LONGEVITY OF AIRWAY GENE THERAPY FOR CYSTIC FIBROSIS: SINGLE AND REPEAT LENTIVIRAL DOSING

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PhD Thesis

November, 2012

Research conducted in the Department of Respiratory and Sleep Medicine

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TABLE OF CONTENTS

TABLE OF CONTENTS III

LIST OF FIGURES XV

LIST OF TABLES XVIII

LIST OF APPENDICES XIX

LIST OF ABBREVIATIONS XX

ABSTRACT XXVIII

DECLARATION XXX

ACKNOWLEDGEMENTS XXXI

CHAPTER 1 INTRODUCTION 3

1.1 CYSTIC FIBROSIS – EARLY HISTORY 3

1.2 INCIDENCE 4

1.3 DISCOVERY OF THE CF GENE 6

1.4 THE CFTR PROTEIN 7

1.4.1 CFTR Structure 7
1.4.2 CFTR Function 8
1.4.3 CFTR Mutations 11
1.4.3.1 Class I Mutations 11
1.4.3.2 Class II Mutations 12
1.4.3.3 Class III Mutations 12
1.4.3.4 Class IV Mutations 12
1.4.3.5 Class V Mutations 13
1.4.3.6 Class VI Mutations 13
1.4.4 Diagnostic Genetic Testing for CF 15

1.5 CLINICAL SYMPTOMS OF CF 17

1.5.1 Sweat Glands 17
1.5.1.1 Diagnostic Sweat Test 18
1.5.2 Gastrointestinal Tract 19
1.5.2.1 Pancreas 19
1.5.2.1.1 Exocrine function 19
1.5.2.1.1.1 Fat malabsorption diagnostic test 20
1.5.2.1.2 Endocrine function 21
1.5.2.2 Intestinal Tract 21
1.5.3 Liver 22
1.5.4 Reproductive Tract 23
1.5.5 Respiratory System 24

1.6 CF AIRWAY DISEASE 26

1.6.1 Airway Anatomy 26
1.6.2 Defective Ion Transport 27
1.6.2.1 Transepithelial Potential Difference 29

1.6.3 Airway Surface Liquid Layer 29
1.6.4 Airway Infections 31
1.6.4.1 Staphylococcus aureus 32
1.6.4.2 Haemophilus influenzae 33
1.6.4.3 Pseudomonas aeruginosa 33
1.6.4.4 Burkholderia cepacia 35
1.6.4.5 Other Bacterial Species 36
1.6.4.6 Fungal Infections 36
1.6.4.7 Viral Infections 37
1.6.5 Current Airway Treatments 37
1.6.5.1 Airway Clearance 38
1.6.5.2 Novel Therapies 38
1.6.5.2.1 Ion regulation 38
1.6.5.2.2 Potentiators 39
1.6.5.2.3 Correctors 39
1.6.5.3 Lung Transplantation 40

1.7 GENE THERAPY 41

1.7.1 Barriers to Gene Therapy 42
1.7.1.1 Extracellular Barriers 42
1.7.1.2 Intracellular Barriers 43
1.7.1.3 Immunological Barriers 46
1.7.2 Non-Viral Vectors 48
1.7.2.1 Cationic Lipid Complexes 48
1.7.2.2 Polymer Complexes 50
1.7.3 Viral Vectors 50
1.7.3.1 Adenoviral Vectors 51
1.7.3.1.1 Helper-dependent adenoviral vectors 53

1.7.3.2 Adeno-associated Virus Vectors 54
1.7.3.3 Sendai Virus Vectors 56
1.7.3.4 Lentiviral Vectors 57
1.7.3.4.1 Lentiviral clinical trials 59
1.7.3.4.2 Pre-clinical LV gene therapy for CF 61

1.8 CF ANIMAL MODELS 62

1.8.1 CF Mouse Model 62
1.8.2 CF Pig Model 64
1.8.3 CF Ferret Model 65

1.9 TARGETS FOR CF GENE THERAPY 66

1.9.1 Therapeutic Levels of CFTR 67
1.9.2 Longevity of Expression 68

1.10 AIMS OF THESIS 70

CHAPTER 2 METHODS AND MATERIALS 75

2.1 MATERIALS 75

2.1.1 Chemicals and Suppliers 75
2.1.2 Consumables and Suppliers 78
2.1.3 Bacterial Strains and Media 79
2.1.4 Cell Lines 79
2.1.5 DNA Plasmids 80
2.1.5.1 Plasmid Kits and Buffers 80
2.1.5.2 DNA Plasmids 80
2.1.6 Real Time qPCR Assay 81
2.1.6.1 PCR Kits 81

2.1.6.2 Primers and Probes....81
2.1.7 LV LacZ Titre Assay....82
2.1.8 Animal Models....82
2.1.8.1 Anaesthesia....82
2.1.8.2 Luciferase Imaging....83
2.1.9 Processing of Mouse Heads....83
2.1.10 ELISA Assay....83
2.1.10.1 p24 ELISA....83
2.1.10.2 ELISA for Serum Antibodies....84
2.1.11 Nasal TPD....84

2.2 METHODS - *in vitro*....85

2.2.1 DNA Plasmid Preparation....85
2.2.1.1 Large Scale Plasmid Preparation....85
2.2.1.2 Agarose Gel Electrophoresis....85
2.2.2 Cell Culture Techniques....85
2.2.3 LV Vector Preparation....86
2.2.4 Quantification of Virus Titre....87
2.2.4.1 Xgal Staining for LacZ Titre....87
2.2.4.2 qPCR for Virus Titre....87
2.2.4.2.1 Sample preparation for real time qPCR for virus titre....88
2.2.4.2.2 qPCR for virus titre....88
2.2.5 Replication Competent Viral Assay....89
2.2.5.1 Sample Preparation for p24 ELISA....89
2.2.5.2 p24 ELISA for Replication Competent Virus....90
2.2.6 qPCR for Transgene Presence in Tissue Samples....90
2.2.6.1 Tissue DNA Extraction....90
2.2.6.1.1 DNA extraction via Wizard SV purification method....90

2.2.6.1.2 DNA extraction via direct tail lysis method 90
2.2.6.2 qPCR for Transgene Presence in Tissues 91
2.2.7 Presence of Circulating Antibodies 91

2.3 METHODS - *in vivo* 93

2.3.1 Animal Care and Maintenance 93
2.3.2 Lentiviral Nasal Instillation 94
2.3.2.1 Pre-treatment Preparation 94
2.3.2.2 Lentiviral Vectors 94
2.3.2.3 Nasal Instillations 94
2.3.3 Gene Transfer Assessment 95
2.3.3.1 Reporter Gene Transfer 95
2.3.3.1.1 LacZ expression via Xgal staining method 95
2.3.3.1.2 Histological processing for LacZ expression 95
2.3.3.1.3 Luciferase gene expression via biophotonic imaging 96
2.3.3.2 CFTR Gene Expression 97
2.3.3.2.1 Nasal transepithelial potential difference measurements 97
2.3.4 Submandibular Blood Sampling 100
2.3.5 Nasal Septum Dissection 101
2.3.6 Tissue Removal 101

2.4 STATISTICAL ANALYSIS 102

CHAPTER 3 LENTIVIRAL PRODUCTION AND INITIAL *IN VIVO* TESTING 105

3.1 LENTIVIRAL PRODUCTION 105

3.1.1 Introduction 105
3.1.2 Aims 105
3.1.3 Methods 106

3.1.3.1 Lentiviral Vector Titres....106
3.1.3.2 Replication Competent Virus....106
3.1.4 Results....107
3.1.4.1 Lentiviral Vector Titres....107
3.1.4.2 Replication Competent Virus....107
3.1.5 Discussion....108

3.2 TESTING OF VIRUS PREPARATIONS – *in vivo*....110

3.2.1 Introduction....110
3.2.2 Methods....110
3.2.2.1 LacZ Gene Transfer....110
3.2.2.2 Luciferase Gene Expression....110
3.2.2.3 CFTR Gene Expression....111
3.2.3 Results....111
3.2.3.1 LacZ Gene Transfer....111
3.2.3.2 Luciferase Gene Expression....115
3.2.3.3 CFTR Gene Expression....122
3.2.4 Discussion....126

3.3 CHAPTER SUMMARY....129

CHAPTER 4 LONG TERM LENTIVIRAL GENE TRANSFER....133

4.1 LONG TERM GENE EXPRESSION IN NORMAL MICE....133

4.1.1 Introduction....133
4.1.2 Aims....134
4.1.3 Methods....134
4.1.3.1 Luciferase Gene Expression – Normal Mice....134
4.1.3.2 LacZ Gene Expression – Normal Mice....135

4.1.3.3 Circulating Antibodies – Normal Mice 135
4.1.3.3.1 Anti-VSV-G protein 136
4.1.3.3.2 Anti-Luciferase 136
4.1.3.3.3 Anti-β-galactosidase 136
4.1.3.4 Genomic DNA in Other Tissues – Normal mice 137
4.1.4 Results 137
4.1.4.1 Animal Weights – Normal Mice 137
4.1.4.2 Luciferase Gene Expression – Normal Mice 138
4.1.4.2.1 Nasal Luminescence 140
4.1.4.2.2 Lung Luminescence 140
4.1.4.2.3 Nasal Luciferase Expression – LPC vs PBS 140
4.1.4.2.4 Lung Luciferase Expression – LPC vs PBS 141
4.1.4.3 LacZ Gene Expression – Normal Mice 144
4.1.4.4 Circulating Antibodies – Normal Mice 146
4.1.4.4.1 Anti-VSV-G protein 146
4.1.4.4.2 Anti-Luciferase 147
4.1.4.4.3 Anti-β-galactosidase 147
4.1.4.5 Presence of Genomic Reporter DNA in Tissues 149
4.1.4.6 Survival Demographics of Normal Mice 149
4.1.5 Discussion 151

4.2 LONG TERM GENE EXPRESSION IN CYSTIC FIBROSIS MICE 156

4.2.1 Introduction 156
4.2.2 Aims 156
4.2.3 Methods 156
4.2.3.1 Luciferase Gene Expression – CF Mice 156
4.2.3.2 Therapeutic CFTR Gene Expression – CF Mice 157
4.2.3.3 Circulating Antibodies – CF mice 157

4.2.3.3.1 Anti-VSV-G protein 158
4.2.3.3.2 Anti-Luciferase 158
4.2.3.3.3 Anti-PDZK1 peptide for presence of the CFTR protein 158
4.2.3.4 Presence of Genomic CFTR 159
4.2.3.4.1 Presence of genomic transgenes in other tissues 159
4.2.4 Results 159
4.2.4.1 Body Weights – CF Mice 159
4.2.4.2 Luciferase Gene Expression - CF Mice 161
4.2.4.2.1 Nasal Luminescence 161
4.2.4.2.2 Lung Luminescence 162
4.2.4.2.3 Nasal Luciferase Expression – LPC vs PBS 165
4.2.4.2.4 Lung Luciferase Expression – LPC vs PBS 165
4.2.4.3 Therapeutic CFTR Gene Expression - CF Mice 167
4.2.4.4 Circulating Antibodies – CF Mice 170
4.2.4.4.1 Anti-VSV-G protein 170
4.2.4.4.2 Anti-Luciferase 171
4.2.4.4.3 Anti-PDZK1 peptide for presence of the CFTR protein 171
4.2.4.5 Presence of Genomic CFTR 173
4.2.4.5.1 Presence of genomic transgenes in other tissues 173
4.2.4.6 Survival Demographics of CF Mice 174
4.2.5 Discussion 179

4.3 CHAPTER SUMMARY 187

CHAPTER 5 REPEAT LENTIVIRAL GENE TRANSFER 193

5.1 MULTIPLE LENTIVIRAL GENE TRANSFER 193

5.1.1 Introduction 193

5.1.2 Aims 194
5.1.3 Methods 194
5.1.3.1 Short Term Multiple LV Dosing 194
5.1.3.2 Long Term Repeat LV Dosing 195
5.1.3.2.1 Circulating antibodies 195
5.1.3.3 Repeat Transgene Dosing Studies 195
5.1.3.3.1 Circulating antibodies after repeat transgene dosing 196
5.1.4 Results 196
5.1.4.1 Short Term Multiple Dosing 196
5.1.4.2 Long Term Repeat LV Dosing 197
5.1.4.2.1 Circulating antibodies 198
5.1.4.2.1.1 Anti-VSV-G protein 198
5.1.4.2.1.2 Anti-β-galactosidase 199
5.1.4.3 Repeat Transgene Dosing Studies 200
5.1.4.3.1 Nasal luminescence gene expression 200
5.1.4.3.2 Nasal LacZ gene expression 201
5.1.4.3.3 Circulating antibodies after repeat transgene dosing 202
5.1.4.3.3.1 Anti-VSV-G protein 202
5.1.4.3.3.2 Anti-Luciferase 203
5.1.4.3.3.3 Anti-β-galactosidase 203
5.1.5 Discussion 205

5.2 CHAPTER SUMMARY 210

CHAPTER 6 IMMUNOSUPPRESSION FOR LV GENE EXPRESSION 213

6.1 IMMUNOSUPPRESSION 213

6.1.1 Introduction 213

6.1.2 Aims....214
6.1.3 Methods....214
6.1.3.1 Immunosuppression Studies....214
6.1.3.2 Circulating Antibodies....215
6.1.4 Results....215
6.1.4.1 Nasal Luminescence....215
6.1.4.2 Lung Luminescence....219
6.1.4.3 Circulating Antibodies....222
6.1.4.3.1 Anti-VSV-G protein....222
6.1.4.3.2 Anti-Luciferase....222
6.1.5 Discussion....224

6.2 CHAPTER SUMMARY....227

CHAPTER 7 CONCLUDING REMARKS....230

7.1 FINAL DISCUSSION....230

7.2 CONCLUSION....237

CHAPTER 8 APPENDICES....241

8.1 INDIVIDUAL NASAL LUMINESCENCE – NORMAL MICE....241

8.2 INDIVIDUAL LUNG LUMINESCENCE – NORMAL MICE....242

8.2.1 LPC/LV-Luc+LacZ....242
8.2.2 LPC/LV-Luc+LacZ continued....243
8.2.3 PBS/LV-Luc+LacZ....244

8.3 INDIVIDUAL NASAL LUMINESCENCE – CF MICE 245

8.4 INDIVIDUAL LUNG LUMINESCENCE – CF MICE 246

8.4.1 LPC/LV-Luc+CFTR 246

8.4.2 LPC/LV-Luc+CFTR Continued 247

8.4.3 PBS/LV-Luc+CFTR 248

8.5 LACZ COUNTS AND VSV-G ANTIBODIES 249

8.6 LACZ COUNTS AND LACZ ANTIBODIES 250

CHAPTER 9 BIBLIOGRAPHY 253

LIST OF FIGURES

Figure 1-1 Location of the CFTR Gene....6
Figure 1-2 CFTR Protein Structure....8
Figure 1-3 Relationship between CFTR and Other Ion Channels....10
Figure 1-4 Classes of Mutations of CFTR....14
Figure 1-5 Abnormalities in Chloride Transport in the Sweat Gland....18
Figure 1-6 Severity of CF Disease Related to CFTR Activity....23
Figure 1-7 Cascade of CF Lung Disease....25
Figure 1-8 Cell Type Composition of the Airway Surface Epithelium....27
Figure 1-9 Model of Ion Transport across Epithelial Cells in Normal and CF Airways.....28
Figure 1-10 Airway Surface Liquid Layer in Airway Epithelia....31
Figure 1-11 Age-related Prevalence of Airway infections in CF Patients.....32
Figure 1-12 Stages of Biofilm Formation....35
Figure 1-13 Extracellular Barriers to Gene Transfer Vectors....43
Figure 1-14 Intracellular Barriers to Gene Transfer Vectors....45
Figure 2-1 Diagrammatic Representation of the Nasal TPD Setup.....98
Figure 2-2 Schematic Representation of TPD Traces....100
Figure 3-1 LacZ (blue staining) Transduction....112
Figure 3-2 Titres LV-LacZ Gene Transfer – Dose Response....113
Figure 3-3 LV-LacZ Gene Transfer – Volumes....114
Figure 3-4 LacZ Transduced Cell Types....115

Figure 3-5 LV-Luc Gene Expression – One Week after Gene Delivery 117

Figure 3-6 LV-Luc Gene Expression – ROI Calculations 118

Figure 3-7 Nasal Luminescence – LPC/LV-Luc 119

Figure 3-8 Lung Luminescence – LPC/LV-Luc 119

Figure 3-9 Nasal Luminescence – One Week 120

Figure 3-10 Lung Luminescence – One Week 121

Figure 3-11 Nasal TPD Traces 123

Figure 3-12 Nasal TPD Measurements 124

Figure 3-13 Repeated Nasal TPD's 125

Figure 4-1 Body Weight – One week 137

Figure 4-2 Body Weight over Time 138

Figure 4-3 Lung Luciferase Imaging – LPC/LV-Luc+LacZ 139

Figure 4-4 Lung Luminescence – LPC/LV-Luc+LacZ 139

Figure 4-5 Nasal and Lung Luminescence 142

Figure 4-6 Average Luciferase Expression – LPC vs PBS 143

Figure 4-7 Long Term en face LacZ Transduction 145

Figure 4-8 Presence of Circulating Antibodies 148

Figure 4-9 Survival Curves 150

Figure 4-10 CF Mice Weights – One week 160

Figure 4-11 Body Weight of CF Mice over Time 161

Figure 4-12 Nasal and Lung Luminescence in CF mice 163

Figure 4-13 Bioluminescence over Time in a LPC/LV-Luc+CFTR treated CF mouse 164

Figure 4-14 Average Luciferase Expression in CF mice – LPC vs PBS 166

Figure 4-15 Nasal ΔPD_{Na+} of the Sodium Channel Response in CF mice 167

Figure 4-16 Nasal ΔPD_{Cl-} of the Chloride Channel Response in CF mice 169

Figure 4-17 Correlation of Luminescence vs ΔPD in CF mice 170

Figure 4-18 Circulating Antibodies – CF Sera 172

Figure 4-19 Presence of Genomic CFTR DNA from Nasal Septa 173

Figure 4-20 Survival Curve – CF Mice 175

Figure 4-21 Number of Anaesthetics 176

Figure 4-22 Time after Procedure 177

Figure 4-23 Unexplained Mice Deaths 178

Figure 5-1 LacZ Gene Expression from Multiple Doses of LPC/LV-LacZ 197

Figure 5-2 LacZ Gene Expression from Long Term LPC/LV-LacZ Re-dose 198

Figure 5-3 Circulating Antibodies to Repeat LV Dosing 200

Figure 5-4 Nasal Luminescence from Repeat LV dosing 201

Figure 5-5 LacZ Reporter Gene Expression after LV Re-dosing 202

Figure 5-6 Circulating Antibodies to Repeat Transgene Dosing 204

Figure 6-1 Nasal Luminescence – Immunosuppression Treatment 216

Figure 6-2 Nasal Luminescence – With and Without Immunosuppression 218

Figure 6-3 Lung Luminescence – Immunosuppression Treatment 219

Figure 6-4 Lung Luminescence – With and Without Immunosuppression 221

Figure 6-5 Circulating Antibodies – Immunosuppression 223

LIST OF TABLES

Table 1-1 Impact of New Born Screening between Australia and USA..... 16

Table 1-2 Adenoviral Gene Therapy Clinical Trials for CF..... 52

Table 1-3 Adeno-associated Gene Therapy Clinical Trials for CF 55

Table 1-4 Current LV Gene Therapy Clinical Trials 60

Table 3-1 LV Preparations 107

Table 4-1 Necropsy Results..... 177

Table 6-1 Sirolimus Dosing Schedule 215

LIST OF APPENDICES

Appendix 8.1-1 Individual Nasal Luc Expression in LPC/LV-Luc+LacZ Treated Mice 241

Appendix 8.2-1 Individual Lung Luminescence in LPC/LV-Luc+LacZ Treated Mice.....242

Appendix 8.2-2 Individual Lung Luminescence in LPC/LV-Luc+LacZ Treated Mice cont. 243

Appendix 8.2-3 Individual Lung Luminescence in PBS/LV-Luc+LacZ Treated Mice 244

Appendix 8.3-1 Individual Nasal Luc Expression in LPC/LV-Luc+CFTR Treated Mice.....245

Appendix 8.4-1 Individual Lung Luminescence in LPC/LV-Luc+CFTR Treated CF Mice.....246

Appendix 8.4-2 Individual Lung Luminescence in LPC/LV-Luc+CFTR Treated CF Mice cont. 247

Appendix 8.4-3 Individual Lung Luminescence in PBS/LV-Luc+CFTR Treated CF Mice 248

Appendix 8.5-1 Individual LacZ Counts vs anti-VSV-G 249

Appendix 8.6-1 Individual LacZ Counts vs anti-LacZ 250

LIST OF ABBREVIATIONS

293T cells (HEK)	Human embryonic kidney cells
A549 cells	Carcinomic human alveolar basal cells
AAV	Adeno-Associated virus
ABC	Adenosine triphosphate binding cassette
ADA-SCID	Adenosine deaminase deficiency–severe combined immunodeficiency disorder
AdV	Adenovirus
AIDS	Acquired immunodeficiency syndrome
Amil	Amiloride
ANOVA	Analysis of variance
APC	Antigen presenting cell
ASL	Airway surface liquid
ATP	Adenosine triphosphate
AUS	Australia
BMI	Body mass index
°C	Degrees Celsius
^{13}C	Isotopic labelled carbon
cAMP	cyclic adenosine monophosphate

