc.  Illinois, United State of America
Thermo Fisher Scientific  Massachusetts, United State of America
United Biosciences  Queensland, Australia
Vector Laboratories  California, United States of America
Wescor Inc.  Utah, United States of America
Appendix 2: Solutions & Buffers

1mM MgCl2 in PBS

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<tr>
<th>Component</th>
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<tr>
<td>MgCl2</td>
<td>0.1015 g</td>
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<tr>
<td>PBS</td>
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4% Paraformaldehyde

<table>
<thead>
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<tbody>
<tr>
<td>Paraformaldehyde</td>
<td>40 g</td>
</tr>
<tr>
<td>PBS</td>
<td>750 mL</td>
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</table>

Stir and heat to 60 °C to dissolve
Add 10N NaOH drop wise until solution goes clear
pH to 7.4 with hydrochloric Acid
Bring to 1 L with PBS
Filter solution through filter paper
Store at 4 °C for up to 1 month

2% Paraformaldehyde / 0.5% Glutaraldehyde

<table>
<thead>
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<th>Component</th>
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<tbody>
<tr>
<td>4% Paraformaldehyde</td>
<td>500 mL</td>
</tr>
<tr>
<td>25% Glutaraldehyde</td>
<td>20 mL</td>
</tr>
<tr>
<td>PBS</td>
<td>480 mL</td>
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Store at 4 °C

0.9% NaCl

<table>
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</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>9 g</td>
</tr>
<tr>
<td>MilliQ Water</td>
<td>1 L</td>
</tr>
</tbody>
</table>
2 x HeBS (HEPES Buffered Saline)
NaCl 16.4 g
HEPES 11.9 g
Na2HPO4 0.21 g
Water 1 L
pH to 7.4 with 5M NaOH
Filter sterilize and store at -20°C

Bromodeoxyuridine (BrdU)
Bromodeoxyuridine 40 g
1N NaOH 130 μL
Vortex until dissolved
1N HCL 120 μL
Ensure pH is between 7 and 8
Make up to 5 mL with PBS. 125 μL dose = 2mg BrdU
Store at -20°C

Carnoy’s Fixative
Acetic Acid (glacial) 10 mL
Chloroform 30 mL
Ethanol 60 mL
Make in fume hood

Decal
EDTA 15 g
MilliQ Water 930 mL
HCl 70 mL
Stir and heat until solution goes clear
**Ethanol (75%)**

- Ethanol (100%)  75 mL
- MQ H2O  25 mL

Store at -20°C

**Lysophosphatidylcholine (LPC) 2%**

- LPC  0.002 g
- PBS  100 μL

Store at 4 °C (discard after 1 month)

**Lysophosphatidylcholine (LPC) 1%**

- 2% LPC  50 μL
- PBS  50 μL

Store at 4 °C (discard after 1 month)

**Lysophosphatidylcholine (LPC) 0.3%**

- 2% LPC  45 μL
- PBS  255 μL

Store at 4 °C (discard after 1 month)

**Lysophosphatidylcholine (LPC) 0.1%**

- 1% LPC  10 μL
- PBS  90 μL

Store at 4 °C (discard after 1 month)

**Lysophosphatidylcholine (LPC) 0.03%**

- 0.3% LPC  10 μL
- PBS  90 μL

Store at 4 °C (discard after 1 month)
Phosphate Buffered Saline (Dulbecco’s PBS)

(Ca²⁺, Mg²⁺ free, pH 7.4, 10x)

NaCl 80.0 g
Na₂HPO₄ 11.6 g
KH₂PO₄ 2.0 g
KCl 2.0 g

Initially make up to 1L to make 10x stock, dilute 1:10 and adjust pH to 7.4

Pre-X-Gal

Potassium Ferricyanide (K₃Fe(CN)₆) 0.8233 mL
Potassium Ferrocyanide (K₄Fe(CN)₆) 1.0560 mL
1mM MgCl₂ 500 μL
PBS 500 mL