CAUV	Congenital absence of the uterus and vagina
CBAVD	Congenital bilateral absence of the vas deferens
CF	Cystic fibrosis
CFLD	Cystic fibrosis liver disease
CFRD	Cystic fibrosis related diabetes
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
CHO-K1 cells	Chinese Hamster Ovary – K1 epithelial cells
Cl^-	Chloride
cm	centimetre
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CpG	Cytosine-phosphate-guanine
Ct	Cycle threshold
CTL	Cytotoxic T Lymphoctes
ΔF508	Delta F508
Da	Daltons
DIOS	Distal intestinal obstruction syndrome
DMEM	Dulbecco's Modified Eagle's Medium
DMF	N, N, Dimethyl formamide
DNA	Deoxyribonucleic acid

ΔPD	Change in Potential Difference
DPX	Distyrene-tricresyl-phosphate-xylene
EDTA	Ethylene diamine tetraacetic acid
EF1-α	Human elongation factor 1-*alpha*
EGTA	Ethylene glycol tetraacetic acid
EIAV	Equine infectious anaemia virus
ELISA	Enzyme linked immunosorbent assay
ENaC	Epithelial sodium channel
env	Envelope
EYFP	Enhanced yellow fluorescent protein
FABp	Fatty acid binding protein
FCS	Fetal calf serum
FE-1	Faecal elastase-1
FEV_1	Forced expiratory volume in 1 second
FIV	Feline immunodeficiency virus
g	grams
gag	Group-specific antigen
Glut	Glutaraldehyde
GTP	Guanosine triphosphate
dH_2O	Deionized water
HCl	Hydrochloric acid

$HCO3^-$	Bicarbonate
HD-AdV	Helper-dependent adenovirus
H&E	Haematoxylin and Eosin
Het	Heterozygote (-/+)
HIV	Human immunodeficiency virus
hr	Hour
HRP	Horseradish peroxidase
Ig	Immunoglobulin
i.n.	Intranasal
i.p.	Intraperitoneal
i.u.	Infectious units
K^+	Potassium
KRB	Krebs-ringers buffer
L	Litre
LacZ	β-galactosidase
LPC	Lysophosphatidylcholine
LTR	Long terminal repeat
Luc	Luciferase
LV	Lentivirus
$\bar{X}$	Mean
m	month(s)

M	Molar
mA	milliamps
mM	millimolar
MCC	Mucociliary clearance
MCT	Mucociliary transport
MHC	Major histocompatibility complex
MI	Meconium ileus
min	minutes
ml	millilitre
µl	microlitre
mRNA	messenger ribonucleic acid
MSD	Membrane-spanning domain
MT	Empty vector
mTransferrin	mouse transferrin
mV	millivolts
MW	Molecular weight
Na^+	Sodium
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NBD	Nucleotide-binding domain
NBF	Neutral buffered formalin

Nef	Negative regulatory factor
NFQ	Non-fluorescent quencher
NIH3T3 cells	Mouse embryonic fibroblast cells
nls	Nuclear localised
nm	nanometres
OPD	o-Phenylenediamine dihydrochloride peroxidase substrate
ORCC	Outwardly rectifying chloride channel
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween
PC2	Physical containment level 2
PCL	Periciliary Layer
PD	Potential difference
PEG	Polyethylene glycol
Pen-Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
Ph	Photons
PI	Pancreatic Insufficiency
PKA	Protein kinase A
PKC	Protein kinase C
pol	Polymerase
PS	Pancreatic Sufficiency

qPCR	Real time quantitative polymerase chain reaction
Rev	Regulator of virion protein expression
RM	Repeated Measures
ROI	Region of Interest
ROMK	Renal outer medullary potassium channel
rpm	Revolutions per minute
RSV	Respiratory syncytial virus
RT	Room temperature
Rx	Treated
SafO	Safranin O
SCID-X1	X-linked severe combined immunodeficiency disorder
SEM	Standard error of the mean
SeV	Sendai virus
SFM	Serum free medium
SIN	Self inactivating
SIV	Simian immunodeficiency virus
SKMPBST	Skim milk PBS + Tween
SMG	Submucosal gland
SV-40	Simian virus type 40
T	Temperature
Tat	Trans-activator of transcription

TPD	Transepithelial potential difference
Treg	T -Regulatory cell
TU	Transducing units
Tween 20	Polyoxyethylene sorbitan monolaurate
UnRx	Untreated
USA	United States of America
UV	Ultraviolet
v	Volume
Vif	Virion infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VSV-G	Vesicular stomatitis virus glycoprotein G
w	Weight
WCH	Women's and Children's Hospital
wk	Week(s)
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ABSTRACT

The promise of gene therapy as a treatment and/or cure for cystic fibrosis (CF) airway disease is yet to be fulfilled. Lentiviral (LV) vectors possess many of the properties that would satisfy the requirements for an effective clinical gene correction treatment; the capacity to hold the large CF transmembrane conductance regulator (CFTR) gene, pseudotyped envelopes that provide broad tropism for a range of cells and tissue types, the ability to transduce dividing and non-dividing cells, the potential for long-term gene expression from genomic integration, and the lack of pre-existing blocking antibodies for majority of the CF population.

To determine the persistence of LV gene expression, the same mice were repeatedly assessed throughout their lifetimes. The utilization of the biological compound lysophosphatidylcholine (LPC) as a pre-treatment enhanced nasal airway gene expression of the HIV-1 based LV vector containing reporter genes, or the functional CFTR gene, in normal and CF mice *in vivo*.

Nasal luciferase (Luc) gene expression from a single LPC/LV nasal dose was sustained for the life time of normal mice, possibly suggesting an involvement of stem/progenitor cells or long-lived terminally differentiated cells. In contrast, stable long-term Luc gene expression was detected in the lung airways without the requirement of LPC pre-treatment. The loss then re-emergence of lung luminescence in CF mice demonstrated that stem/progenitor cells were transduced.

This was the first examination of persistence of LV reporter gene and functional gene expression, in individual CF mice over their lifetimes. CF mice treated with LPC/LV-CFTR demonstrated a significant

partial functional correction of the nasal CFTR electrophysiological defect that was sustained for up to 1 year. Importantly, this significantly increased survival, close to that observed in normal mice.

Since the level of functional expression diminished over time in CF mice the ability to re-dose and evade blocking host immune responses was addressed. Multiple doses of a LV vector over a short time frame were feasible but did not significantly increase expression compared to a single dose. Circulating antibodies to both the vector envelope and the transgene protein were detected after repeat dosing conducting over a longer time frame. The timing of additional LV vector doses may be crucial for effective boosting of waning gene expression.

The addition of a transient immunosuppressive treatment did not significantly enhance the level of gene expression produced by a single dose, but did reduce circulating antibodies to both the delivered foreign transgene and to the pseudotyped envelope protein.

The demonstration of longevity of gene expression, the functional correction of the CFTR defect, the substantial increase in CF animal survival, the ability to re-dose and the use of immune-suppression to reduce antibody production provides strong and specific support for the continued investigation of LV CFTR gene transfer towards a clinical gene therapy treatment 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 Patricia Cmielewski 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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Signed:

Patricia Cmielewski

Date:

xxx

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Dr David Parsons for his unfailing optimism, enthusiasm, encouragement and support throughout my PhD. If I could have 1/10 of his energy and positivity I would be so thankful.

To my co-supervisor Associate Prof Don Anson (retired) for help with all molecular biological techniques and constructive instruction throughout this long process. To my co-supervisor Dr Simon Barry for stepping in at the final year, providing the curly questions during seminar presentations and with the only one stipulation of "just submit".

Special thanks to all the various staff from different departments outside and at the WCH that without their support this work would not have been possible. All in the Respiratory and Sleep Medicine; (the former) Gene Technology Unit; University of Adelaide Department of Paediatrics; Histopathology; Women's and Children's Health Research Institute; Adelaide Microscopy and the Animal Care Facility (and all the mice who graciously contributed).

To the Adelaide Cystic Fibrosis Gene Therapy Group: Martin (I'm not tech support) Donnelley, Nigel (Do you know what I mean?) Farrow, Corrine (I feel like I'm Penny / Nespresso coffee) Reynolds. Also, many thanks to the former members: Karlea Kremer, Stanley Tan, Sue Lim, Sin Lay Kang, Julie Bielicki, Richard Bright and Darren Miller for their help and input crucial to this thesis.

To all my friends who replied "It's about bl**dy time you did your PhD" or "Haven't you finished that yet?" The answer is finally YES!

Finally to my family for all their support and the ultimate in distraction therapy “it was the best of times, it was the worst of times” with health, both mental and physical, happiness and a good sense of humour being the most important things in life.

Thank-you, one and all.

CHAPTER 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 CYSTIC FIBROSIS – EARLY HISTORY

The term cystic fibrosis (CF) was first coined in the 1930s by the pathologist Dr. D. Andersen[1]. Andersen described in detail the neonatal intestinal obstruction, gastrointestinal and respiratory complications and most importantly the characteristic pancreatic histology in affected infants as "fibrocystic disease of the pancreas". The name CF refers to the formation of cysts and scarring (fibrosis) within the pancreas. Previous to this seminal paper physicians did not identify CF as a separate clinical condition and it was vastly under diagnosed. CF (also known as mucoviscidosis) is a multi-organ disease in which the lung, liver, gastrointestinal tract, pancreas, sweat glands and reproductive organs are affected[2].

The majority of our knowledge about possible CF cases reported in early history comes from Busch[3, 4] and reviewed by Quinton[5]. In medieval times (1595) Prof Pieter Pauw[3] in Leiden of the Netherlands, performed possibly the first autopsy on an "bewitched" infant describing the pancreatic pathology attributed to CF. The excessive salt characteristic of CF has references in late medieval times from German and Swiss children's rhymes and games[3] in which the "salty when kissed" child is said to be "hexed" and an early death is predicted. In early 1600, Alonso y de los Ruyzes de Fonteca, a professor of medicine in Spain, described the salty forehead of a child[5]; indeed the high salt concentration in sweat continues to be a useful clinical diagnostic tool for early diagnosis of infants with CF.

The term meconium ileus (MI), an intestinal obstruction in a proportion of newborns with CF, was first reported in 1838 by Viennese pathologist K von Rokitansky[5], but it has been attributed to a report in 1905 by K. Landsteiner[6, 7]as the first with histological pancreatic changes[8] associated with CF.

In the 1930-40s the majority of the symptoms associated with CF were described, including the term mucoviscidosis (thickened mucus), a term that is still used in non-English speaking nations coined by Farber[9, 10]. The presence of steatorrhea[11] (fatty stools) at birth in CF individuals was distinct from infants with celiac disease[12] and the predominant respiratory symptoms[11, 13] associated with CF were also defined.

In the 1950s P. di Sant' Agnese demonstrated the increased salt concentration in the sweat of CF patients[14] and that "CF is in reality a generalized disease affecting many or perhaps all exocrine glands" and its probable genetic origin[15] with a significant proportion of affected heterozygotes (Het, - / +) in the adult population.

1.2 INCIDENCE

CF is one of the most common lethal autosomal recessive genetic disorders in Caucasian populations of European descent, with an incidence of 1 in 3,000 live births in the Australian population[16]. The incidence of CF varies considerably among different countries, regions within countries and ethnic background, ranging from 1:1,400 in Ireland to an incidence of 1:4,500 estimated in France and Italy[17]. This equates to 1:25 ratio of Caucasians that are heterozygous (Het) for the CF gene.

It has been hypothesized that the continued high frequency of the fatal recessive homozygote of the genetic disorder of CF may be due to a selective advantage of being a Het over a normal homozygous individual. In the literature comparisons have been made of the protective effect of Het individuals with the disease sickle-cell anaemia over the high mortality of malaria in endemic regions[18]. Possible explanations for a Het advantage in CF are an increase in fertility of Hets, random genetic drift or balanced polymorphism where a proportion of the defective gene in Hets provides protection against an otherwise virulent disease.

Using statistical modelling of the condition, enhanced fertility is unlikely to account for the observed Caucasian CF gene frequency[19]. Many infectious diseases, especially pandemics that have devastated populations throughout history, have been suggested as a possible reason for an evolutionary advantage for Hets. Diseases such as malaria[20], influenza[21], typhus[22, 23], the bubonic plague[24] and tuberculosis[25] have been postulated as linked with a Het advantage but not scientifically confirmed.

The most likely infectious disease that may explain an advantage of Hets over normal is cholera[26, 27]. Cholera is an infection caused by the bacteria *Vibrio cholera* affecting the small intestine resulting in severe diarrhoea and vomiting leading to dehydration and electrolyte imbalances. It has been suggested that as Hets for CF have 50% of normal chloride ion transport due to the mutant gene, this reduction in apical chloride conductance lessens the immense loss of salt associated with cholera[28, 29]. This hypothesis has found support using transgenic CF mice where chloride secretion was positively correlated with the number of CF alleles in response to the cholera endotoxin[30].

Due to the prevalence of CF throughout the ages, the notion that famous people may have suffered from the disease has also been investigated since documentation of their life and death including autopsy reports are in the public domain. The Polish composer Frederic Chopin seems to have suffered from CF disease[31-33] and not from tuberculosis[34] or α_1-antitrypsin deficiency[35, 36]. Chopin's persistent cough, emaciated appearance, frailty, recurrent chest infections and "barrel-chested" appearance[31], possible infertility, as well as the early death of his sister Emily at 14, are all trademarks associated with CF. George Orwell the author of "1984" may also have had CF but tuberculosis may have been the cause of death[37].

1.3 DISCOVERY OF THE CF GENE

In the mid-1980s the use of extensive linkage analysis of CF families provided evidence for a single CF locus that was located on the long arm (q) of the human chromosome 7[38-41] (Figure 1-1). With three landmark papers published in 1989 by lead authors Kerem[42], Riordan[43] and Rommens[44], the positive identification and cloning of the gene responsible for CF and the definition of its gene protein product was elucidated. The predicted CF protein product was named after other membrane-associated proteins as the cystic fibrosis transmembrane conductance regulator (CFTR) gene[43].

Figure 1-1 Location of the CFTR Gene
The CFTR gene is found in region q31.2 on the long arm of human chromosome 7. Adapted from NCBI Gene Library.

The CFTR membrane-bound glycoprotein is composed of 1480 amino acids and has a molecular weight of 168,173 Da. The normal CFTR gene is composed of 250,000 base pairs and contains 27 exons.

CFTR is classified as an adenosine triphosphate (ATP)-binding cassette (ABC) transporter and transports chloride (Cl^-) ions across the membranes of cells in the lungs, liver, pancreas, intestinal tract, reproductive tract and skin.

1.4 THE CFTR PROTEIN

1.4.1 CFTR Structure

The CFTR protein is primarily located on the apical surface of epithelial cell membranes, acting as a Cl^- ion channel where the transport of salt and fluid are regulated through and across the cell's epithelium (i.e. transepithelial transport). The structure of CFTR was based on that proposed by other ABC transporter proteins that are transmembrane proteins which utilize ATP hydrolysis to carry out various biological processes such as transport of substrates[43].

The CFTR protein is composed of two motifs, containing a membrane-spanning domain (MSD) and a nucleotide-binding domain (NBD) in the cytoplasm. In CFTR the MSD comprises of usually 6 transmembrane α-helix sequences connected to a NBD that contain sequences that can interact with ATP. These two MSD-NBD motifs are connected by a regulatory (R) domain, unique to the CFTR protein, at the first NBD site and second MSD site. The R domain contains multiple phosphorylation sites and amino acids[45]. The carboxyl or C-terminal end of the CFTR protein is anchored to the cell cytoskeleton by a PDZ-interacting domain[46] that holds together the signalling complexes (Figure 1-2).

Figure 1-2 CFTR Protein Structure
CFTR protein structure comprising two Membrane-Spanning Domains (MSD), two Nucleotide-Binding Domains (NBD), Regulatory (R) domain attached to PDZ domain at the C-terminus (Modified from [47]).

1.4.2 CFTR Function

CFTR is the only member of the ABC superfamily that acts as a Cl^- channel that is open and closed by ATP hydrolysis. The structure of the CFTR protein defines the MSD's as the formation of a low conductance Cl^- channel "pore", the NBD's hydrolyze ATP to regulate channel gating (opening and closing) following initial phosphorylation of the R domain (distinctive to CFTR) that controls the Cl^- channel activity[48]. The Cl^- channel activity increases greatly when phosphoryl groups are transferred from ATP to side chains of the protein, catalyzed by cyclic adenosine monophosphate (cAMP) - dependent protein kinase.

After the initial phosphorylation of the R domain, the Cl^- channel is “closed” but requires activation by ATP. After activation of ATP to either one of both of the NBD sites, the channel can remain closed, but can also open spontaneously. Therefore, there are at least two ATP bound states, open and closed. This is the model of CFTR gating and activation by ATP[47, 48]. The initial phosphorylation of the R-domain is also reversible. The regulation of the CFTR Cl^- channel activity is by controlling the balance of intracellular protein kinase and phosphatase. The major kinase responsible for Cl^- activity is protein kinase A (PKA), but other kinases such as PKC and cyclic guanosine monophosphate (cGMP) can also play a role[47, 49, 50].

CFTR also regulates other membrane proteins, such as the down-regulation of the amiloride sensitive epithelial sodium channel (ENaC)[51]; up-regulation of an alternate (outwardly rectifying) Cl^- channel (ORCC)[52, 53] and the (inwardly rectifying) renal outer medullary potassium channel (ROMK)[54] (Figure 1-3).

Following cloning of the α,β,γ-ENaC subunits, the ability of CFTR to influence other ion transport proteins was identified[55]. Of the 3 subunits, α-ENaC is required for a functional channel. Regulation of ENaC can occur through a wide variety of hormonal and non-hormonal mechanisms and co-transfection with CFTR resulted in reduced sodium transport.

One explanation for the CFTR-ENaC interaction is that the inhibition of the ENaC activity is directly related to the rising magnitude and direction of intracellular Cl^- ions controlled by CFTR. Other possibilities are indirect methods that CFTR affects ENaC through intermediary proteins. The presence of CFTR may reverse PKA activation, which would lead to inhibition of ENaC. Alternatively, CFTR could utilize other binding receptors to influence the activity of ENaC, such as the PDZ-binding domain of the C-terminal end of the CFTR protein, as well as adaptor proteins that bind to and regulate both CFTR and ENaC[56].

CFTR and ORCC are both activated by cAMP and depolarizing voltages of transepithelial potentials; and that phosphorylation of CFTR is the dependent role in activation of the ORCC[52]. Guanosine triphosphate (GTP)-binding proteins may also be involved as the NBDs of CFTR have been shown to hydrolyze not only ATP but also GTP.

In summary, CFTR regulates water and ion content of luminal secretions. The direction of ion movement through the ion channels is governed by chemical and electrical gradients. Ions flow passively through the channel down a chemical gradient which are potently controlled by the gating mechanisms.

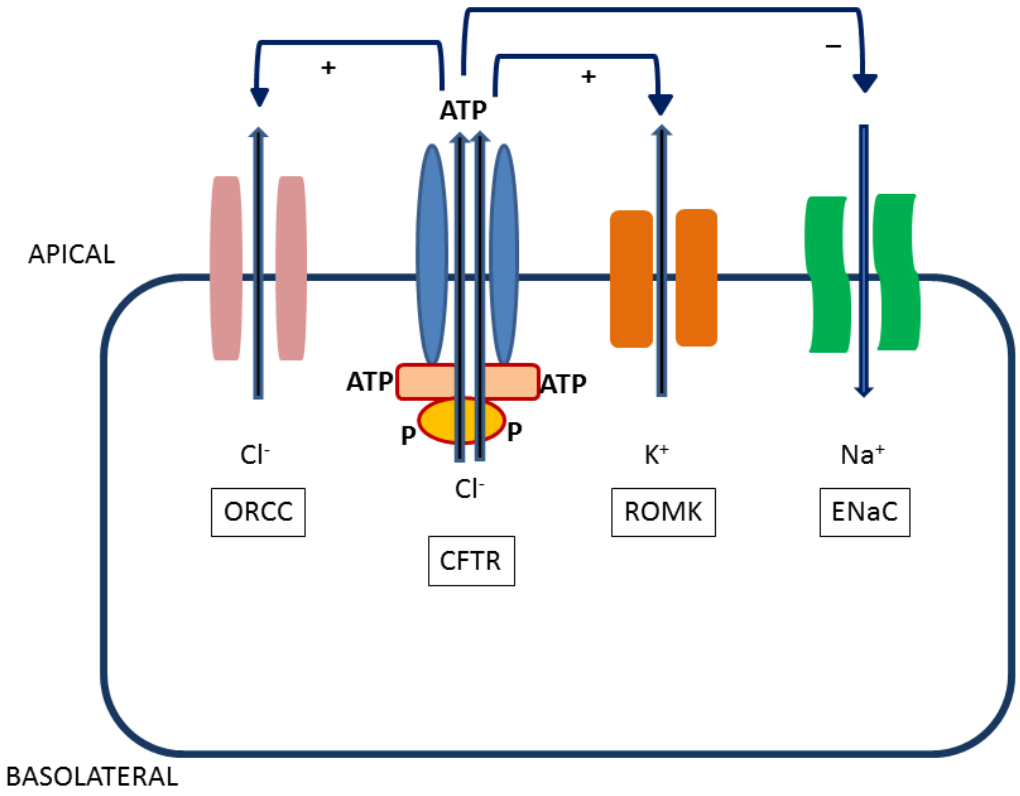

Figure 1-3 Relationship between CFTR and Other Ion Channels
CFTR regulated chloride (Cl^-) transport after phosphorylation (P) and activation by intracellular ATP. The outwardly rectifying Cl^- channel (ORCC) and the renal outer medullary potassium (K^+) channel (ROMK) are activated by the release of ATP from CFTR, while ATP negatively regulates the epithelial sodium (Na^+) channel (ENaC) (Adapted from[54, 57]).

1.4.3 CFTR Mutations

CF is caused by mutations in the CF gene that encodes the CFTR protein, which leads to defective ion transport. Currently there are more than 1900 mutations listed in the CF Mutation database[58] known to be responsible for CF disease. CFTR mutations have been found in the coding sequence, messenger ribonucleic acid (mRNA) splice signals and other regions. The vast array of mutations of genes and protein defects can be categorized into classes according to the mechanisms known to cause the disease state (Figure 1-4). There are 5 classes of mutations for CF, with a 6th Class also being proposed[59]. Class I mutations being the most severe form of the disease ranging to milder phenotypes as a result of Class IV and V mutations.

Class I are categorized as defective or absence of protein production often due to premature stop codon mutations; Class II mutations, the most common type, results in defective protein maturation due to mis-folding and premature degradation; Class III mutations cause defective ion channel activity due to disordered regulation such as impaired channel opening; Class IV exhibit only partial CFTR ion channel function due to reduced conduction; Class V results in reduced mRNA and/or protein levels and therefore reduced transcripts due to a promoter or abnormal splicing; and Class VI mutation results in defective CFTR stability at the cell surface due to accelerated turnover from the cell surface[59-61].

1.4.3.1 Class I Mutations

Mutations in this class are characterized as defective protein synthesis resulting in the complete absence or abnormal variations of the CFTR protein. Abnormalities include nonsense mutations, truncations due to a premature stop codon in the mRNA or frame-shift or mis-splicing mutations in which the resultant mRNA is unstable and is rapidly degraded. The overall effect is that no functional CFTR is

presented to the apical surface of epithelial cells, resulting in the most severe phenotype of CF disease. Approximately 2-5% of CF mutations are Class I mutations, for example the nonsense mutations that are designated with an X. The nonsense mutation G542X where the glycine in position 542 is replaced by a stop codon, and W1218X are both highly prevalent in the Ashkenazi Jewish populations[62].

1.4.3.2 Class II Mutations

Class II mutations result in either the premature degradation or defective maturation of the CFTR protein. The in-frame deletion of phenylalanine at position 508 (the ΔF508 mutation) the most common type of CF mutation associated with more than 70% of defective CFTR alleles, results in mis-folding and defective trafficking of CFTR to the apical cell surface[63]. In the case of ΔF508 mutations, the defective protein may still retain some residual chloride channel activity and may lead to a milder affected phenotype.

1.4.3.3 Class III Mutations

Defective regulation of the CFTR function associated with Class III mutations are the next most common mutation type. Mutations prevent ATP binding and the hydrolysis of the NBDs resulting in impaired gating of the chloride channel activity. The missense mutation G551D, where glycine at position 551 is replaced with aspartic acid, may also affect the CFTR regulation of other channels such as the ORCC and the ROMK.

1.4.3.4 Class IV Mutations

Class IV mutations are associated with a milder CF phenotype due to reduced conductance, that is there are normal amounts of CFTR with some residual activity at the apical membrane[64]. The missense

mutation R117H, where arginine is replaced with histidine at residue 117, affects the MSD responsible for the pore formation of the chloride channel[65], which results in some CFTR function meaning these CF patients are usually pancreatic sufficient (PS).

1.4.3.5 Class V Mutations

Mutations associated with reduced amounts of CFTR due to abnormal promoter, splicing, or ineffective trafficking are designated as Class V. These mutations include A455E, common in CF individuals from the Netherlands, are characterised with preserved pancreatic function and residual Cl^- secretion across the apical membrane[66] resulting in milder lung disease.

1.4.3.6 Class VI Mutations

The newly categorized Class VI mutations result in unstable CFTR due to truncation of the C-terminus on the CFTR protein[59] leading to accelerated turnover of mature CFTR from the cell surface. These are usually nonsense or frameshift mutations, such as Q1412X, which results in the shortest truncation of the C-terminus, and produces a severe CF phenotype in both pancreatic insufficiency (PI) and lung function.

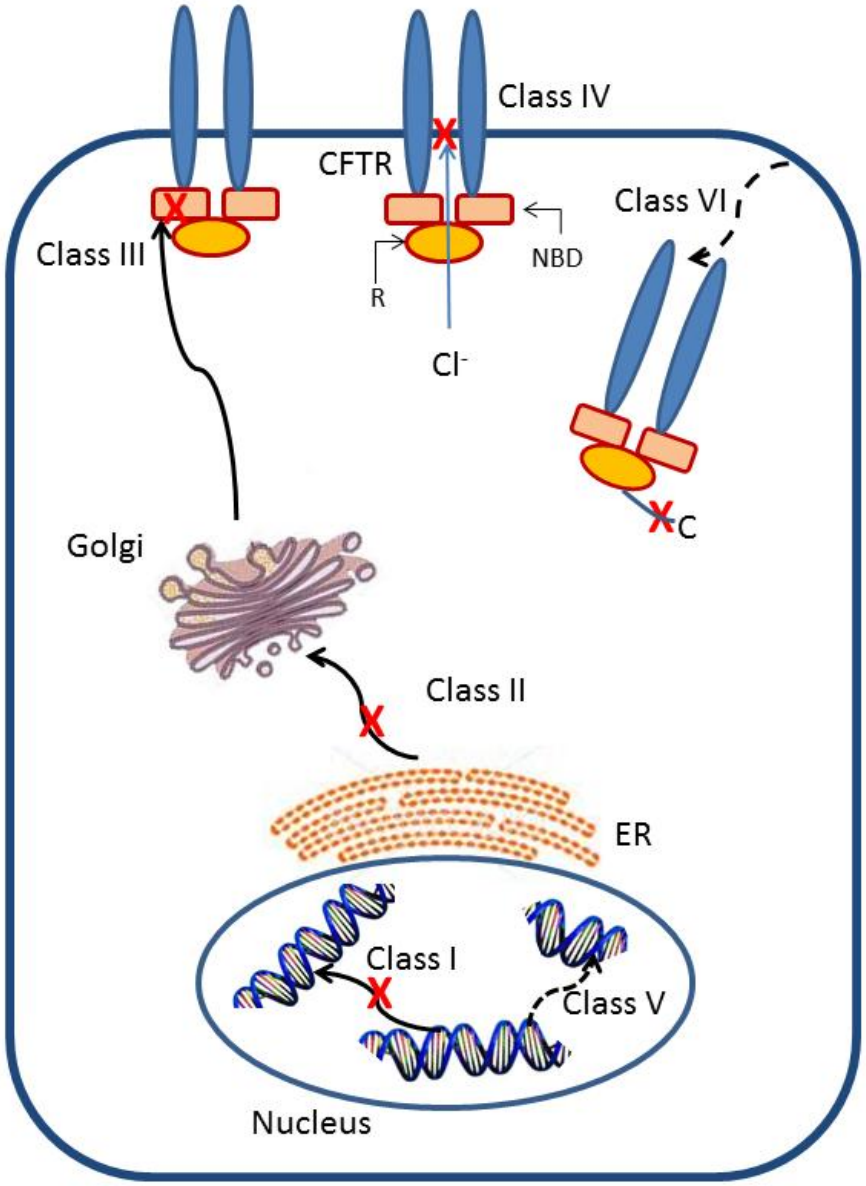

Figure 1-4 Classes of Mutations of CFTR
Classes of mutations of the CFTR gene include:
Class I: absence of synthesis;
Class II: defective protein maturation and premature degradation;
Class III: disordered regulation, due to reduced ATP binding;
Class IV: reduced conductance or channel gating;
Class V: reduced mRNA due to a promoter or splicing abnormality; and
Class VI: accelerated turnover from the cell surface.
(Modified from [60, 61]).

1.4.4 Diagnostic Genetic Testing for CF

All Australian babies are screened for CF at birth by heel prick blood sample performed between 2-3 days after birth. An elevated level of immunoreactive trypsinogen (IRT)[67] in the serum from the neonatal screening test provides evidence of pancreatic dysfunction and is a predictor of CF. However due to low sensitivity and suboptimal cut off values another repeat diagnostic test is usually performed on the same sample[68]. A second elevated IRT increases the sensitivity of positive CF diagnosis to 95%[69] or an initial high IRT with a following positive sweat test increases sensitivity to 85%[70].

Most newborn screening programs now use a combination of IRT and deoxyribonucleic acid (DNA) mutational analysis (usually for the most common mutation ΔF508) using a single dried blood sample[71]. Babies with two copies of the mutation are positive for CF, one copy present are designated either a CF or a carrier and therefore directed for a sweat test, and those with no mutations are designated negative for CF. The detection rate for CF using the IRT/DNA screening program is 95%[68].

The inclusion of multi-mutational panels that can screen a range of CFTR mutations and variants, such as the polymerase chain reaction (PCR) / oligonucleotide ligation assay, to detect over 30 mutations, (including 24 of the most common CF mutations worldwide[72, 73]), greatly enhances the sensitivity and predictability of early CF diagnosis.

The benefits of early CF diagnosis are improved health outcomes relating to nutrition and growth[74], early detection of pancreatic dysfunction and subsequent treatment with pancreatic enzyme supplements[75] and early prevention of respiratory infections[76] (Table 1-1). These benefits in health and respiratory symptoms have the potential to slow disease progression and improve quality of life for CF individuals.

Table 1-1 Impact of New Born Screening between Australia and USA.
Significant differences between screening programs resulted in higher nutritional status in Australian CF populations. Body mass index (BMI), Forced Expiratory Volume in 1 sec (FEV_1), (Adapted from[77]).

1.5 CLINICAL SYMPTOMS OF CF

Cystic fibrosis results from greatly reduced or absent Cl^- conductance across epithelial cells due to mutations in the CFTR gene. This defect in the ion transport across the majority of tissues gives rise to the traits and wide variety of symptoms associated with CF. As stated earlier CF is a multi-organ disease affecting the sweat glands, pancreas, liver, gastrointestinal tract, reproductive organs with the majority of morbidity and mortality due to lung disease.

1.5.1 Sweat Glands

The absence of functioning CFTR leads to the high concentration of sodium chloride (NaCl) in the sweat of CF patients[78]. The sweat glands of CF individuals have no secondary morphological anomalies, are unlikely to become obstructed, but have noticeable abnormalities in NaCl homeostasis. In normal sweat glands, NaCl ions are rapidly reabsorbed from the luminal side of the ducts by the activity of the apical Na^+ and Cl^- channels and the basolateral Na/K pump driven by ATP. This creates an electrochemical gradient for Na^+ transport and with Cl^- passively following through the CFTR channels (Figure 1-5).

In the CF sweat glands where CFTR is not functioning, Cl^- is unable to be reabsorbed as this is the only effective pathway for chloride reabsorption[61]. Sodium uptake is also reduced due to the lack of associated chloride and the net result is an increase in NaCl levels in the sweat on the surface of the skin.

Due to the impermeability of chloride across the apical membrane of the sweat duct, the potential gradient across the lumen is more negatively charged[79]. This ductal transepithelial potential difference is 2-3 times more negative than the normal sweat values[78].

Figure 1-5 Abnormalities in Chloride Transport in the Sweat Gland
NaCl transport across the sweat glands in normal and CF. When CFTR is defective Cl^- cannot be reabsorbed across the lumen and results in a higher salt concentration in the sweat on the skin surface (- - - = greater negative charge in the ductal lumen) (Modified from[2]).

1.5.1.1 Diagnostic Sweat Test

The heat wave of the late 1940s in the USA led di Sant' Agnese to demonstrate that the severe dehydration was due to the high concentration of salt in the sweat of CF patients[14]. The sweat chloride test relies on an electrical and pharmacological stimulation of the skin by pilocarpine iontophoresis and the resultant sweat is then analysed for Cl^- concentration[80]. A concentration of chloride >60mM/L reliably predicts CF in both infants and older individuals[2]. A sweat Cl^- concentration of 30-59 mM/L and 40-59 mM/L for infants <6 months and >6 months of age respectively, implies the diagnosis of CF is likely. The sweat test still remains the gold standard for diagnosing CF and provides an accurate diagnosis of CF in over 98% of cases[79, 81].

1.5.2 Gastrointestinal Tract

1.5.2.1 Pancreas

Cystic fibrosis refers to the formation of cysts and scarring (fibrosis) within the pancreas[1]. The CF phenotype is widely variable; however the severity of disease can be correlated with the function of the pancreas (Figure 1-6). The level of pancreatic function is known to be associated with classes of mutation and hence severity of disease[82]. Mutations in the CFTR gene are classified as either severe or mild based on their particular pancreatic phenotype[83]. Those individuals with close to normal levels of pancreatic function (PS), have a milder form of the disease, whereas those patients that are diagnosed as insufficient pancreatic function (PI), have very low levels <1% of normal, and tend to develop a more severe form of CF symptoms[84] that correlates to mutation genotype. CF patients that inherit two severe mutant alleles will have PI whereas patients with one or two milder mutations are more likely to be PS[83] however some PS patients can become PI over time[85]. Around 85 - 90% of CF mutations produce PI[83] and patients are treated with a high caloric diet and pancreatic enzyme supplements[86].

1.5.2.1.1 Exocrine function

The main function of the pancreas is to secrete digestive enzymes (exocrine) and hormones (endocrine). The cells of the pancreatic duct secrete a fluid rich in sodium bicarbonate ($NaHCO_3$) that dilutes digestive enzymes and mucins from the ducts into the duodenum. The CFTR channel is located on the apical surface in the proximal duct of the pancreas[87] and the HCO_3/Cl exchanger is also located on the luminal surface[88]. In CF the pancreatic secretions are more concentrated and less alkaline[89] due to the defective Cl^- permeability and the reduced exchange of HCO_3^-. Pancreatic enzymes are released from the acinar cells but do not enter the duodenum due to the lack of fluid, acids are not inactivated and mucus plugging of the pancreatic ducts also occurs[87]. The consequence of the compromised CF

pancreas incites an inflammatory response as wells as auto-digestion of the gland itself, leading to eventually destruction of the exocrine tissue and pancreatitis.

Destruction and loss of pancreatic function in CF occurs at birth or early infancy and others gradually lose function over time. The progression of the disease occurs in distinct stages from eosinophilic infiltration in the lumen, widening of the ducts with lobular fibrosis and microcysts due to obstruction, acinar and ductal atrophy, scarring surrounding the islets, with eventual complete loss of acinar and duct structures that results in insulin and glucagon insufficiency[5].

1.5.2.1.1.1 Fat malabsorption diagnostic test

The combination of pancreatic and intestinal abnormalities in CF leads to malnutrition mainly due to the malabsorption of fats. Patients that are PI have measureable steatorrhea (fatty stools) that occurs when only 1-2% of pancreatic function remains[84]. CF patients that are PS, still have abnormal pancreatic function but have enough enzyme secretion to avoid steatorrhea. The gold standard indirect method for pancreatic function has been the three-day faecal fat collection. A high fat diet is ingested for 72 hours and faeces collected and analysed for fat excretion[90]. Results are expressed as co-efficient of fat absorption, grams of fat excreted per day.

More recent developments in in-direct measurements for pancreatic function have been the non-invasive mixed triglyceride breath test. The use of a non-radioactive, naturally occurring labelled carbon isotope (^{13}C) in a long chain fatty acid (triolein) and presence of ^{13}C in the exhaled carbon dioxide (CO_2) allows for detection of lipid digestion[91]. Those patients who are PI have virtually no ^{13}C detected in exhaled breath over 6 hours and the ^{13}C breath test can also be useful in monitoring the efficacy of pancreatic enzyme replacement therapy[92].

Another method for pancreatic function is the fecal elastase-1 (FE-1) measured by ELISA in a spot stool sample[93]. As the elastase-1 enzyme is not degraded during intestinal transit it correlates well with direct more invasive pancreatic tests such as duodenal aspirates from endoscopy[94]. PS patients have a FE-1 of >200μg/g and those CF patients with FE-1 <100 μg/g is a positive predictor of PI[93].

1.5.2.1.2 Endocrine function

Progressive pancreatic disease and scarring affect the pancreatic islets and CF-related diabetes (CFRD) is frequent in older CF patients[95]. The eventual loss of the islets cells of Langerhans of the pancreas, which is responsible for insulin hormone secretion and the regulation of blood glucose; results in a unique form of diabetes – CFRD – that consists of both type 1 and type 2 diabetes mellitus[96].

1.5.2.2 Intestinal Tract

Meconium ileus (MI), obstruction of the intestine of CF patients occurs in approximately 10-15% of infants[97]. In neonates MI presents with abdominal distension, bilious vomiting and failure to pass meconium. In complicated cases of MI neonates have a palpable abdominal mass and may also present with volvulus (twisting of the bowel), atresia (malformation), necrosis, perforation, pseudocyst formation and giant cystic meconium peritonitis. Less severe forms of MI can be treated by conservative enema therapies, however for complicated MI, surgical intervention is usually required for survival[98].

The CFTR defect in the intestinal tissues results in abnormal ion transport in the CF colon[99]. Defects in the intestinal crypt chloride and fluid secretions produces increased viscous secretions in the CF intestine. The consequence of pancreatic dysfunction and abnormal intestinal secretions in 15% of CF adolescent and adult patients[100] is distal intestinal obstruction syndrome (DIOS). DIOS, the adult

equivalent of MI in neonates, is characterized by abnormally viscous mucus faecal matter in the terminal ileum and ascending colon[101]. Intestinal dysmotility may also be a factor.

1.5.3 Liver

The improvement in life expectancy of CF patients has led to an increase in the prevalence of liver disease in the CF population[102]. In the liver CFTR is located on the apical membrane of the cholangiocytes (bile duct cells) and the epithelial cells of the gall bladder but not expressed in the hepatocytes[103]. CFTR is responsible for the regulation of secretion, acid concentration and flow of bile through the regulation of Cl^-, $HCO3^-$, Na^+ and water transport[104]. The absence or dysfunction of CFTR in CF can lead to varying degrees of liver abnormalities. Defective ion transport results in thickened epithelium, thick abnormal viscosity of mucus secretions in the bile ducts which can cause obstruction and eventual injury and cell death due to inflammatory processes[105]. Collagen depositions around the bile ducts, fatty infiltrations and increased fibrosis in the portal tracts are also a consequence of bile duct plugging and necrosis, which may progress to the development of multi-lobular cirrhosis and portal hypertension in severe cases.

Some of the main factors to be associated with severe CF liver disease (CFLD) include PI, a severe CF genotype, predominance in males, an early diagnosis of CF[106] as well as other environmental and modifying genetic factors[107]. CFLD usually develops before puberty, is often asymptomatic and progresses slowly but in 10% of cases can progress to severe liver disease with portal hypertension established in adolescence.

1.5.4 Reproductive Tract

The reproductive tissue most affected by reduced CFTR is the epididymis and the vas deferens[108]. Congenital bilateral absence of the vas deferens (CBAVD) or atrophy is responsible for infertility in males with CF[109, 110]. Impaired CFTR function has been implicated in obstructive azoospermia (no live sperm in semen), and severe oligozoospermia (low sperm count in semen) in men[111] as well as hypofertility in CF women[112]. The reduced fertility in CF females has been related to delayed puberty and amenorrhoea as a consequence of malnutrition as well as evidence of viscous mucus plugs in the cervix impeding sperm motility[113]. CFTR mutations are also associated with congenital absence of the uterus and vagina (CAUV)[114].

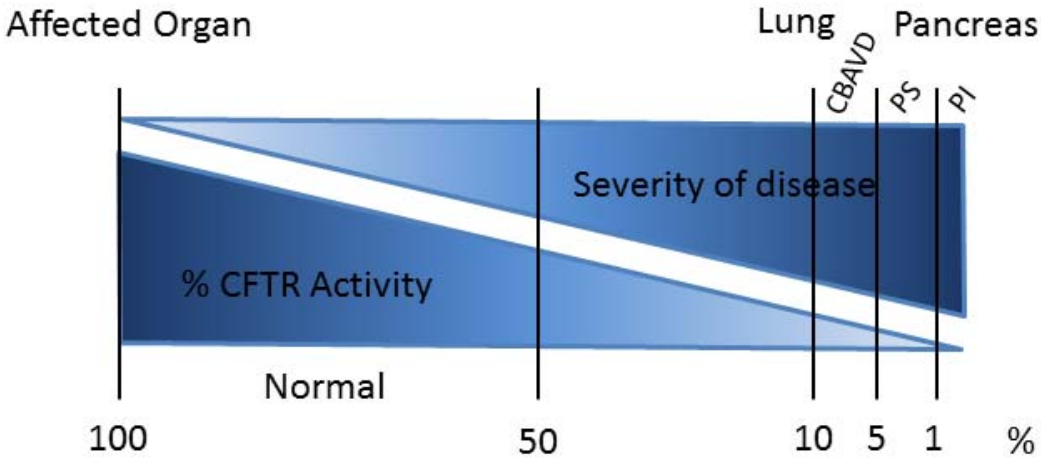

Figure 1-6 Severity of CF Disease Related to CFTR Activity
Decreased levels of CFTR are associated with more severe CF phenotypes of congenital bilateral absence of the vas deferens (CBAVD), with pancreatic sufficiency (PS) and pancreatic insufficiency (PI) (Modified from [81, 95]).