Store at room temperature, covered in foil

TPD - Basal Solution

KH₂PO₄ 0.163 g
K₂HPO₄ 0.052 g
CaCl₂ 0.088 g
MgCl₂ 0.122 g
HEPES 1.191 g
NaCl 3.942 g
MilliQ Water 500 mL

Keep at 4 °C for maximum of 4 weeks

TPD - Basal Plus Amiloride Solution

Basal TPD Solution 500 mL
0.1 mM Amiloride 0.012 g

Keep at 4 °C for maximum of 4 weeks
TPD - Low Chloride Solution

KH₂PO₄  0.163 g
K₂HPO₄  0.052 g
CaCl₂  0.088 g
MgCl₂  0.122 g
HEPES  1.191 g
NaOH  14.722 g
MilliQ Water  500 mL

Keep at 4 °C for maximum of 4 weeks

TPD - Low Chloride Plus Amiloride Solution

Low Chloride TPD Solution  500 mL
0.1 mM Amiloride  0.012 g

Keep at 4 °C for maximum of 4 weeks

X-Gal Stock (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

X-Gal    1 g
Dimethylformamide  50 mL

Make in fume hood and store at -20 °C

Cell lines

A549 American Type Culture Collection (ATCC), CCL 185
293T ATCC, CRL 11268
NIH3T3 ATCC, CRL 1658
CHO-K1 ATCC, CRL 61
## Solutions

<table>
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<tr>
<th>Solution</th>
<th>Supplier</th>
<th>Catalog Number</th>
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<tr>
<td>Dulbecco’s Modified Eagle Medium (DMEM) with 4500 mg/L Dextrose and</td>
<td>SAFC Biosciences (Lexana, KS, USA)</td>
<td>51441C</td>
</tr>
<tr>
<td>4.0 mM L-Glutamine without Sodium Pyruvate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foetal Calf Serum (FCS)</td>
<td>JRH Biosciences (Lenexa, KS, USA)</td>
<td>12103-500M</td>
</tr>
<tr>
<td>L-Glutamine Solution 200mM</td>
<td>SAFC Biosciences (Lexana, KS, USA)</td>
<td>59202C</td>
</tr>
<tr>
<td>Penicillin G 5000 U/mL, Streptomycin Sulphate 5000 μg/mL</td>
<td>JRH Biosciences (Lenexa, KS, USA)</td>
<td>59620</td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS) without calcium and magnesium</td>
<td>SAFC Biosciences (Lenexa, KS, USA)</td>
<td>59321C</td>
</tr>
<tr>
<td>OptiPRO™ SFM</td>
<td>Invitrogen Corporation (Carisband, CA, USA)</td>
<td>12309-019</td>
</tr>
<tr>
<td>Trypsin/EDTA solution (0.12% (w/v) Trypsin, 0.2% (w/v) EDTA)</td>
<td>SAFC Biosciences (Lexana, KS, USA)</td>
<td>59430C</td>
</tr>
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</table>

All disposable cell culture plasticwares were supplied by Costar (Corning, NY, USA), NUNC (Nalgene Nunc International, Rochester, NY, USA), Sarstedt (Numbrecht, Germany) and Greiner Labortechnik (Solingen, Germany).
# Real Time PCR Plastics & Reagents

<table>
<thead>
<tr>
<th>Item</th>
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<th>Catalog Number</th>
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<tr>
<td>ABI PRISM™ Optical Adhesive Cover</td>
<td>Applied Biosystems (Foster City, CA, USA)</td>
<td>Cat # 4311971</td>
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<tr>
<td>ABI PRISM™ optical caps (8 wells/strip)</td>
<td>Applied Biosystems (Foster City, CA, USA)</td>
<td>Cat # 4323032</td>
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<td>GAPDH control reagents (human)</td>
<td>Applied Biosystems (Foster City, CA, USA)</td>
<td>Cat # 402869</td>
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<td>MicroAmpR Optical 96 well reaction plates</td>
<td>Applied Biosystems (Foster City, CA, USA)</td>
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<tr>
<td>TaqMan MGB Probe (50,000 pmol)</td>
<td>Applied Biosystems (Foster City, CA, USA)</td>
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<td>20 x TaqMan Universal PCR Master Mix</td>
<td>Applied Biosystems (Foster City, CA, USA)</td>
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All primers were manufactured by Invitrogen Corporation (CA, USA).
### ANIMAL MONITORING SHEET