1.5.5 Respiratory System

By far the most significant effect on morbidity and mortality due to CF is attributed to the characteristics of lung disease. However unlike other organs such as the intestine and pancreas, the lung at birth is essentially functionally normal[115]. The pathogenesis of CF lung disease occurs in progressive stages, a cascade of events, leading to lung transplantation or eventual death. The cascade of events can began weeks, months, years or decades after birth with a vicious cycle of bacterial infection, exhibiting inflammation which cannot be resolved and leads to eventual bacterial colonization and destruction of lung airways (Figure 1-7). The defect in ion transport due to absent or reduced CFTR activity results in increased mucus and dehydration of the airways allowing bacterial colonization of the airways due to ineffective clearing of bacterial pathogens. The persistent bacterial infections and inflammation of the airways cause injury and structural changes in the lung such as bronchiectasis[116] (irreversible dilation and scarring of the bronchial walls), atelectasis (alveoli collapse), cyst formation, mucoid impaction and fibrosis each leading to a decline in pulmonary function.

CF lung disease is associated with the epithelial cells that line the conductive airways, and first presents as an upper lobe disease[117] with eventual lower lobe involvement as disease progresses. Infants with CF have more dilated airways with thickened walls compared to normal infants[116] with structural changes in the smaller and larger airways. Airway dilation increases with age and leads to bronchiectasis.

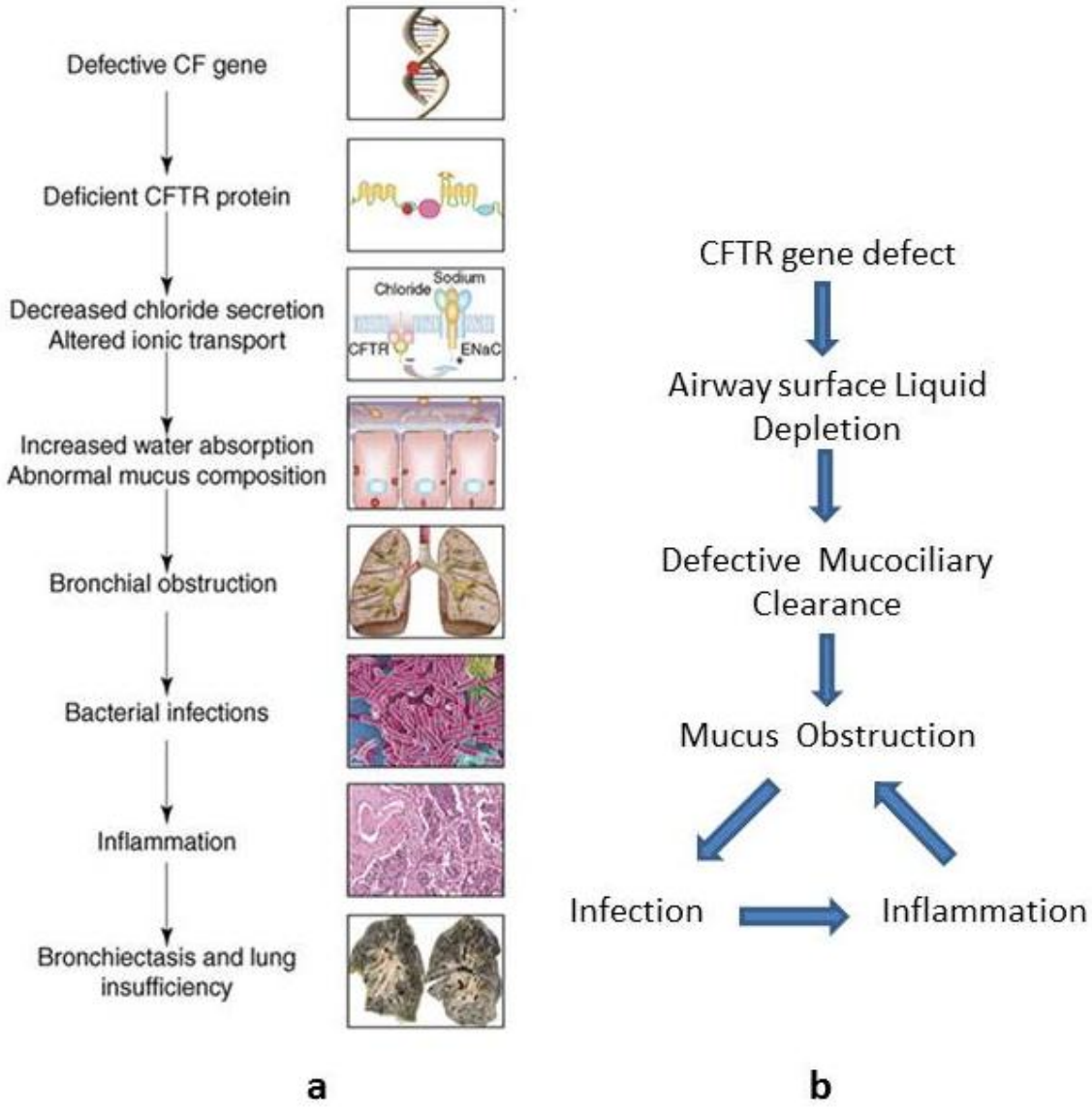

Figure 1-7 Cascade of CF Lung Disease

Defect in the a) CF gene results in defective CFTR protein and abnormal chloride transport and dehydration of the airways, abnormal mucus secretion and obstruction of airways. Colonization of the lung with bacterial infections, due to impaired clearance, leads to chronic inflammation, structural changes and lung destruction. The cascade of CF lung disease b) from defective mucociliary clearance results in a vicious cycle of mucus obstruction, chronic infection and inflammation (Adapted from [60, 118]).

1.6 CF AIRWAY DISEASE

1.6.1 Airway Anatomy

Pulmonary epithelial cells can be classified as belonging to three broad regions: proximal airways, distal airways and alveolar epithelia[119]. The proximal airways are the nasal and cartilaginous lung conducting pseudostratified epithelia that are comprised of predominantly ciliated cells, with non-ciliated cells, secretory or goblet cells, basal cells and submucosal glands to a lesser extent. The distal lung airways are non-cartilaginous consisting of ciliated and non-ciliated bronchiolar cells or Clara cells. Alveolar epithelia consist of Type I (simple squamous alveolar cells) and Type II (glandular and cuboidal cells) pneumocytes, comprising a very large surface area of approximately 70m^2 for gas exchange and secretion of surfactants[120]. A two compartment layer, the airway surface liquid (ASL) layer, overlies the epithelia as a barrier to the environment. The ASL comprises two layers: first, the periciliary layer (PCL), a low viscosity liquid layer that covers the surface of the airway epithelia that allows for effective movement of cilia, hydration of the airway surface and in conjunction with a net like structure, the glycocalyx[121], provides a protective barrier from deleterious particles. Secondly, overlying the PCL layer is a film of mucus responsible for particle retention and effective mucociliary clearance (MCC) of particles out of the airway (Figure 1-8).

The main function of the airway epithelia is to defend the airway against foreign materials, remove unwanted debris from the airway, support homeostasis of electrolyte transport and replenish liquid lost during effective gas exchange. The lungs produce approximately 100 ml of mucus daily, mostly secreted by the submucosal glands, with 10% removed by coughing and the other 90% is reabsorbed by surface epithelia[122]. The physical action of moving mucus out of the lung requires co-ordinated beating of the airway cilia, known as mucociliary transport (MCT). The synchronised co-ordination of

cilial beat is not only defined by the full length of the cilia, with the height of the PCL at 6-7 μm[123], but also the hydration of PCL and the overlying viscosity of the thin mucus gel layer[124].

This fine balance of the ASL and MCC is regulated by the transport of ions across the airway via the CFTR protein. The defective CFTR function in CF airways results in abnormal epithelial cell ion transport, which is a main diagnostic tool of CF disease.

Figure 1-8 Cell Type Composition of the Airway Surface Epithelium
Airway epithelium is composed of ciliated (C), non-ciliated (NC), goblet (G), basal (B) cells and submucosal glands (SMG). Overlying the epithelial layer is the airway surface liquid (ASL) layer, comprising the periciliary layer (PCL), the glycocalyx and the mucus layer (Modified from[125]).

1.6.2 Defective Ion Transport

Cystic fibrosis is a disease of defective epithelial ion transport. The role of CFTR is to regulate Cl^- and Na+ ion transport and as a consequence hydration of the airways[126]. Sodium ions enter the cell

passively from the apical surface through the amiloride-sensitive ENaC. Sodium is then actively transported out across the basal membrane via the Na^+/K^+ pump and K^+ can be either recycled or secreted into the interstitial space[127]. Chloride ions can be secreted by the apical Cl^- channels (CFTR and calcium) and the basolateral $N^+/K^+/2Cl^-$ co-transporter from which the driving forces for Cl^- secretion is generated and water is either secreted or absorbed to maintain homeostasis, hydrated PCL and effective MCC across the airway surface (Figure 1-9).

For CF disease, not only is Cl^- transport inhibited due to absent or defective CFTR, but Na^+ absorption is also increased. The net result is the dehydration of the ASL, with increased passive removal of water (H_2O) from the PCL and an increase in viscosity of the mucus layer.

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Figure 1-9 Model of Ion Transport across Epithelial Cells in Normal and CF Airways.
Loss of CFTR function results in reduced Cl^- transport towards the lumen, luminal Na^+ absorption is increased and passive absorption of water (H_2O) from the lumen is increased, resulting in dehydration of the airway surface (Modified from[128]).

1.6.2.1 Transepithelial Potential Difference

The CFTR electrolyte transport can be measured electrically as the transepithelial potential difference (TPD). As a consequence of the Cl^- defect in CF airways, a raised TPD measurement can be used as a diagnostic tool[129]. The characteristic of CF is a greater negative (hyperpolarized) potential across the airway at baseline, a large depolarization (more positive) in response to the sodium channel blocker amiloride and a less or absent response to the effect of a chloride free environment[130] compared to normal and Het/carriers for CF.

Similar to nasal airways, there is also an impaired response to chloride stimulation in the presence of the β-adrenergic agonist isoproterenol, a finding noted in the lower airways in both paediatric and adult CF populations[131].

1.6.3 Airway Surface Liquid Layer

The defective epithelial ion transport characteristic of CF disease leads to dehydration of the PCL airway surfaces and thickening of the mucus secretions that can inhibit the MCC function of the airways. The failure to clear thickened mucus facilitates the initiation of bacterial infection and inflammation that precipitates the pathogenesis of CF lung disease.

The composition of the PCL (i.e. near cell layer) is mainly water and salts and includes tethered mucins and glycolipids, provides movement of water and the liquid environment for the synchronisation of cilial beat[132]. The overlying mucus layer is comprised of 97% water, 1% salt and consists of long-stranded gel-forming mucins (2%) that interact with globular proteins which provide the viscoelastic properties necessary for particle adhesion and movement[124, 133]. The mucus layer also acts as a reservoir to receive or provide liquids to the PCL.

The absence of CFTR produces unrestricted Na^+ absorption and reduced Cl^- secretion, and results in a reduction in the volume of both layers[123]. The water component of the mucus layer is reduced and becomes more viscous and sticky and the liquid content of the PCL also depletes causing a reduction in volume and a loss of the lubricating properties that separates the two layers of the ASL. This dual loss of fluid collapses the PCL depth and causes stasis of cilial movement, and the mucus layer becomes more adhesive to the PCL and airway surfaces[134]. The resultant dehydrated, viscous and sticky mucus layer forms mucus plugs that cannot be removed from the airways by the cough reflex. Bacterial infections are then more adherent, cannot be dislodged or cleared, causing inflammation of the airways. This triggers hypersecretion of mucins from the submucosal glands and the immovable mucus plugs are further impacted and the density, volume and viscosity of the mucus layer increases (Figure 1-10). Hence a vicious cycle of mucin hypersecretion, bacterial infection and inflammation, can begin further dehydrating the ASL and greatly reducing MCC.

The hallmarks of CF lung disease are established, usually very early in life. The persistent infection of the mucus of CF airways leads to large dense bacterial colonisations known as biofilms[135] and the secondary defence such as infiltration of neutrophils to remove these biofilms are hindered by the thickened, gel mucus layer. The pH of the ASL is more acidic in CF airways due to the defective bicarbonate transport[136] and this compromises the antibacterial defences, interfering with the killing of bacteria and contributing to the pathogenesis of CF lung disease.

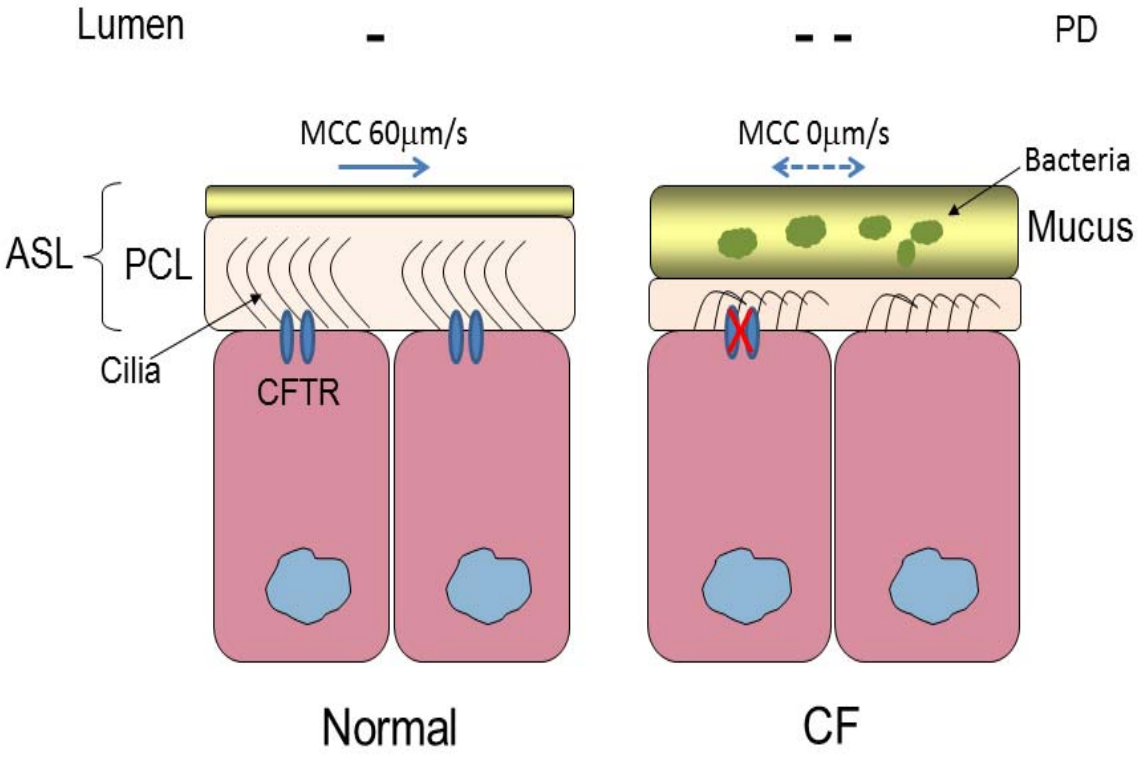


Figure 1-10 Airway Surface Liquid Layer in Airway Epithelia
Loss of CFTR results in abnormal ion transport, more negative electrical potential difference (PD) of the luminal airways; dehydration of the airway surface liquid (ASL) layer; loss of cilia function in the periciliary layer (PCL); increase in concentration and viscosity of the mucus layer. Mucociliary clearance (MCC) is defective and accumulation of bacteria as biofilms infect the airways (Modified from[124, 132]).

1.6.4 Airway Infections

The majority of morbidity and mortality of CF is due to lung disease that is associated with respiratory infections. The majority of pulmonary bacterial infections start early in childhood, with acute infections, that once established can become chronic by adolescence and early adulthood. The most common bacterial species found in CF sputum are *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Haemophilus influenzae* with other species such as *Burkholderia spp, Stenotrophomonas maltophilia,*

and Mycobacterium spp. colonized to a lesser extent (Figure 1-11). Fungal infections and viruses also contribute to CF lung disease pathophysiology.

Figure 1-11 Age-related Prevalence of Airway infections in CF Patients.
The prevalence and acquisition of common respiratory pathogens in CF patients from the US data registry (Modified from Gibson et al 2003[137]).

1.6.4.1 Staphylococcus aureus

Staphylococcus aureus was the bacterial species that was responsible for the mortality of the majority of CF children in the pre-antibiotic era[1]. It still remains a pulmonary pathogen in CF children under 10 years of age[138]. The ability of *S. aureus* to adhere to the mucus of CF lungs, rather than to respiratory

cell membranes, results in the colonization of the lower respiratory tract[139]. The reduced MCC and viscous mucus of the ASL associated with CFTR dysfunction exacerbates this colonization. The virulence of *S. aureus* due to persistent adherence; the phenotypes of small colony variants[138]; and antibiotic resistant strains, lead to chronic infections, pulmonary destruction and priming of the airways for co-infection with other bacterial species such as *P. aeruginosa and H. influenzae.*

1.6.4.2 Haemophilus influenzae

Haemophilus influenzae is more commonly found as acute and chronic pulmonary infections in infants and young children with CF[140]. The persistence of *H. influenzae* in the respiratory tract is due to its genetic diversity or hyper-mutability that allows the bacteria to evade immune defences and adapting to a changing environment. The high frequency of hypermutable strains of *H. influenzae* that have been isolated in CF sputum have evolved due to antimicrobial resistance over time[141, 142]. The biofilm formations of non-typeable (non-capsulated) *H. influenzae* enable the bacteria to survive the multiple antibiotic therapies in young CF patients[143] and cause chronic upper and lower respiratory tract infections. However, the co-infection with *P. aeruginosa* is the most likely cause of severe and chronic lung infections and is ultimately responsible for destructive lung disease in CF.

1.6.4.3 Pseudomonas aeruginosa

Pseudomonas aeruginosa is the most common pathogen found in CF sputum from infancy and early childhood since the introduction of anti-staphylococcal antibiotics. *P. aeruginosa* causes acute and chronic lung infections that are associated with a rapid decline in lung function. If the onset of chronic *P. aeruginosa* infection can be delayed with the use of anti-pseudomonals antibiotics, life expectancy for CF patients is increased[144].

P. aeruginosa has a wide genetic diversity that allows it to persist in both aerobic and anaerobic conditions, it expresses a range of virulence factors, is inherently resistant to antimicrobials and its ability to form biofilms provides the basis for recurrent acute and chronic infections in the CF lung.

The strains of *P. aeruginosa* found in CF airways are hyper mutable as the bacteria react promptly to changes in the environment by eliciting mutational events in the genome. The conversion from a non-mucoid to a mucoid phenotype[145] is responsible for the worsening of lung function in CF patients. The mutant *P. aeruginosa* over produces exopolysaccharide alginate (mucoid) that protects the bacteria from MCC, host immune responses, antibiotics and is itself inflammatory.

The other major cause for persistence and resistance to *P. aeruginosa* in the CF lung is its ability to form biofilms[146] (Figure 1-12). Biofilm formation occurs in distinct stages, first with attachment to the mucus overlying the airway surface by normal planktonic strains. Adherence to the mucus then becomes irreversible, allowing for an increase in bacterial colonies and load. Once these microcolonies reach a critical mass, they are surrounded by a matrix that protects the colony formation from phagocytosis and anti-microbials, and the mushroom like stalk structures are comprised of a mixture of different strain phenotypes.

The final stage of the biofilm structures is the breakdown of the biofilm to disperse bacteria and smaller biofilm structures to colonise other areas of the lung, and this process maybe be the cause of respiratory exacerbations[144].

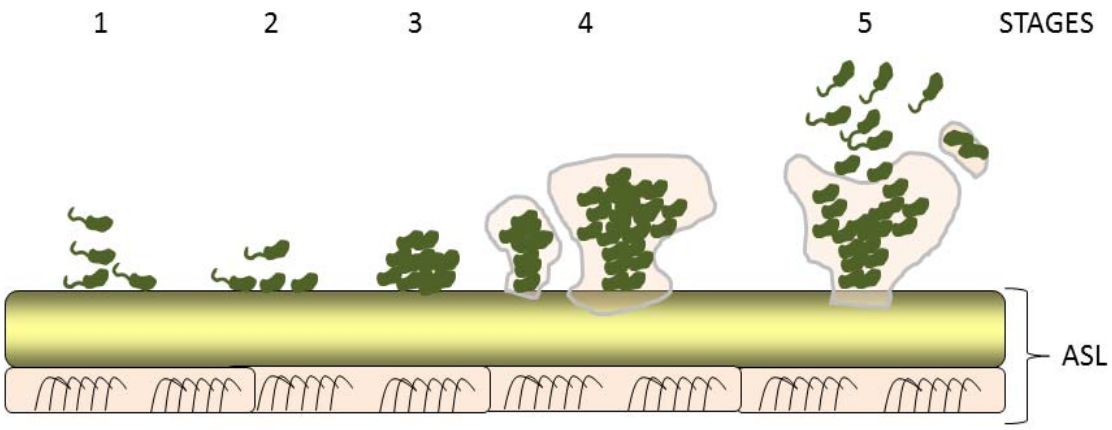


Figure 1-12 Stages of Biofilm Formation
Stage 1: Bacterial attachment to mucus layer of the airway surface liquid layer (ASL) which is reversible; Stage 2: Irreversible adherence; Stage 3: Microcolony development; Stage 4: Mature biofilm formation and Stage 5: Dispersal of biofilms and bacteria (Modified from[147]).