<table>
<thead>
<tr>
<th>AEC number:</th>
<th>Investigator name and contact/s:</th>
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<tbody>
<tr>
<td>Animal ID number:</td>
<td>Species/Strain:</td>
</tr>
<tr>
<td>Animal Details (sex/age etc.):</td>
<td>Comments:</td>
</tr>
</tbody>
</table>

- Each animal is to be examined and observed at each time point (daily or weekly as appropriate)
- Normal clinical signs are recorded as "N"
- Abnormalities are recorded as "A". Severity is recorded as a score eg "A 3" Abnormality score charts are available in holding rooms and from Animal House staff.
- Any abnormalities must be brought to the attention of the Animal House Manager so appropriate action may be taken.

#### CLINICAL OBSERVATION CHECKLIST

<table>
<thead>
<tr>
<th>DATE</th>
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<th>ON HANDLING</th>
<th>OTHER</th>
<th>COMMENTS</th>
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<tr>
<td></td>
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<td>Alert</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Activity</td>
<td>Body condition</td>
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<tr>
<td></td>
<td>Breathing</td>
<td>Bodyweight</td>
<td>Eyes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Movement/gait</td>
<td>Dehydration</td>
<td>Faeces</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eating</td>
<td>Dehydration</td>
<td>Nose</td>
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<td></td>
<td>Drinking</td>
<td>Eyes</td>
<td>Breathing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alertness/sleeping</td>
<td>Eyes</td>
<td>Vocalisation</td>
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**INITIALS:**

Signature Chief Investigator

Date
### CLINICAL SIGNS SEVERITY SCORE – RODENTS

<table>
<thead>
<tr>
<th>SIGN</th>
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<tr>
<td><strong>Activity</strong></td>
<td>normal</td>
<td>Isolated, abnormal posture</td>
<td>Huddled/inactive or overactive</td>
<td>Moribund or fitting</td>
</tr>
<tr>
<td><strong>Alertness/sleeping</strong></td>
<td>normal</td>
<td>Dull or depressed</td>
<td>Little response to handling</td>
<td>Unconscious</td>
</tr>
<tr>
<td><strong>Body condition</strong>*</td>
<td>normal</td>
<td>Thin</td>
<td>Loss of body fat, failure to grow</td>
<td>Loss of muscle mass</td>
</tr>
<tr>
<td><strong>Body weight</strong>*</td>
<td>normal</td>
<td>Reduced growth rate</td>
<td>Chronic weight loss &gt; 15% or failure to grow</td>
<td>Acute weight loss &gt; 20%</td>
</tr>
<tr>
<td><strong>Breathing</strong></td>
<td>normal</td>
<td>Rapid, shallow</td>
<td>Rapid, abdominal breathing</td>
<td>Laboured, irregular, skin blue</td>
</tr>
<tr>
<td><strong>Coat</strong></td>
<td>normal</td>
<td>Coat rough</td>
<td>Unkempt, wounds, hair thinning</td>
<td>Bleeding or infected wounds or severe hair loss or self mutilation</td>
</tr>
<tr>
<td><strong>Dehydration</strong></td>
<td>none</td>
<td>Skin less elastic</td>
<td>Skin tenting</td>
<td>Skin tenting and eyes sunken</td>
</tr>
<tr>
<td><strong>Drinking</strong></td>
<td>normal</td>
<td>Increase/decrease intake over 24 hours</td>
<td>Increase/decrease intake over 48 hours</td>
<td>Constantly drinking or not drinking over 24hrs</td>
</tr>
<tr>
<td><strong>Eating</strong></td>
<td>normal</td>
<td>Wetness or dullness</td>
<td>Discharge</td>
<td>Eyelids matted</td>
</tr>
<tr>
<td><strong>Faeces</strong></td>
<td>normal</td>
<td>Slight incoordination or abnormal gait</td>
<td>Loose, soiled perineum or abnormally dry +/- mucus</td>
<td>Running out on handling or no faeces for 48 hrs or blood on faeces</td>
</tr>
<tr>
<td><strong>Movement/gait</strong></td>
<td>normal</td>
<td>Slight incoordination or abnormal gait</td>
<td>Uncoordinated or walking on tip toe or reluctant to move</td>
<td>Staggering or limb dragging or paralysis</td>
</tr>
<tr>
<td><strong>Nose</strong></td>
<td>normal</td>
<td>Wetness</td>
<td>Discharge</td>
<td>Coagulated</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td>normal</td>
<td>Abnormal colour/volume</td>
<td>No urine 24 hrs or incontinent, soiled perineum</td>
<td>Abnormal vocalisation</td>
</tr>
<tr>
<td><strong>Vocalisation</strong></td>
<td>normal</td>
<td>Squeaks when palpated</td>
<td>Struggles and squeaks loudly when handled</td>
<td></td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* these criteria may not apply in some situation (eg tumour growth, obesity/metabolic studies)
Appendix 4: Challenges Associated with Mouse Nasal TPD Recordings

The nasal epithelium of the CFTRtm1UNC mouse has been used extensively in CF research because it exhibits ion transport defects similar to those of human CF airways.

TPD measurements are technically difficult and trace quality and accurate scoring can have a significant impact on the results obtained. Griesenbach and colleagues have reported that large numbers of mice are required to detect small changes in baseline and low Cl δTPD values, thus the TPD may have limited value when assessing chloride channel function with anticipated small treatment effects (Griesenbach, Smith et al. 2008). This is particularly relevant in the study presented in Chapter 5 as there were a large number of traces rejected for various reasons. TPD traces were rejected on the basis of

- Electrical interference that produced unstable TPD readings
- Insufficient time (< 2 minutes) allowed for a plateau to be reached after solution change
- Anaesthesia maintenance issues, e.g. if the anaesthesia beings to wear off, the emerging movement of the mouse can displace the cannula tip position, and possibly cause damage to the epithelium
- Bubbles present in perfusion tubing unable to be cleared via brief high flow purges of the fluid system. Bubbles can affect TPD measurement stability, alter the overall circuit resistance, and produce an open-circuit condition
- TPD readings reaching levels above circuit zero (i.e. showing positive mV readings greater than the usual variation in a stable voltage reading)
- Irregular cyclic patterns present without evidence of an underlying plateau being reached
- Unstable basal TPD value, e.g. if the TPD reading had not settled within 15 minutes of the start of recording with a given fluid perfusion
- Physical errors, e.g. cannula knocked inadvertently during a solution change procedure; the assumption was made that a change in cannula tip position will have occurred, thus invalidating the relationship between the present and earlier TPD readings on the epithelium

Examples of rejected traces are shown in Figure A.1

In order to assess the potential reasons why there was such a large number of TPD traces rejected, various aspects of the experiment were investigated.
Figure A.1: Examples of Rejected TPD Traces. Traces rejected for electrical interference (A), cyclic readings (B), bubbles in the cannula (C), potential difference going positive (D) and a combination of positive readings, bubbles and the mouse waking up (E), plateau too short (F) and because of a knocked cannula (G). Compared to accepted CF-knockout mouse TPD trace (H).
Appendix 5: Publications

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.  
It is also available online to authorised users at:  

http://dx.doi.org/10.1002/jgm.1368

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1002/jgm.803
Bibliography


Barlow-Stewart, K. (2007). Newborn Screening for Genetic Conditions, Centre for Genetics Education.