1.6.4.4 Burkholderia cepacia

During the 1980s an outbreak of *Burkholderia cepacia* complex, comprising 11 different *Burkholderia spp*, severely affected the morbidity and mortality of CF sufferers[148]. Due to effective infection control techniques and procedures in CF clinics the occurrence has stabilised to around 3-4% of the CF population affected.

B. cepacia spp was originally misclassified as *P. cepacia* due to similarities between both bacteria. *B. cepacia* complex is inherently resistant to antimicrobials, effectively evades the hosts' immune responses, can survive within macrophages[149] and produces virulence factors that are pro-inflammatory. *B. cepacia* also forms biofilms which are variable in their genetic phenotype and are

highly multi-drug resistant. The onset of *B. cepacia* complex colonization can be responsible for a rapid decline in clinical lung health, or a "cepacia syndrome" leading to multi-organ failure and early death, but can also be non-symptomatic depending on the *B. cepacia* species[150].

1.6.4.5 Other Bacterial Species

The acquisition of *Stenotrophomonas maltophilia* in CF increases with age and antibiotic usage. As CF patients with *S. maltophilia* are older and usually already have progressive lung disease, the detection of the bacterial species is not a predictor of lung health prognosis[151].

A similar gram negative bacteria *Achromobacter xylosoxidans* is also found in CF sputum, usually co-infected with other bacterial species and pathogens, and infection can range from transient to chronic. However, *A. xylosoxidans* doesn't appear to be associated with major decline in lung health[146].

Non-tuberculous *Mycobacteria spp.* has been colonized in the lower respiratory tract of older CF subjects. CF patients with repeated positive *M. avium*, *M. abscessus* or more than one species cultured from sputum had no short term effect on lung function (Forced Expiratory Volume over 1 second, FEV1) but showed signs of clinical lung disease on high resolution chest X-ray. The clinical findings of cysts or cavities, areas of consolidation, and nodules are more prevalent in CF patients with *Mycobacteria spp.,* and are a predictor of the progression of lung disease[152].

1.6.4.6 Fungal Infections

The major fungal infection in CF airways is *Aspergillus fumigatus* which may be responsible for allergic bronchopulmonary aspergillosis which comprises of bronchoconstriction, pulmonary infiltration, eosinophilia, and increased serum immunoglobulin E levels[153, 154]. Current treatments using corticosteroids and antifungals are usually successful. *Candida albicans* has also been isolated in CF

patients primarily due to overgrowth from excessive antibiotic therapies, but has little implication in clinical severity of respiratory symptoms of CF[155].

1.6.4.7 Viral Infections

The major viruses in CF include respiratory syncytial virus (RSV), the common cold virus (adenovirus), parainfluenza virus, influenza and rhinovirus. Respiratory viral infections pre-dispose the lung to colonization of bacterial infections; RSV infection enables *P. aeruginosa* colonization[156] and is associated with decline in respiratory health and the increase in the amount of hospitalizations for CF patients[157]. The consequences of viral infections have more significant impact on respiratory health in the CF population that in the normal population. An acute infection of Influenza A virus has also been associated with more severe respiratory clinical symptoms[158].

1.6.5 Current Airway Treatments

The majority of the current treatments for CF have not been tailored to the genetic origin of the disease but to deal with the development of respiratory symptoms of the disease. Lung function is maintained by treatments that minimise infection, inflammation, airway obstruction and removal of viscous mucus. Clearance of airways using nebulisation or physiotherapy techniques, and a variety of drug therapies to improve or delay respiratory symptoms, are a constant feature of life for CF patients. The aggressive treatments of pulmonary exacerbations are the mainstay of CF therapy and can delay the inevitable decline in lung health, but often ultimately lead to death or to lung transplantation as the only therapeutic option for survival.

1.6.5.1 Airway Clearance

Chest physiotherapy with postural positioning using percussion and vibration techniques, as well as mechanical devices such as the flutter valve and the positive expiratory pressure mask, greatly improve airway clearance and cough production, and have beneficial outcomes in lung function[159].

Drug treatments using mucolytic agents that improve the hydration of the airways such as inhaled hypertonic saline[160] and those that can reduce mucus viscosity with for example nebulised treatment of recombinant human deoxyribonuclease I[161], have been shown to reduce pulmonary exacerbations and produce improvements in lung function.

Bronchodilators such as the short acting β2 agonists (e.g. salbutamol) and long acting β2 agonists (e.g. salmeterol) can improve airway obstruction and MCC, have also shown improvements in lung function on their own[156], or as adjunct therapies with physiotherapy, hypertonic saline[160] and antibiotic treatments[157,158].

Anti-inflammatories such as ibuprofen[162] and steroids[163, 164] have also been shown to slow the progression of lung disease by improvements in lung function of CF patients with mild to moderate disease. To date, effective antibiotic therapy[165, 166] is the most common and useful treatment for improvements in respiratory health and delaying the onset of chronic airway disease.

1.6.5.2 Novel Therapies

1.6.5.2.1 Ion regulation

Treatment to inhibit the sodium hyper-absorption of the airways with the short acting sodium channel blocker amiloride has also been trialed[167], however no significant improvement in lung function has been shown over standard treatments.

Other treatments for the chloride dysfunction in CF have been to stimulate chloride secretion through alternative channels to CFTR, such as provided by the drug denufusol which is a purinergic receptor $P2Y_2$ agonist. This drug demonstrated improvement in lung function at 24 weeks[168] but not at 48 weeks[169] and there was no reduction in the incidence of respiratory exacerbations compared to placebo in the long term.

Triphosphate nucleotides such as ATP and uridine triphosphate interact with purigenic receptor P2 have been shown to improve chloride secretion in human nasal epithelial[170], but the use of these nucleotides has not been correlated to improvement in lung health.

1.6.5.2.2 Potentiators

The development of new small molecule modulators (potentiators and correctors) for enhancing chloride transport responsible for certain types of CF mutations have recently been trialled and successfully translated into the clinic. Class III mutations, such as GD551 (~4-5 % of the CF population[171]) produce sufficient amounts of CFTR, but due to defective gating the chloride channel does not remain open enough to allow for effective transport. The potentiator drug Ivacaftor (Kalydeco™) restores the gating defect of CFTR at the cell surface to allow effective chloride transport[172]. Twice daily oral doses of Ivacaftor were associated with sustained improvement in lung function for up to 48 weeks, produced less respiratory exacerbation events, and displayed improvements in nasal PD measurements and sweat chloride[173, 174].

1.6.5.2.3 Correctors

CFTR correctors restore protein folding and membrane trafficking and increase the cell surface density of the CFTR protein. The small molecule drug VX-809 has been shown to improve sweat chloride in

patients homozygous for the ΔF508 mutation compared to placebo in the short term after daily oral administration, however no discernible improvements in lung function parameters were observed in Phase II studies[175].

1.6.5.3 Lung Transplantation

Ultimately, the only current viable option for treatment of end-stage CF lung disease is heart-lung or lung transplant to prolong life. Suitability for transplantation, worsening of disease symptoms, provisional and active waiting lists, the availability of appropriate donors, long-term immunosuppressive therapy, post-transplantation infections and rejection of organs are all limiting factors, especially for the paediatric population in which more than half will die before transplantation is possible[176]. In rare cases the survival post-transplant can be over 20 years. However the majority of transplant survivors have improved quality of life, albeit for a limited time, typically between 3-5 years[177].

In summary, the multidisciplinary treatment for CF from the respiratory, physiotherapy, gastroenterology and pharmacology arenas has extended the median survival to late 30's. The new pharmacotherapy treatments such as the small molecule modulators currently emerging provide exciting options for the CF population. However treatments are still continuous, expensive and remain an extensive burden on those with CF as well as their families. Current treatments only slow the progression of the disease and are not considered a cure.

The ultimate goal is to provide a cure for CF by correcting the defect at its genetic source. A successful gene therapy would insert a functioning copy of the CFTR gene into the appropriate cell types in sufficient numbers to correct the genetic defect. A gene therapy cure could be long term or permanent if started prior to the progression of the pathophysiological cascade of the disease. Gene therapy should also provide a cure for all class type mutations associated with CF.

1.7 GENE THERAPY

The early gene therapy trials for CF were unsuccessful primarily due to poor efficiency and transient gene expression, and the inability to repeat dose due to immunological responses[178]. In overcoming the physical and cellular barriers, gene therapy delivery packages (vectors) are needed to deliver the desired nucleic acid sequence into the nucleus of the cell for functional expression, as naked DNA is degraded within minutes by nucleases in the cytoplasm[179]. Vectors can be either non-viral (e.g. cationic liposomes) or viral (e.g. adenovirus (AdV), adeno-associated virus (AAV), or retroviruses including Lentivirus (LV)) to facilitate DNA entry to airway cells. More than 25 clinical trials for CF have been conducted utilizing both viral and non-viral gene transfer vectors and these have highlighted the many challenges associated with current gene therapy methods[180].

Other factors such as distribution of vector particles to the airways by different administration routes and techniques also affect efficacy of transduction[180, 181]. Instillation by bronchoscopy results in limited deposition that requires anaesthesia and multiple instillations for more widespread distribution of the lung airways. Spray devices inserted through the bronchoscope can improve vector distribution[182]. Less invasive aerosolisation of vector particles via nebulisation techniques can result in enhanced distribution throughout the lung but also have major limitations. Shear forces from the nebulisation process can impede non-viral vectors[183] and viral vectors[184] and the nebulisation process is relatively inefficient as only a small portion of the total dose vector reaches the lower airways.

The difficulties include delivery to the airway cells along with the associated anatomical and immunological barriers, the particular cells to be targeted, and the amount of CFTR required for a therapeutic effect. The safety aspects of gene therapies as well as the appropriate outcome measures

designed to ascertain effective improvements in lung function and ion transport have yet to be fully addressed.

1.7.1 Barriers to Gene Therapy

As stated earlier in section 1.6.1 and 1.6.3, the function of the upper airways is to prevent foreign particles from entering the lung. The complex defensive networks of not only extracellular barriers, but also intracellular barriers, have evolved over time as effective mechanisms to maintain respiratory health. The added complications of reduced MCC, low volume of the ASL and increased mucus viscosity in the airways of CF individuals further compounds the challenges of delivery for an effective airway gene transfer therapy.

1.7.1.1 Extracellular Barriers

The first barrier to gene vectors is the mucus layer that binds and traps inhaled gene vectors with the vectors removed from the airway surface by MCC mechanisms[124]. In CF airways the delivered gene vector can be trapped in the far more viscous adherent mucus layer which contains actin, DNA and inflammatory products that prevent penetration of the gene vector to the desired cell targets. The PCL contains electrolytes, immunoglobulins, cytokines, antimicrobial proteins, inhibitors, surfactants and secretions that inhibit introduced gene transfer vectors[185].

If the gene vectors can overcome the first barriers of the ASL, the next barrier is the glycocalyx that can also bind the vectors and prevent attachment to the luminal cell surface[121]. The glycocalyx is composed of glycoproteins consisting of tethered mucins, glycolipids and many carbohydrate molecules.

Airway epithelia are polarised, well differentiated columnar cells, which are relatively resistant to both non-viral and viral vectors[186, 187].

Viral receptors are predominantly located on the basolateral surface of the airways[188, 189]. For topically administered gene vectors to adhere to these receptors the tight junctions between epithelial cells must be breached (Figure 1-13).

The apical surfaces of the well differentiated airway epithelial cells also have a slow rate of endocytosis that limits the uptake of vector molecules into the desired cell[190].

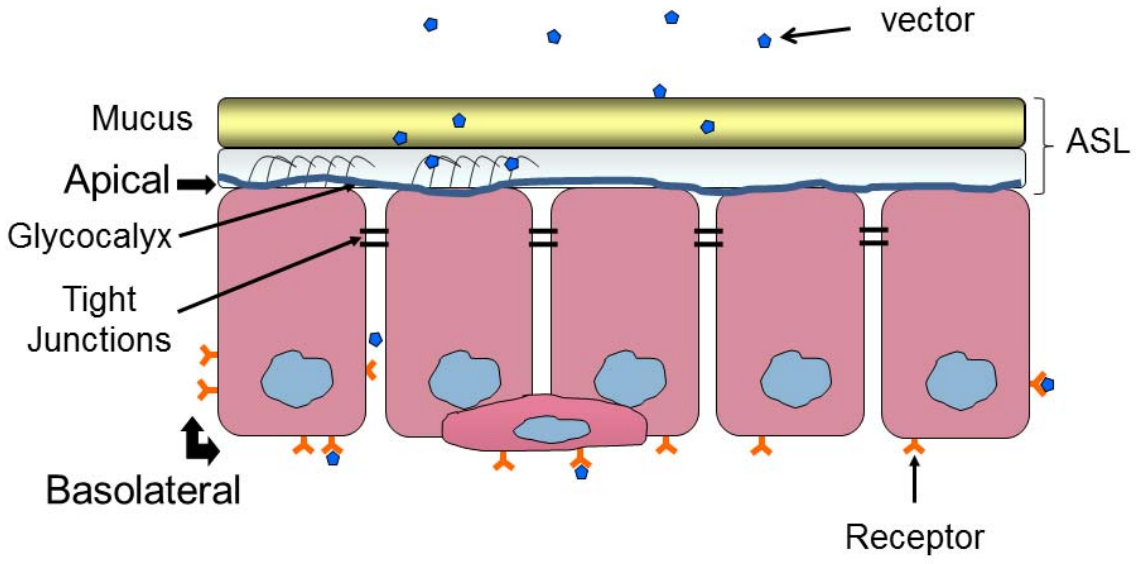


Figure 1-13 Extracellular Barriers to Gene Transfer Vectors
Barriers of the airway epithelia include the mucus layer, mucociliary clearance mechanisms, glycocalyx and tight junctions between cells that can all limit access to receptors located on the basolateral membrane.

1.7.1.2 Intracellular Barriers

The next barriers to gene transfer of the introduced DNA into the desired cell are the surface and intracellular barriers of the cell. For successful uptake of non-viral and viral agents into the cell, both require specific targeting of the vector packaging.

The DNA must first enter the cell, then traffic through the cytoplasm, and enter the nucleus all prior to transcription, translation, and modification. Many physical and chemical approaches are used to deliver the required DNA into the cytoplasm of the cell, but delivery of the DNA to the nuclear compartment for successful genomic transcription and then translation of the expressed protein[191] is still required.

Gene transfer vectors usually enter cells by endocytosis (including phagocytosis and pinocytosis), which is the process of internalisation and engulfing of extracellular particles into the cell. The process of internalisation with the cell membrane can occur in a variety of methods. The vector must first bind to the surface receptor (or by charge-mediated interactions on the cell membrane for non-viral vectors), and results in fusion with the cell membrane. Chemical (e.g. positively charged cationic complexes) modification of the vector, pseudotyping of vector envelopes and physical methods (e.g. ultrasound, electroporation, hydroporation) can all result in delivery of the DNA across the plasma membrane and deposition into the cytoplasm. Internalisation of gene transfer vectors occurs via the endosome complex.

The gene transfer agent must then escape the endosome, traverse the cytoplasm and the cytoskeletal network, be transported into the nucleus and enter by the nuclear pore complex for successful transcription of the delivered DNA (Figure 1-14) which is then processed, exported out of the nucleus and translated into the functional protein. The main intracellular barrier to gene transfer is degradation of the contents of the endosome complex by the lysosome.

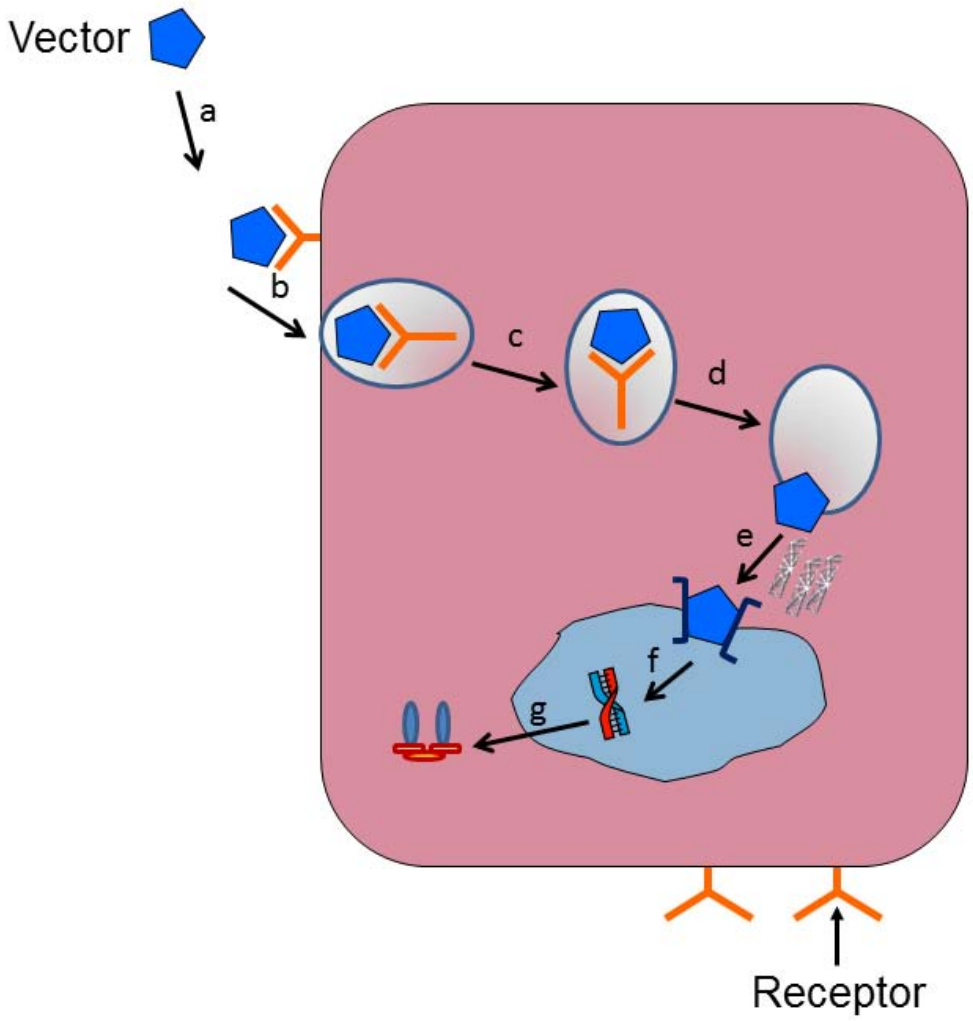


Figure 1-14 Intracellular Barriers to Gene Transfer Vectors
Gene transfer vectors a) attach to receptors, b) undergo endocytosis of vector, c) traffic by the endosomal complex through the cytoplasm, d) escape from the endosome and e) transported by cytoskeleton network to bind to the nuclear pore complex, f) transcription of DNA/mRNA in the nucleus and g) translation of encoded gene expression (Modified from[191, 192]).

Exogenous plasmids do not move through the cytoplasm to the nucleus by simple diffusion but by the active or facilitated transport of the cytoskeletal network system and various transcription factors. Viruses such as human immunodeficiency virus (HIV) utilise cytoplasmic dynein, a motor protein, and

microtubules for facilitated co-transport to the nucleus[193]. The nuclear envelope is the next barrier to gene transfer as the membrane regulates all macromolecules that traffic from the cytosol to the nucleus. In non-dividing cells the nuclear membrane is intact and is a major hindrance to gene transduction, but this is not the case for actively dividing cells[194]. However, many retroviruses have evolved to overcome the nuclear envelope barrier and can infect non-dividing cells by utilizing complex signalling processes that control the uptake to the nucleus via the nuclear pore complex[195].

1.7.1.3 Immunological Barriers

Immunological host responses are another major contributor to the lack of success observed in gene therapy trials. Innate or cellular immune responses can block initial gene expression, the duration of expression can be reduced by both cellular and humoral (adaptive) immune responses, and the adaptive immune response can also prevent expression arising from any subsequent/repetitive dosing events. The cellular non-specific immune response is the first mechanism of defence and involves phagocytic cells, such as airway macrophages[196], neutrophils, dendritic cells[197] and leukocytic natural killer cells to identify and eliminate vector particles. Dendritic cells are activated by pathogen-recognition receptors that detect molecular patterns found in microbes[198]. Acute inflammatory responses to vector delivery result in cytokine production, such as tumour necrosis factor-alpha and type I interferon, that can down regulate gene expression and promote removal of dead cells and clearance of the foreign vector[199]. The cellular immune response also mediates the adaptive immune response by activation of antigen-presenting cells (APC).

If a pathogen evades the innate immune system, the humoral immune response is triggered. This immune response confers long term immunity by specific recognition of ‘non-self’ pathogens. The leukocyte cells of the adaptive immune response are mainly B-cells and some T-cell lymphocytes. The

primary function of B-cells is to produce antibodies to antigens, perform the role of APC, and develop into memory B-cells to provide long-term immunity to the specific antigen. B-cells recognise their matching antigen in their naïve form via the B-cell receptor or membrane bound immunoglobulin, whereas T-cells recognise their corresponding antigen in a processed form: T-cells recognise a presented peptide bound by the major histocompatibility complex (MHC) molecule of the APC to the T-cell receptor.

B-cells do not produce antibodies until they are activated. Activation occurs when the B-cell receptor protein binds to its particular antigen and with additional signalling from T-helper cells ($CD4^+$, as they express the CD4 protein) can proliferate, secrete antibodies and differentiate into different B-cell types, plasma and memory B-cells. Memory B-cells are formed from activated B-cells that are long-lived and produce an enhanced immune response to the second exposure of the same pathogen/antigen. The B-cell receptor evolves over its lifespan, mutating the variable region of its immunoglobulin gene that increases its antibody affinity to specific antigens.

T cells mature in the thymus and are distinguished by their T-cell receptor on the cell surface. CD4+ T-helper cells have regulatory functions whereas $CD8^+$ T-cells or cytotoxic T lymphocytes (CTL) destroy virally infected cells and are also responsible for the hosts' rejection of foreign tissue used in transplantation medicine. T-helper cells become activated when antigens are presented to MHC Class II molecules, which are expressed on the surface of APCs. Once stimulated they produce cytokines that regulate or assist in the required immune response. CTL cells are activated by antigens presented as peptides on the MHC Class I molecules on mature dendritic cells. CTL cells can readily eliminate the cells expressing the transgene protein product or the viral vector vehicle itself[200].

Memory T-cells can be both $CD4^+$ and $CD8^+$ subsets and are responsible for long term immunity to re-exposure of antigens. Subsets of T-cells that have an immunosuppressive function are T regulatory cells (Treg), such as the naturally occurring $CD4^+CD25^+$ $FoxP3^+$ cells. Tregs terminate the immune response after successful elimination of the antigen and prevent autoimmunity[201]. The opportunity to target Tregs to either the vector vehicle or the transgene product could overcome many of the immunological barriers to both non-viral and viral gene therapy strategies. The introduction of donor antigens (gene vector) into the thymus prior to a gene transfer treatment could induce recipient central tolerance in newly-formed T cells to those donor antigens and produce antigen-specific Tregs, allowing longevity of gene expression and the possibility of effective re-dosing airway gene protocols.

1.7.2 Non-Viral Vectors

Non-viral vectors for CF gene therapy have the potential to avoid some of the problems associated with viral vectors, since they produce reduced host immune responses, have improved safety from undesired mutagenesis, and have the ability to deliver DNA of any size. However, currently the transduction efficiency of non-viral therapies is too low for therapeutic benefits mainly due to inefficient nuclear delivery and the transient duration of expression[202, 203].

Synthetic vectors are commonly used as carriers to enable delivery of plasmid DNA to the nucleus of the cell. Non-viral vectors are mainly modified liposomes that include complexes involving cationic lipids, polymers and peptides.

1.7.2.1 Cationic Lipid Complexes

Most non-viral clinical trials for CF using cationic lipid liposome complexes (lipoplexes) have demonstrated the safety profile is less immunogenic than viral vectors, that compounds can be

produced to manufacturing standards, and that a variety of cell types can be transfected. Clinical trials employing the cationic liposome DC-Cholesterol:DOPE containing the human CFTR cDNA under control of different promoters, have demonstrated safety but only transient gene expression. DC-Cholesterol:DOPE utilizing the simian virus (SV-40) promoter and delivered into the nasal airways of CF patients via nasal spray demonstrated a 20% correction of the ion channel bioelectrical defect at day 3, but a return to pre-treatment levels by day 7[204]. The same cationic liposome but under control of the Rous Sarcoma virus promoter, showed transient gene correction towards normal in 6 out of 8 CF patients from 7-15 days post CFTR delivery[205], and in a repeat dose study verified that while safe, the method did not display significant functional correction of nasal PD measurements[206].

The DNA-liposome complex *p*-ethyl-dimyristoylphosphadityl choline (EDMPC) cholesterol under the cytomegalovirus (CMV) promoter was also ineffective in producing CFTR functional expression that was significantly different from pre-treatment[207].

Another trial utilising the cationic liposome DOTAP under the CMV promoter showed no adverse inflammatory responses and correction of 30-45% of the nasal bioelectrical defect towards normal in 2 of 8 CF treated patients, an effect that extended to 4 weeks after administration[208].

More recent trials with the second generation cationic lipid GL-67 complexed with the neutral lipid DOPE resulted in significant correction of the CFTR defect in nasal airways[209]. The lipid complex GL-67/DOPE/DMPE-PEG_{5000} delivered to the lungs of CF patients resulted in significant (25%) partial correction of the chloride defect in lower airways 2 days after instillation[210], however with accompanying flu-like symptoms. Flu symptoms may have been related to the unmethylated cytosine-phosphate-guanine (CpG) sequences present in the bacterial plasmid DNA, which was also observed in an escalation dose study using the same liposome[211].

1.7.2.2 Polymer Complexes

Another form of non-viral gene therapy is the use of compacted DNA with cationic polymers to form polyplexes. Polymer complexes such as polyethylene glycol (PEG), polyethylenimine, poly-L-lysine and chitosan can be custom made and non-toxic, can enhance delivery of DNA, evade extracellular and intracellular barriers, target specific cell types and aid transport to the nucleus to increase efficacy of airway gene transfer at lower doses, while still producing a potential therapeutic effect[212, 213].

A clinical trial involving compacted DNA with the polyplex of PEG, complexed with a lysine residue, demonstrated a partial to complete correction of the chloride defect in nasal airways for a subset of treated CF patients that was sustained for 2 weeks[214].

Despite advances in non-viral gene therapy and with the potential to allow repeat dosing, a necessary requirement for a life-long treatment for CF, the low efficiency and transient nature of gene expression has emphasised there are still major obstacles to overcome before a non-viral airway gene therapy for CF could become a realistic clinically effective option.

1.7.3 Viral Vectors

The earliest and most common clinical approach for CF gene therapy has been viral vector based. As previously discussed in this section many extracellular and intracellular barriers to gene therapy must be overcome for any effective therapeutic benefit. From an evolutionary perspective, viruses have already adapted to bypass these obstacles and make them the obvious potential candidates for airway gene therapy. Viral mediated gene therapy for CF has focussed on AdV, AAV and retroviruses.

1.7.3.1 Adenoviral Vectors

Adenoviruses, which are responsible for upper respiratory infections in humans, are able to transduce non-dividing cells, have enough capacity to carry large amino acid gene sequences, can be genetically manipulated to increase safety, and the outer capsid can be modified to target particular cell types. High titres of a large gene such as CFTR with 300 kb of DNA can be produced stably for effective gene therapy. The translation of *in vitro* studies that demonstrated functional correction of the chloride defect employing an AdV vector containing CFTR[215] was followed by *in vivo* animal models in which efficacy was much lower, with only partial correction observed despite the use of high doses[216].

Early clinical trials (Table 1-2) provided proof-of-principle of therapeutic gene expression levels for correction of the CFTR defect in respiratory epithelia of people with CF *in vivo.* However, the low efficacy, inflammatory responses, the transient expression due to non-integrating AdV vectors – plus the inability to re-dose due to pre-existing immunity – has seen AdV gene therapy clinical trials for CF overtaken by other vectors.

Inefficient transduction was due to the location of the receptors for AdV. The coxsackie-adenovirus receptor required was predominantly on the basolateral membrane of airway epithelial cells, where it was difficult to access[188]. Retargeting of the adenoviral vector to apical receptors did not improve transduction, in part due to the physical barrier of the glycoclayx layer of the ASL[217, 218]. The use of chemicals (e.g. the non-ionic detergent polidocanol[219], sodium caprate[220] and the calcium chelator ethylene glycol-bis (*b*-aminoethyl ether) -*N,N,N*9 ,*N*9 -tetraacetic acid (EGTA)[221]) to transiently disrupt the tight junctions to allow for basolateral access has increased AdV efficiency but has only been tested in animal models.

TRIAL	VIRAL VECTOR	TARGET	GENE TRANSFER	ADVERSE EVENTS
Zabner et al (1993)[222]	AdV serotype 2 E1 deleted	Nasal	Partial correction of PD for 3 weeks	None noted
Crystal et al (1994)[223]	AdV serotype 5 E1 & E3 deleted	Nasal / Lung	DNA detected for 15 days	AdV antibodies, fever
Hay et al (1995)[224]	AdV serotype 5 E1 & E3 deleted	Nasal	Partial correction of PD for 2 weeks	None noted
Knowles et al (1995)[225]	AdV serotype 5 E1 & E3 deleted	Nasal	DNA detected for 1 week, no change in PD	Inflammation, Neutralizing antibodies
Zabner et al (1996)[226]	AdV serotype 5 E1 & E4 deleted	Nasal	Partial correction of PD for 1 week	Neutralizing antibodies
Bellon et al (1997)[227]	AdV serotype 5 E1 & E3 deleted	Nasal / Lung	RNA detected for 15 days	None
Zuckerman et al (1999)[228]	AdV serotype 5 E1 & E4 deleted	Lung	RNA detected for 3 days	Neutralizing antibodies
Harvey et al (1999)[229]	AdV serotype 5 E1 & E3 deleted	Lung	RNA detected for 3 days	None
Perricone et al (2001)[230]	AdV serotype 2 E1 & E4 deleted	Lung	DNA detected for 2-7 days	Mild inflammatory response at high dose

Table 1-2 Adenoviral Gene Therapy Clinical Trials for CF
(Modified from[231, 232])

The major hurdle to AdV gene therapy is the acute inflammatory response[233, 234] and subsequent immune response to the proteins on the AdV vector[233] which limits the ability to repeat dose, a necessity since AdV gene expression is transient. The large setback for AdV vectors was in a pilot safety trial of liver gene therapy for ornithine transcarbamylase deficiency. The use of a high dose of an AdV vector was directly attributed to the death of a young patient from the systemic immune response to the AdV vector and resultant multiple organ failure[235].

1.7.3.1.1 Helper-dependent adenoviral vectors

To improve the safety of AdV vectors the development of "gutless" or helper-dependent AdV (HD-AdV) vectors, in which all viral protein-coding sequences are removed[236], resulted in reduced toxicity and immunogenicity. HD-AdV vectors have the capacity for larger gene sequences, little risk of random integration and mutagenesis, and can be produced at high titres for efficient *in vivo* delivery. Transgene expression is also extended over the first generation AdV vectors. Nasal instillation of HD-AdV under the cytokeratin 18 promoter, after a chelating agent ethylene glycol tetraacetic acid (EGTA) pre-treatment, targeted lung conducting airways and submucosal glands with sustained reporter gene expression lasting for up to 28 days without significant immune responses[237]. This same protocol using HD-AdV vector containing the CFTR gene when delivered to CF mice challenged with *B. cepacia* resulted in less severe pulmonary symptoms and lower bacterial counts[238]. In another study with a HD-AdV vector, reporter gene expression in the lungs of mice was observed 3 weeks after delivery and for up to 3 months but at a significantly reduced level[239].

Due to the episomal delivery of DNA via HD-AdV vectors, expression was still transient and subsequent gene vector doses were required for extended gene expression. Repeat administration of HD-AdV vectors with reporter or CFTR genes in mice airways was achievable[240] and improved when

administered with an immunosuppressive agent[241]. Significantly lower antibodies to the AdV protein were detected in mice that received HD-AdV. However the vector did elicit a local pro-inflammatory cytokine response and neutralizing antibodies to the transgenes have remained an issue.

Aerosolisation of HD-AdV with the surfactant lysophosphatidylcholine (LPC), as a pre-treatment for opening of tight junctions, in the lungs of other animal models including rabbits[242] and non-human primates[243] demonstrated high efficiency of lung transduction with low toxicity.

For future gene therapy trials for CF the combination of safety and efficacy of the treatment has always been paramount, and the development of new "safer" viral vectors continues.

1.7.3.2 Adeno-associated Virus Vectors

Adeno-associated virus vectors are derived from non-pathogenic, defective human parvoviruses that are capable of stable integration and transduction of the airway epithelia. They are attractive for gene therapy due to their safety, broad tissue tropism and their ability to transduce quiescent cells. There are at least 12 serotypes of human AAV which can provide extended gene expression using multiple dosing events with different serotypes, therefore reducing immune responses. The genomic capacity of AAV is relatively small and to allow for the large CFTR gene to be packaged effectively, techniques that condense the CFTR sequence without losing the ability to produce the chloride channel function[244] have been developed.

Extended gene transfer with recombinant AAV with CFTR has been demonstrated in a variety of animal species, including mice[245], rabbits[246, 247] and non-human primates[248, 249] for up to 6 months from single and repeat dosing. However functional correction of the chloride channel defect has not been

demonstrated *in vivo* in a CF model, although administration of rAAV5-CFTR vector had reduced histopathological signs of lung inflammation in a *Pseudomonas* infected CF mouse model[245].

The majority of AAV based gene therapy clinical trials for a wide variety of diseases have used AAV2[250] due to the tropism of the vector for many tissues, its low immunogenicity and efficacy as demonstrated from pre-clinical animal models, including the successful gene expression from repeat dosing. The first clinical trials to use AAV vectors were for CF and were therefore development trials focussed mainly on safety data and detection of molecular endpoints for CFTR distribution to airways (Table 1-3).

TRIAL	VIRAL VECTOR	TARGET	GENE TRANSFER	ADVERSE EVENTS
Wagner et al (1998, 1999)[251, 252]	AAV serotype 2	Maxillary Sinus	Nasal PD and DNA detected for 14 days. DNA in 1 subject for 70 days	AAV antibodies
Aitken et al (2001)[253, 254]	AAV serotype 2	Lung	DNA dose dependent response for 14 – 60 days	Exacerbations, AAV antibodies
Wagner et al (2002)[254]	AAV serotype 2	Maxillary Sinus	No difference in recurrence of sinusitis	Increase in anti-inflammatory cytokines
Flotte et al (2003)[255]	AAV serotype 2	Nasal / Lung	DNA detected for 1 month in lung	AAV antibodies
Moss et al (2004)[256]	AAV serotype 2 repeat	Lung	Improvements in lung function for 14-30 days	Neutralizing AAV antibodies

Table 1-3 Adeno-associated Gene Therapy Clinical Trials for CF
(Compiled from[250, 257]).

These clinical trials highlight the need for non-invasive endpoints to determine efficacy and inflammatory profiles without compromising clinical outcomes of pulmonary function. Molecular expression of CFTR, using real time quantitative PCR (qPCR) to detect DNA, was technically challenging and mRNA could not be detected via reverse transcription PCR (RT-PCR). The wide variability, low level and short duration of expression could be due to the low number of receptors and co-receptors on the apical airway for AAV vectors, inactivation of AAV2 within the extracellular barriers and loss of expression due to normal turnover of airway epithelia. The increase in circulating antibodies to the AAV2 vector also suggests limitations in efficiency of gene expression from repeat dosing may also arise.

Recent preclinical studies using AAV for airway reporter gene expression have involved other serotypes e.g AAV5[245, 258], AAV6[259, 260] and AAV9[261, 262] that have a preference for airway epithelia. Further development of AAV airway gene transfer is still required before sufficient levels and longevity of gene expression for the translation into clinical applicability can be achieved.

1.7.3.3 Sendai Virus Vectors

Sendai virus (SeV), also known as the murine parainfluenza virus, is a single-stranded RNA virus of the *Paramyxoviridae* family that efficiently infects respiratory epithelia[263]. Recombinant SeV delivery produces effective gene transduction, in part due to the location of the receptors on the apical cell surface and because SeV replication and protein production occurs in the cytoplasm so the need to breach the nuclear membrane is not a barrier to transduction efficacy[264].

Effective *in vivo* reporter gene transduction with a recombinant SeV vector delivered to the respiratory epithelia of mice and ferrets occurred even after a short contact time, and no mucus altering agents were necessary for gene transduction of sheep trachea *ex vivo*[265].

Second generation non-transmissive SeV was successful in reporter gene transduction of nasal and lung epithelia in mice[266, 267] and SeV containing the CFTR gene demonstrated partial functional correction of the nasal bioelectrical defect in CF mice[268], but expression was only transient lasting 2-7 days.

Since gene expression was only short term, the ability to repeat dose SeV to provide an effective long term therapy for CF is a necessity. Repeat dosing was ineffective and even the induction of immune tolerance to the T-cell epitopes of SeV did not increase gene expression nor reduced anti-SeV neutralizing antibodies[269].

Sendai virus may be effective for strategies that require short term expression, rather than persistent expression that is required for CF airway disease. The efficacy of apical transduction of the SeV envelope is now being explored as pseudotyping for other viral vectors as gene therapy strategies for CF airway disease[270].

1.7.3.4 Lentiviral Vectors

The unique ability of LV vectors of the *Retroviridae* family to infect non-dividing cells, to provide long-term stable gene expression and do so with low immunogenicity makes them the most efficient of the vectors for gene therapy. LV vectors are enveloped viruses that can be pseudotyped to target particular cell types and this increases their attractiveness as a tool for gene therapy for many diseases. Many LV species have been used in airway gene transfer studies such as feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV), equine infectious anaemia virus (EIAV) and HIV-Type I (HIV-1).

The prototype for LV based vector systems is the HIV-1. HIV-1 is composed of two copies of single-stranded RNA enclosed by a conical capsid comprising the viral protein p24 characteristic of LV. The

viral elements of the LV vector system consist of four components i) the packaging helper proteins and enzymes consisting of at least *gag-pol* genes, ii) post-transcriptional regulator for group-specific antigen (*gag)* and polymerase (*pol)* expression, iii) LV vector RNA containing the transgene expression cassette and iv) the heterologous glycoprotein for pseudotyping of the envelope[271].

The packaging helper constructs essential for replication of all retroviral vectors include *gag* which directs synthesis of structural proteins; *pol* is responsible for RNase H, integrase activity and reverse transcriptase taking genomic RNA to double-stranded DNA pre-integration form; and the envelope (*env)* gene responsible for envelope protein that dictates viral tropism. Particular to LV are the regulatory proteins Trans-activator of transcription (Tat), the Regulator of virion protein expression (Rev) and the Viral protein U (Vpu) that indirectly assists in assembly and assists in viral particle release from the host cell. Other minor accessory genes of LV are the Negative regulatory factor (Nef) protein that alters antigen presentation to evade destruction from cytotoxic T-cells, Virion infectivity factor (Vif) that suppresses the innate anti-viral response of the host and the Viral protein R (Vpr) which acts as a cytoplasmic-nucleus shuttle protein[272].

All these elements allow HIV-1 to effectively infect cells *in vivo*, by integration into the host genome, evading both the intracellular barriers to infection and the host immune system. To improve safety of LV vectors for gene therapy a number of replication-defective versions of HIV-1 have been developed[273]. Second generation LV vectors retained the essential packaging components but deleted the virulence accessory proteins such as Vif, Vpr, Nef. The resultant LV vector provided stable integration of the transgene into the genome of the host cell, but it is unable to direct production of replication-competent viral particles. Third generation LV vectors have the *tat* sequence replaced by a heterologous promoter sequences and expression of *rev* supplied *in trans*. The biosafety of LV vectors was increased by the

creation of a self-inactivating (SIN) vector by abolishing the transcriptional activity of the long terminal repeat (LTR).

The major hindrance to LV gene therapy is the extracellular barriers that prevent vectors from attaching to cell receptors for transfection of the target cells. LV vectors have been pseudotyped with a variety of envelope proteins to increase efficiency of attachment to the desired cell type needed. LV vectors have been pseudotyped with the Vesicular Stomatitis Virus G glycoprotein (VSV-G) that provides broad tropism including respiratory epithelial cells for CF airway gene therapy. Other pseudotypes for LV vectors envelopes that have been developed include the glycoprotein of the Ebola envelope, Murine leukaemia virus, Mokola virus, influenza-hemagglutinin, RSV[274], SeV[270] and baculovirus GP64 glycoprotein[275].

1.7.3.4.1 Lentiviral clinical trials

Early clinical trials using γ-retroviral vectors demonstrated long term clinical efficacy from gene therapy for inherited genetic disorders such as X-linked severe combined immunodeficiency disorder (SCID-X1) and adenosine deaminase deficiency–severe combined immunodeficiency disorder (ADA-SCID)[276]. However vector-induced insertional mutagenesis has been associated with SCID-X1[277], chronic granulomatous disease[278] and Wiskott-Aldrich syndrome[279].

To address this serious and adverse side-effect from insertional mutagenesis, LV vectors with increased safety were developed. New generations of SIN LV are less capable of activating genes across the genome, as the viral enhancer elements in these vectors have been deleted[280]. Many pre-clinical studies of SIN LV vectors have been very encouraging, for example in murine models of β-thalassemia, sickle cell disease and muscular dystrophy, and a primate model of Parkinson's disease[281]. This success has led to clinical testing of LV vectors for gene therapy. Since the first LV based trials were

successful for the treatment of HIV / Acquired Immunodeficiency Syndrome (AIDS), many other diseases have now been explored as a long term therapeutic option (Table 1-4).

TRIAL	DISEASE	VECTOR	DELIVERY	RESULTS
Bank et al, (2005)[282]	β-thalassemia	SIN LV	*ex vivo* infusion	No Adverse events Sustained clinical benefits
Levine et al (2006)[283]	AIDS	VSV-G LV (full LTR of HIV)	Repetitive autologous *ex vivo* infusion	Long term safety Full therapeutic effect
Cartier et al (2009)[284]	X-linked adrenoleukodystrophy	LV with internal viral LTR	*ex vivo* infusion	Safe Good clinical benefits
Ingram et al (2009)[285]	Acute Myeloid Leukaemia	HIV-1	Autologous leukocytes	Awaiting results
Chen et al (2009)[286]	Melanoma	HIV-1	Autologous cytotoxic T-lymphocytes	Awaiting results
DiGiusto et al (2010)[287]	AIDS/AIDS related lymphoma	SIN LV	*ex vivo* infusion	Safe, Insufficient
Galy et al (2011)[288]	Wiskott-Aldrich syndrome	VSV-G LV	autologous *ex vivo* infusion	Awaiting results
Oxford Biomedica (2012)[286, 289]	Parkinson's Disease	LV derived from EIAV	Intraputaminal delivery	Long term safety Significant clinical improvement

Table 1-4 Current LV Gene Therapy Clinical Trials
(Adapted from[276, 288, 290, 291]).

Of the current vectors used in gene therapy clinical trials approximately 2.9% are LV vector based, however none are currently directed towards CF airway disease. Encouragingly, there have been sustained clinical benefits and long term safety with no serious adverse events or incidence of insertional mutagenesis in the various trials using LV vectors, whereas oncogenesis has been observed in some clinical trials employing γ-retroviral vectors.

The potential for LV gene therapy for CF airway disease may be realised with further development of successful pre-clinical gene therapy studies for CF.

1.7.3.4.2 Pre-clinical LV gene therapy for CF

The use of a LV vector pseudotyped with different glycoproteins from other enveloped viruses helped improve the effectiveness of transduction of airway epithelia from either apical or basolateral entry. Apical attachment for airway LV gene therapy occurs using a baculovirus GP64 envelope[275] and Ebola-Zaire virus envelope[274, 292] in murine models. To improve transduction efficacy increased contact time of the apical surface GP64 pseudotyped FIV was achieved with the addition of a viscoelastic methylcellulose gel that reduced MCC[275], one of the extracellular barriers to airway gene transfer. Also this GP64-FIV vector could be repeatedly dosed to the nasal epithelia to produce long term reporter gene expression without significant host immune response[293].

For basolaterally targeted LV vector, envelopes such as the VSV-G pseudotype, pre-conditioning agents that transiently disrupt tight junctions between cells, along with agents that reduce mucus viscosity and MCC, can greatly enhance gene transduction efficacy. Pre-treatment with chelating agents such as EGTA in a hypotonic buffer also enhanced reporter gene expression with a FIV vector in trachea of rabbits[294], and inhalation injury with sulphur dioxide boosted HIV reporter gene expression in nasal airways of mice and rats and in the trachea of rats[295]. Pre-treatment with detergents such as

polidocanol[296] and the lung surfactant LPC[296-299] enhance not only reporter LV gene transfer in normal mice nasal and lung airways, but provide partial functional correction of the nasal bioelectrical defect in nasal airways of CF mice. LPC can transiently open tight junctions[300], reduce cilial beat, and act as a mucolytic agent[298] that facilitates access to the VSV-G basolaterally located receptors on the airway surface.

For pre-clinical studies to progress to a clinical therapy for CF airway disease the development of animal models of CF disease that can test functional correction of the chloride defect is an obvious requirement for safety and efficacy testing for any new therapy, not just gene therapy.

1.8 CF ANIMAL MODELS

Translations of successful *in vitro* and *ex-vivo* results into animal models of disease have underscored the differences when compared to a fully intact and physiologically relevant *in vivo* model. Differences between animal species also provide valuable insight into the complex nature of disease pathogenesis and development of novel therapeutic strategies for potential human treatments.

1.8.1 CF Mouse Model

Several years after the cloning of the CFTR gene the first CF mouse model was developed[301]. Many mouse models of CF were subsequently created, with mutations that were complete knockouts to block CFTR function entirely or to model clinical mutations such as ΔF508 and G551D[302]. An early handicap of CF mice with absent CFTR function (*cftr* -/-) is that the majority of mice die early after weaning from intestinal obstruction[301]. The addition of a nutrient rich liquid diet[303] or high-fat chow in conjunction with a mild diarrhoeal agent[304] prolongs the life span of CF null mice making them a useful tool for gene therapy testing.

The development of a CF strain with the intestinal defect corrected – by the insertion of human CFTR under the control of the fatty acid binding protein (FABp), expressing CFTR in gut tissue but no other epithelial cells – has allowed for long surviving CF mice without onerous animal husbandry requirements[305].

The pathophysiology of disease in various organs of CF mice is diverse due to the method of mutational generation of murine models as well as strain differences[306]. Despite having severe intestinal disease, the CF airway pathophysiology in CF mice is present in the nasal airways, but unexpectedly, not in the lung airways. This is due to the operation of an alternate chloride channel in the lung epithelia that is regulated by intracellular calcium, independent of CFTR[307]. Reduction of ASL height, increased bacterial adherence, goblet hyperplasia and metaplasia[308] and reduced chloride transport and increased sodium absorption – all characteristics of human CF pathophysiology – are present in the nasal airways of CF mice[302]. Other organs affected include the pancreas, liver, reproductive tract and salivary glands where effects range from mild to absent depending on the mutation[302, 306].

The observation that the incisor teeth of CF mice are soft, chalky and white compared to normal mice that have hard, yellow-brown coloured incisor teeth[309] has allowed for simple visual determination of phenotype of *cftr* -/- compared to Het (*cftr* +/-) littermates as early as 3 weeks of age. The enamel of the front incisors is hypomineralised and the lack of pigmentation is due to abnormal enamel development that undergoes premature degeneration in CF mice regardless of mutational type.

The CF mouse nose is the standard site for functional testing of gene therapy protocols and utilises nasal TPD measurements to measure the chloride defect. Hyperabsorption of sodium ions across the nasal mucosa is indicated as a significantly more negative basal nasal PD and a greater depolarization response to perfusion by the amiloride-sensitive component of the basal PD in all CF mice models,

compared to normal mice[310]. CF mice also lack the functional chloride transport in the response to low chloride perfusion that is observed as in either no change in mucosal PD, or a slight depolarization in electrical PD compared to the hyperpolarization seen in normal mice.

Recently the applicability of nasal TPD as a functional test for gene therapy strategies has been questioned due to the potential confounding bioelectrics from the olfactory epithelia of the murine nasal airways[311, 312].

The creation of a mouse with spontaneous CF-like lung disease through the over expression of the ENaC ion channel[313] has provided a model with all the hallmarks of CF pathophysiology in the lung airways. In these *Scnn1b* -/- mice that overexpress β-ENaC, accelerated Na^+ transport, even in the presence of Cl^- secretion, results in reduced ASL, increased mucus viscosity, mucus plugging of the airways, goblet cell metaplasia and reduced MCC in the lung airways. Survival can be severely reduced (up to ~ 50% die within 2-3 weeks of age), with corresponding lung abnormalities when compared to normal lung pathology in newborn mice; a similar pathogenesis to human CF lung disease was also reported[314]. These mice models are useful for evaluating novel and therapeutic agents to treat CF lung disease but not for correction of the CFTR chloride defect via gene therapy strategies since there is no change in chloride channel function.

1.8.2 CF Pig Model

The production of a larger CF animal model closer to the lung anatomy, biochemistry, physiology, size and life span of humans was realised with the creation of a CF porcine animal model[315, 316], that displays the characteristics of human CF disease. Newborn *CFTR* -/- pigs display intestinal obstruction including MI that requires surgery for survival, exocrine pancreatic deficiency, gallbladder abnormalities, and early focal biliary cirrhosis. Newborn CF pig lungs lack inflammation but have defective bacterial clearance

that develops hours after birth[317]. Over time the full spectrum of CF lung disease spontaneously develops in the *CFTR* -/- pig mimicking the pathogenesis of human CF lung disease, including defective ion epithelial transport.

The unique opportunity to study the origin of CF lung pathogenesis in a porcine model has elucidated that the loss of Cl^- and not excessive Na^+ absorption due to defective CFTR results in early onset of impaired bacterial eradication followed by the subsequent development of airway disease[318]. This porcine animal model of CF provides great promise for future gene therapy pre-clinical trials and other novel therapeutic strategies for CF disease treatments.

1.8.3 CF Ferret Model

Another promising animal model for CF disease has been created using the domestic ferret *Mustela putorius furo.* There is strong similarity in airway cellular composition, abundant submucosal glands, and CFTR expression in lung biology of both ferrets and humans. The short gestational time and early sexual maturity in 5-6 months in ferrets makes it an attractive animal model for CF studies. The CF ferret was created by recombinant AAV–mediated gene targeting in fibroblasts, followed by nuclear transfer cloning[319].

The early phenotype of *CFTR* -/- ferrets shared the same abnormalities of CF as those found in human infants[320], including the severity in pancreatic function, liver disease, impaired nutrition and the absence or defective vas deferens. Neonatal mortality from intestinal complication of MI is found in the *CFTR* -/- pig (100%) and in *CFTR* -/- ferret (75%) both at higher rates than in humans (15%). To overcome this complication clones of CFTR gut-corrected ferrets, under the control of the FABp promoter used successfully in CF mice, produced one clone that excreted normal faeces and so improved survival[320].

Using *ex vivo* functional studies, newborn *CFTR* -/- ferret tracheas demonstrated abnormalities characteristic of the proximal airways of CF patients, including alterations in chloride permeability and submucosal gland fluid secretion[320]. The progression of CF pathophysiology in adult CF ferrets is eagerly awaited as colony survival improves and should produce another suitable animal model for CF gene therapy.

The presence of three CF animal models offers renewed hope and research opportunities to further the development of the efficacy and safety needed to bring a gene therapy for CF airway disease into a clinical setting.

1.9 TARGETS FOR CF GENE THERAPY

Despite the advances and many lessons learnt from gene therapy for CF airway disease over the last 20 years from *in vitro*, pre-clinical animal models studies and clinical trials, many aspects are still controversial and the complexity of CF lung disease continues to challenge researchers. Current research has emphasized the hurdles to effective gene therapy including ensuring the insertion of the functioning CFTR gene into the correct cell types and determination of the amount of correction required for a therapeutic benefit. The current transient gene expression observed for CF has mainly been due to inefficient initial expression and the immune responses of the host blocking continuing expression. However for a long-term cure the influence of the normal airway cell turnover processes in the host on maintenance of expression must also be addressed. As the goal is a life-long cure for CF airway disease, strategies to achieve these aims are still developing.

1.9.1 Therapeutic Levels of CFTR

In normal respiratory airways CFTR is abundantly expressed in the submucosal glands and the ciliated epithelial cells[321, 322]. Ciliated cells are the predominant luminal epithelial cell type present throughout the upper and lower conducting airways and are responsible for maintaining hydrated ASL and effective MCT. It follows that ciliated conducting airway epithelial cells offer a primary, but not exclusive target, for correction of the CFTR defect in CF airways.

The amount of expression required to correct the CFTR defect in CF airways sufficient to correct lung function is still under debate. Theoretically 50% or less CFTR function is required as Het (+/-) individuals are considered normal and without CF symptoms or physiology. Even amounts as low as 5 – 10 % CFTR have significant benefits, producing less severe symptoms of CF and improvements in overall health and survival[95, 323]. As few as 6-10% corrected cells with CFTR generated similar chloride transport to 100% corrected epithelial sheets *in vitro*[324] and up to 20% CFTR in well differentiated epithelia was required to restore functional chloride transport. Importantly, overexpression of CFTR did not have a detrimental effect[325]. The latter finding is helpful as the development of gene transfer techniques continue to utilise maximal titres to achieve gene transfer *in vivo*. The normalisation of airway physiology (i.e ASL height and MCT), has been demonstrated by the correction of CFTR expression in 25% of human ciliated respiratory epithelia from tracheobronchial tissues in an *in vitro* model of human airway epithelia on an air-liquid interface[326].

Whilst 5 – 25% of ciliated conducting airway cells expressing CFTR may be adequate for correction of the CF defect *in vitro*, a direct translation to the *in vivo* setting is more complex. Restoration of the chloride defect in CF mice has been demonstrated with only 5% CFTR[327] that also resulted in 100% correction of the intestinal defect by direct interbreeding crosses. However for a gene therapy treatment

the minimum level of CFTR needed for success in conducting airways is still unknown. Proposals include low expression of CFTR in many cells, such as the conducting ciliated airway epithelia; or high expression in only a few cells; or a proportion of CFTR also being expressed in the luminal cells of the submucosal glands. Each approach remains in contention as the optimal method to correct CFTR airway function to produce a therapeutic effect.

1.9.2 Longevity of Expression

For a chronic disease such as CF a long-term sustained benefit is required. In the right circumstances longevity of CF correction can theoretically be achieved by a gene therapy since the disease is corrected at its genetic basis. However current potential clinical treatments utilizing non-viral and viral gene transfer vectors have resulted in only transient expression for CF airway disease. To achieve longevity of a therapeutic treatment one option is to achieve effective vector re-dosing over a life time, i.e. without inducing major immune responses. Another paradigm for long-term correction is to transduce the airway's resident stem/progenitor cells, to thus arrange for all "daughter cells" subsequently arising from the original transduced stem/progenitor cells to express adequate levels of CFTR, and so provide the on-going physiological correction able to extend for the lifetime of the individual.

The average life of different cell types of the airway epithelia for steady-state homeostasis with the response to injury and repair is another confounding factor for longevity of a gene therapy approach. The current knowledge of normal cell turnover in airways comes from studies of rodent *lung* airways and may not be applicable to *nasal* airway where the vast majority of CF gene therapy assessments are conducted. The normal turnover rates of nasal epithelia nor the distribution and type of cells that may act as progenitors for the proximal conducting respiratory epithelia have been reported. The expectation

that the nasal airway is the first line of defence from foreign particles, and is subject to frequent injury events, implies that the majority of nasal epithelial cells would possess a faster turn-over rate and a higher proportion of stem/progenitor cell types with proliferative capacity, but this has not been examined directly.

The main target for CF gene therapy is the ciliated conducting airways which are a terminally differentiated population and responsible for general slow turnover rate of the respiratory epithelia of the lung. The average half-life of ciliated epithelia has been demonstrated to be up to 6 months in the trachea and extended to 17 months in the deep lung airways of mice[328]. The proliferative capacity of the lung airways for other cell types that are potential stem/progenitors has been suggested to have a half-life of approximately 3 months[329].

Possible stem/progenitor cells for the airways reside in protective niches in the epithelia[330]. Stem/progenitor cells are usually slow-cycling and give rise to transient amplifying cells which are responsible for the majority of tissue renewal after injury[331]. Stem/progenitor cells possess an unlimited proliferative capacity, whereas transient amplifying cells have a limited capacity to divide and cannot proliferate indefinitely. A proportion of basal cells that line the basal membrane of tracheal and bronchial epithelia are thought to be the primary stem/progenitors of the upper airways[332, 333]. For more distal lung airways a sub-population of Clara/secretory cells are possible stem/progenitors or transient amplifying cells[334] capable of renewing bronchiole airways.

Elucidation of the stem/progenitor cells of the conducting airways that can be targeted for long-term homeostasis of a CF gene will be an important facet of successful gene transfer treatments designed to cure CF airway disease.

1.10 AIMS OF THESIS

The potential for gene therapy as a clinical treatment for CF has not eventuated after 20 years of research. After the cloning of the CFTR gene, the expectation was that a gene therapy cure for CF was imminent. However, numerous hurdles to airway-directed gene therapy that arose relate to the transient and low efficiency of gene expression due to the extracellular and intracellular barriers for a variety of vector systems, and the inability to effectively re-dose to produce a long term therapy due to blocking of gene expression by the host immune system[335]. These are the primary challenges that must be conquered for significant clinical progress to occur.

Our group has previously demonstrated partial functional correction of the CFTR defect in CF murine nasal airways for up to 3[269] and 12 months[297] after a single LV dose. However gene expression was inconsistent over time and in the long term study was measured in individual mice at one time point only. In addition, the dynamics of long term expression on the overall health of individual animals had not been elucidated.

Bioluminescence imaging techniques allow repeat non-invasive assessments of luciferase (Luc) gene expression to be made over a mouse lifetime. In contrast, the LacZ reporter gene (expressing β-galactosidase) requires humane killing for histological analysis, but remains an excellent method to determine gene transduction outcomes; i.e. transduced cell types and locations can be can clearly identified within the airway epithelia.

The gold standard for functional assessment of the electrophysiological defect in CF mice airways is the TPD measurement, but this technique has not been previously applied repeatedly in the same animal to monitor the bioelectrical correction of CFTR over long periods such as a mouse life-time.

The main objective of this thesis project was to determine the longevity of airway gene expression in both normal C57Bl/6 mice and CF knockout mice, utilizing a LV vector containing reporter gene(s) or the therapeutic CFTR gene, respectively.

Chapter 3 – Lentiviral Production: Most gene therapy treatments using a LV vector have been hampered by low titre production methods that result in poor *in vivo* gene transduction.

Aim i) The ability to produce large volumes of an endotoxin-free LV vector containing a single transgene, while retaining high titres was first examined and established.

The single-transgene vectors were then mixed physically to enable simultaneous analysis of different reporter genes, or reporter and functional gene expression, in the same animal.

Chapter 4 – Long Term Gene Expression: The main objective was to determine the longevity of airway gene expression in both normal C57Bl/6 mice and CF knockout mice.

Aim ii) The second aim was to utilize a repeated-measure design to determine the persistence and longevity of airway reporter gene expression, after a single LV vector dose in normal C57Bl/6 mice.

Gene expression was measured by bioluminescence at various time intervals over the animals' lifetime. To determine which cell types were transduced, LacZ reporter gene expression was assessed by histochemical (X-gal) and histological methods after humane killing.

The ultimate test of the longevity of gene expression was determined by employing the same repeated-measure design as Aim 2, but in a CF knockout mouse model.

Aim iii) Longevity of both reporter gene expression and functional CFTR gene expression was examined in CF mice over their lifetime after a single LV dose.

Luciferase reporter gene transduction was assessed via bioluminescence and nasal TPD measurements were used to assess the success of functional correction of the CFTR gene defect, with both assessment techniques applied over the lifetime of individual animals. For the first time this has allowed the progression of both LV reporter gene and functional CFTR gene expression longevity to be followed in individual CF mice.

Chapter 5 – Repeat Lentiviral Gene Transfer: As noted, the goal for CF airway gene therapy is for a long term effective treatment, and this may require repeated dosing protocols. A further aim of this thesis was to determine if these LV vectors could be re-dosed without inducing blocking immune responses.

Aim iv) These studies were designed to determine if a LV gene vector could be successfully re-dosed to achieve improved initial gene expression levels or boost long term gene expression in the nasal airways of mice, compared to our standard single two step gene treatment regime.

Chapter 6 – Immunosuppression for LV Gene Expression: The host immune response to the introduced protein has been shown to hamper the efficacy and longevity of a gene therapy treatment. The use of an immunosuppressive treatment regime – as used in transplantation medicine to prevent organ rejection – was applied to prevent possible rejection of the introduced foreign transgene protein.

Aim v) The final aim of this thesis was to determine if administration of an immune suppression therapy could not only achieve higher initial levels of airway gene expression compared to our standard single airway gene dose, but also produce a sustained benefit in expression and reduction in host immune responses in the long-term.

CHAPTER 2

MATERIALS

&

METHODS

CHAPTER 2 METHODS AND MATERIALS

2.1 MATERIALS

2.1.1 Chemicals and Suppliers

Agarose, type C, gelling T° 40-43°C	Calbiochem (USA) Cat # 121852
Amiloride HCl hydrate	Sigma Aldrich (USA) Cat # A7410
Anti-mouse IgG HRP, from sheep	GE Healthcare (AUS) Cat # NXA931
Antisedan® (atipamazole HCl 5 mg/ml)	Pfizer (USA)
Bacto™-Agar	Becton, Dickinson (USA) Cat # 214010
Bacto™-tryptone	Becton, Dickinson (USA) Cat # 211705
Bacto™-yeast extract	Becton, Dickinson (USA) Cat # 212750
Boric acid	Sigma Aldrich (USA) Cat # D6768
Bromophenol Blue	BDH Chemicals (USA) Cat # H3392-2
Calcium chloride ($CaCl_2$)	Sigma Aldrich (USA) Cat # 223506
Chloroform	Sigma Aldrich (USA) Cat # 288306
ColonLyetly®	Dandy Pharmaceuticals (AUS)
Dimethylformamide (DMF)	Sigma Aldrich (USA) Cat # D4551
DMEM	SAFC Biosciences (USA) Cat # 51441C
Domitor® (medetomidine HCl 1 mg/ml)	Pfizer (USA)
DPX	Sigma Aldrich (USA) Cat # 317616
EDTA	Sigma Aldrich (USA) Cat # E5134

Eosin Y	ProSciTech (AUS) Cat # C097
Ethanol	Ajax Chemicals (AUS) Cat # 1045
Ethidium Bromide	Sigma Aldrich (USA) Cat # E7637
F12 Hams Media	SAFC Biosciences (USA) Cat # 51655
Fetal calf serum (FCS)	JRH Biosciences (USA) Cat # 12103-500M
Formalin	Fronine Laboratories (AUS) Cat #JJ018B
β-Galactosidase from E.Coli	Sigma Aldrich (USA) Cat # G5635-1KU
Gentamycin (1mg/ml)	Sigma Aldrich (USA) Cat # 48757
Glacial acetic acid	Sigma Aldrich (USA) Cat # A9967
D-Gluconic acid	Sigma Aldrich (USA) Cat # G9005
L-Glutamine (200mM)	SAFC Biosciences (USA) Cat # 59202C
Glutaraldehyde, grade II (25%)	Sigma Aldrich (USA) Cat # G6257
Glycerol	Sigma Aldrich (USA) Cat # G5516
Haematoxylin (Mayer's)	ProSciTech (AUS) Cat # AMH
Hydrochloric acid (HCl, 37%)	Sigma Aldrich (USA) Cat # 258148
Hepes	Sigma Aldrich (USA) Cat # H3375
Indian Ink	Windsor and Newton (UK) Cat # 1005754
Ketamine (100 mg/ml)	Parnell Laboratories (AUS)
Luciferase	Promega (USA) Cat # E1700A
D-Luciferin	Caliper Life Sciences (USA) Cat # XR-1001
α-LPC, Type I from egg yolk	Sigma Aldrich (USA) Cat # L4129

Magnesium Chloride hexahydrate ($MgCl_2$)	Sigma Aldrich (USA) Cat # M9272
OPD	Sigma Aldrich (USA) Cat # P9187
OptiPro SFM	Invitrogen (USA) Cat # 12309-019
Paraformaldehyde	Sigma Aldrich (USA) Cat # P6148
PDZK1 Peptide	Abcam (UK) Cat # AB86227
Penicillin G (5000 U/ml)/Streptomycin (5mg/ml)	Sigma Aldrich (USA) Cat # P4458
Phosphate Buffered Saline Tablets (PBS)	MP Biomedicals (USA) Cat # 2810305
PBS without calcium and magnesium	Sigma Aldrich (USA) Cat # D8537
Polybrene (Hexadimethrine bromide)	Sigma Aldrich (USA) Cat # H9268
Potassium Chloride (KCl)	Sigma Aldrich (USA) Cat # P1597
Potassium Ferricyanide ($K_3Fe(CN)_6$)	Sigma Aldrich (USA) Cat # P8131
Potassium Ferrocyanide ($K_4Fe(CN)_6$)	Sigma Aldrich (USA) Cat # P3289
Potassium Phosphate monobasic (KH_2PO_4)	Sigma Aldrich (USA) Cat # P9791
Potassium Phosphate dibasic (K_2HPO_4)	Sigma Aldrich (USA) Cat # P3786
Proteinase K	Promega (USA) Cat # V3021
Rapamune® (sirolimus 1mg/ml)	Wyeth (AUS) Cat# NDC-0008-1030-06
Safranin O	ProSciTech (AUS) Cat # C138
Skim milk	Diploma Instant Skim Milk Powder
0.9% Sodium Chloride (Saline)	Baxter Healthcare (AUS)
Sodium Chloride (NaCl)	Sigma Aldrich (USA) Cat # S1679
Sodium Hydroxide (NaOH)	Sigma Aldrich (USA) Cat # S5881

Sodium Phosphate (Na_2HPO_4)	Sigma Aldrich (USA) Cat # S7907
Sulphuric Acid (H_2SO_4)	Sigma Aldrich (USA) Cat # 320501
Tail Lysis Buffer	Viagen Biotech (USA) Cat # AB102-T
TE Buffer	Usb Corp (USA) Cat # 75834
Trizma base	Sigma Aldrich (USA) Cat # T1503
Trypsin	SAFC (USA) Cat # 59430
Tween 20	Sigma Aldrich (USA) Cat # P1379
Water for Irrigation (H_2O)	Baxter Healthcare (AUS)
Xgal	Progen Industries (AUS) Cat # 200-0191
Xylene	Merck (Germany) Cat # 410234

2.1.2 Consumables and Suppliers

Cell culture plates and flasks	Costar (Corning Scientific, USA), NUNC (Nalgene, USA), Sarstedt (Germany), Greiner Lab (Germany) and Becton Dickinson (USA)
EIA/RIA 96 well plates	Corning Scientific (USA) Cat # 3590
GELoader Tips	Eppendorf (USA) Cat # 0030.001.222
Glass Syringe Gastight® (1ml)	Hamilton (USA) Cat # 1001
Goldenrod™ Lancets	MediPoint International (USA) Cat # 5 mm, 5.5 mm
Histology Cassettes	ProSci Tech (AUS) Cat # RCH40-G
Microscope Coverglass slips	Menzel-Glazer (Germany) Cat # CS22401GP
Microscope Slides	Menzel-Glazer (Germany) Cat # S41014A

Optical Adhesive Cover	Applied Biosystems (USA) Cat # 4311971
Optical Caps	Applied Biosystems (USA) Cat # 4323032
Optical Tubes	Applied Biosystems (USA) Cat # 4316567
Optical 96 well reaction plates	Applied Biosystems (USA) Cat # N8010560
Ultrafine insulin syringes	Becton, Dickinson (USA) Cat # 326725

2.1.3 Bacterial Strains and Media

Broth	1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 0.5% (w/v) NaCl in dH_2O.
LB Agar	1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 1.5% (w/v) Agar, 0.5% (w/v) NaCl in dH_2O.
E. coli	*E. coli* DH10β

2.1.4 Cell Lines

293T cells	American Type Culture Collection, CRL 11268
A549 cells	American Type Culture Collection, CCL 185
CHO-K1 cells	American Type Culture Collection, CCL 61
NIH3T3 cells	American Type Culture Collection, CRL 1658

2.1.5 DNA Plasmids

2.1.5.1 Plasmid Kits and Buffers

Agarose Gel	1.2% (w/v) agarose in TBE buffer
Endofree Plasmid Mega Kit	Qiagen (Germany) Cat # 12381
2 x HeBS	0.28 M NaCl, 0.05 M Hepes, 1.5 mM Na_2HPO_4, pH 7.04
TBE Buffer	89 mM Trizma base, 89mM Boric acid, 2mM EDTA

2.1.5.2 DNA Plasmids

CFTR	pHIV-SV40-CFTR
Empty	pHIV-SV40-empty
Gagpol	pHCMVgagpolmllstwhv
LacZ	pHIV-ext4SV40-LacZ
$LacZ_{Co}$	pHIV-1SDMPSv-nlsLacZCo
Luciferase	pHIV-SV40-Luciferase
Luciferase $_{EF1-\alpha}$	pHIV-EF1α-Luciferase
Rev	pHCMVRevmlwhvpre
Tat	pcDNA3Tat101ml
VSV-G	pHCMV-G

2.1.6 Real Time qPCR Assay

2.1.6.1 PCR Kits

TaqMan Universal PCR Master Mix	Applied Biosystems (USA) Cat # 4304437
TaqMan MGB Probe (50,000 pmol)	Applied Biosystems (USA) Cat # 43016032
Wizard SV Genomic DNA System	Promega (USA) Cat # A2361
20 x Assay Mix	18 μM forward primer, 18 μM reverse primer, 5 μM probe in TE buffer

2.1.6.2 Primers and Probes

CHO-CMP-sialic acid 3' primer (CHOK-1)	CTA TGT AAT GTT GTT CTT GTT GAC TTG CT
CHO-CMP-sialic acid 5' primer (CHOK-1)	CTA CTA CAT CAG ACA CAG TTA CTA CTA ACA TTA GA
CHO-CMP-sialic acid Probe (CHOK-1)	FAM-TCC CAG AGA AAA TTT AC-NFQ
Gag Forward primer	AGC TAG AAC GAT TCG CAG TTG AT
Gag Reverse primer	CCA GTA TTT GTC TAC AGC CTT CTG A
Gag Probe	6FAM-CCT GGC CTG TTA GAA AC-NFQ
mTransferrin Forward primer	AAG CAG CCA AAT TAG CAT GTT GAC
mTransferrin Reverse primer	CGT CTG ATT CTC TGT TTA GCT GAC A
mTransferrin Probe	6FAM-CTG GCC TGA GCT CCT-NFQ
NLS-LacZ 3' primer	GCC ACT TCT TGA TGG ACC ACT T
NLS-LacZ 5' primer	CCG CCA CCG ACA TCA TCT

NLS-LacZ Probe	FAM-CAC GCG GGC GTA CAT-NFQ
Luciferase 3' primer	TTG TCG ATG AGA GTG CTC TTA GC
Luciferase 5' primer	GCG CAG CTT GCA AGA CTA TAA G
Luciferase probe	FAM-CTG GTG CCC ACA CTA T-NFQ
CFTR 3' primer	CCT TTA GAG AGA AGG CTG TCC TT
CFTR 5' primer	CGC TGA TGC GAG GCA GTA T
CFTR probe	FAM-CCC TGC TCA GAA TCT-NFQ

2.1.7 LV LacZ Titre Assay

Pre-Xgal	35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in PBS
Xgal	40 mg/ml Xgal in DMF

2.1.8 Animal Models

C57Bl/6	Laboratory Animal Services, University of Adelaide, SA (AUS)
	ARC, Perth, WA (AUS)
CF (*cftr*^{tm1unc})	CF colony stock, Animal Care Facility, WCH, SA

2.1.8.1 Anaesthesia

Domitor:Ketamine Mix	0.1 mg/ml Medetomidine, 7.6 mg/ml Ketamine in sterile H_2O

Antisedan Reversal	0.5 mg/ml Atipamazole in sterile H_2O

2.1.8.2 Luciferase Imaging

D-Luciferin	15 mg/ml D-Luciferin in PBS

2.1.9 Processing of Mouse Heads

Carnoy's Fixative	60% (v/v) Ethanol, 30% (v/v) Chloroform, 10% (v/v) Glacial acetic acid
Decal	7% (v/v) HCl in 1.5% EDTA (w/v) in dH_2O
10% NBF	10% (v/v) Formalin, 0.22 M NaH_2PO_4, 0.45 M Na_2HPO_4 in dH_2O
PFA /Glut	2% (w/v) PFA, 0.5% (v/v) Glut in PBS
Pre-Xgal	5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in PBS
Xgal	20 mg/ml Xgal in DMF

2.1.10 ELISA Assay

2.1.10.1 p24 ELISA

HIV-1 p24 ELISA Kit	Perkin Elmer Life Sciences (USA) Cat # NEK050

2.1.10.2 ELISA for Serum Antibodies

Anti-mouse IgG HRP, from sheep	GE Healthcare (AUS) Cat # NXA931
PBST	0.05% (v/v) Tween-20 in PBS
SKMPBST	5% (w/v) Skim milk powder in 0.05% (v/v) Tween-20 in PBS
OPD substrate	0.4 mg/ml OPD, 0.4 mg/ml urea hydrogen peroxide, and 0.05 M phosphate-citrate, pH 5.0, in dH_2O.

2.1.11 Nasal TPD

Agar Bridges	3% (w/v) Agarose Type C, gelling T 40-43°C in 0.9% NaCl
Calomel Electrodes	Cole-Parmer Instrument Co. (USA) Cat # E-05990-50
Iso milivolt Meter	World Precision Instruments (USA) Cat # ISOMIL-A
Basal KRB	2.4 mM KH_2PO_4, 0.6 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM Hepes, 135 mM NaCl in dH_2O
Basal + Amil KRB	0.1 mM amiloride in Basal KRB
Low Cl + Amil KRB	0.1 mM amiloride, 2.4 mM KH_2PO_4, 0.6 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM Hepes, 135 mM Na-Gluconate in dH_2O
Pasport Sensor	Pasco Scientific (USA) Cat # PS-2147
PE 10 Tubing	SteriHealth (AUS) Cat # I-10338
Perfusion Pump	World Precision Instruments (USA) Cat # SP200 1Z

2.2 METHODS - *IN VITRO*

2.2.1 DNA Plasmid Preparation

2.2.1.1 Large Scale Plasmid Preparation

All plasmids were prepared using the Endofree Plasmid Mega-Kit (Qiagen, Germany) according to the manufacturer's instructions.

2.2.1.2 Agarose Gel Electrophoresis

Confirmation and quantification of plasmid preparations were performed via gel electrophoresis. Various DNA restriction enzyme digestions were visualized on 1.2% - 1.8% (w/v) agarose in 1x TE buffer gel, with the addition of 0.5 μg/ml ethidium bromide. The gel was submerged in 1x TE buffer with the addition of ethidium bromide and run at 100 volts and 400 mA. The purified plasmid was digested in the appropriate buffer and enzyme according to the manufacturer. 300ng/ml of DNA was loaded on to the gel in the presence of bromophenol blue dye. Fluorescence was measured under UV light and photographed. DNA fragment sizes were compared to the Standard DNA molecular weight marker SPP1/*Eco*RI (Geneworks, AUS).

2.2.2 Cell Culture Techniques

Cells were sub-cultured when confluent. Media was aspirated from the cell culture flasks; remaining adherent cells were rinsed in phosphate buffered saline (PBS) and then the PBS was aspirated to remove excess media. To detach cells from the culture plate 10% trypsin in PBS was added and incubated for at least 3 minutes (min). Detachment of cells was confirmed under light microscopy and cells were spritzed to form a single cell suspension. Cells were sub-cultured into required dilution,

usually 1:2 to 1:10, with appropriate media and transferred to fresh flasks. Cells were incubated at 37°C and 5% CO_2 for the appropriate time.

2.2.3 LV Vector Preparation

The LV vector was produced by transient transfection of 293T cells with a five plasmid system using calcium phosphate co-precipitation[296, 336]. Briefly, 293T cells were expanded over 5 days then split when confluent and plated onto 150mm dishes. On day 6 293T cells were harvested and cells counted with a haemocytometer and adjusted to a suspension containing 4.5 x 10^5 cells/ml with the addition of the appropriate volume of Dulbecco's modified Eagle's medium (DMEM) /10% (v/v) fetal calf serum (FCS) / penicillin-streptomycin (Pen-Strep). A total of 4.5 x 10^7 cells/105 ml was slowly poured onto 15 - 20 x 245 mm plates. Plates were incubated for 20-24 hours and transfected with the relevant plasmids with 320 μl of 2.5 M $CaCl_2$.The ratio of plasmids used for all virus preparation (per 245 mm square plate) was 158 μg of the LV vector plasmid, 3.16 μg of pcDNA3 Tat, 3.16 μg of pHCMVRev, 15.18 μg pHCMVgagpol, 7.9 μg pHCMV-G.

For each preparation the LV vector DNA plasmids used were: the reporter genes β-galactosidase (LacZ) and Luciferase (Luc); a no transgene empty vector (MT) and the therapeutic gene (CFTR).

Equal volumes of DNA / $CaCl_2$: 2 x HeBS were mixed by vortexing for 30 seconds and allowed to stand for a further 90 seconds. DNA-$CaPO_4$ precipitate was then slowly pipetted from a low height over the entire 245 mm plate. Plates were then incubated for 8 hours and media changed to serum-free media with glutamine and Pen-Strep. The number of plates ranged from 8- 22 depending on scale of virus production. Forty eight hours later, virus was collected in serum-free medium and processed using a combination of ultrafiltration and ultracentrifugation for medium and large scale virus preparations[336]. Briefly, virus supernatant was filtered through an Amersham 0.45 μm hollowfibre cartridge (CFP-4-E-

4MA) and was then concentrated using an Amersham Quickstand system with an UFP-750-E-4x2MA 750 kDa cut-off cartridge, all at room temperature. The retentate was then passed through a 0.8 µm syringe filter unit (Millipore Millex-AA) before ultracentrifugation at 50,000 *g* for 90 min (Beckman SW40) at 4°C. Virus pellets were resuspended in PBS and all virus preparations were aliquoted into sterile eppendorfs and stored at -70°C until required.

2.2.4 Quantification of Virus Titre

2.2.4.1 Xgal Staining for LacZ Titre

CHO cells or A549 cells were seeded at 0.5 x 10^6 cells/ml in 24 well plates and incubated for 3 hours in F12 Media / 10% (v/v) FCS. Media was aspirated, replaced with F12 / 10% FCS supplemented with 4 µg/ml polybrene and 2 µg/ml gentamycin and cells were transduced with the LV vector. Media was then changed at 24 hours and replaced with F12 / 10% FCS / gentamycin for a further 48 hours. Cells were aspirated, rinsed with PBS and fixed with 0.1% glutaraldehyde (Glut) in PBS for 15 minutes on a rocking platform at room temperature. Cells were washed 3 times in 1 mM $MgCl_2$ / PBS for 10 minutes each. Cells were incubated overnight with 1:40 dilution of Pre-Xgal : Xgal solution (see 2.1.7) at 37°C. The Xgal solution was aspirated, rinsed with PBS, and cells were stored in 80% glycerol. LacZ gene expression was quantified as the number of blue stained cells averaged from 2 fields of 1.88cm^2/well using light microscopy.

2.2.4.2 qPCR for Virus Titre

Genomic DNA was analysed by qPCR for determination of the virus titre for the LV preparations containing the therapeutic gene (CFTR) or no gene (empty (MT)).

2.2.4.2.1 Sample preparation for real time qPCR for virus titre

NIH3T3 cells were seeded at 0.5 x 10^6 cells/ml in 24 well plates and incubated for 3 hours in DMEM / 10% FCS. Media was aspirated, replaced with DMEM / 10% FCS supplemented with 4 μg/ml polybrene and 2 μg/ml gentamycin and cells were transduced with the LV vector for 24 hours. Media was then changed and replaced with DMEM / 10% FCS / gentamycin for a further 24 hours. Cells were then split at 1:4 ratio and cultured for a further 2 days. All LV vector preparations cells were sub-cultured for up to 4 weeks. Media was aspirated and cells were rinsed with PBS. Cells were harvested by addition 0.5ml of 10% trypsin / PBS and the trypsin was neutralized by adding the cell suspension to a tube containing 1% (v/v) FCS / PBS. The samples were then centrifuged at 2,000 rpm for 5 min, the supernatant removed and the remaining pellet resuspended in 3 ml PBS. The cell pellet was washed a further 2 times with PBS, the supernatant was then aspirated and isolation of genomic DNA was performed using the Wizard SV Genomic DNA Purification System (Promega, USA) as per manufacturer's instructions. Genomic DNA was stored at -20°C until analysed.

2.2.4.2.2 qPCR for virus titre

Each 20 μl reaction contained 1 μl of 20 x assay mix, 10 μl of 2 x TaqMan Universal PCR Master Mix and 5 μl of genomic DNA in H_2O. Each assay contained a sample for detecting the Gag sequence and either the control mTransferrin gene or CHOK-1 (see section 2.1.6). All samples were performed in triplicate including a non-template control and a standard. All samples were analyzed on a 7300 real time PCR machine (Applied Biosystems) under the following cycles: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. The fluorescence of each reaction was read at the end of each cycle and the amplification plot was constructed using the 7300 system software. Cycle threshold

(Ct) bar was set in the linear part of the graph and the Ct for each sample calculated. To determine LV titre the following formulas were applied:

ΔCt = Ct of gag – Ct of mTransferrin (or CHOK-1) for a sample

$\Delta\Delta$Ct = ΔCt – 1 (or average of Standard)

$1/ 2^{\Delta\Delta Ct}$ = copy number per cell

Titre = (number of cells initially plated x copy number per cell x 1000 volume in μl)/dilution factor

= infectious units/ml

2.2.5 Replication Competent Viral Assay

This assay for replication competent virus relies on assaying the HIV-1 Gag protein (p24) in the medium of cells transduced with recombinant virus.

2.2.5.1 Sample Preparation for p24 ELISA

293T cells were seeded at 5 x 10^5 cells/ml in DMEM / 10% FCS in 12 well plates and incubated for 4 hours. Media was replaced with DMEM / 10% FCS supplemented with 4 μg/ml of polybrene and 2 μg/ml of gentamycin. An aliquot of the final concentration of virus was added to three wells. A control was also included in triplicate. Cells were incubated for 24 hours and media was then replaced with DMEM / 10% FCS / gentamycin. Cells were split 1:10, and on days 6, 12 and 18 a 450 μl aliquot was removed and stored at -70° C until required. Samples were clarified by centrifugation at 2,000 rpm for 5 min. The supernatant was assayed undiluted for p24 by ELISA.

2.2.5.2 p24 ELISA for Replication Competent Virus

Samples were analysed using the HIV-1 p24 ELISA kit (Perkin Elmer, USA) according to manufacturer's instructions with slight modifications. That being the standard curve was diluted to a lower range of concentrations (6.25-800 ρg/ml) than was recommended. All samples were assayed in triplicate. The reaction was stopped after 10-15 min and the absorbance was read at 490 nm. Concentration of p24 in the virus was calculated from the standard curve.

2.2.6 qPCR for Transgene Presence in Tissue Samples

2.2.6.1 Tissue DNA Extraction

2.2.6.1.1 DNA extraction via Wizard SV purification method

The isolation of genomic DNA from mouse tissues such as spleen and liver was performed using the Wizard SV Genomic DNA Purification System (Promega, USA) as per manufacturer's instructions. Genomic DNA was stored at -20°C until analysed.

2.2.6.1.2 DNA extraction via direct tail lysis method

The isolation of genomic DNA from mouse tissues such as nasal septum and lung was performed by DirectPCR Tail Lysis Reagent (Viagen Biotech, USA) method. A 1:50 ratio of tail lysis buffer to Proteinase K (20 mg/ml) was added to either whole nasal septum or 20 mg of lung tissue. Samples were mixed by vortexing and then incubated at 55°C overnight. Tissue samples were then heated to 80°C for 1 hour to inactivate RNA. The crude lysates were stored at -20°C until analysed.

2.2.6.2 qPCR for Transgene Presence in Tissues

Genomic DNA or crude lysates from various tissues were assayed by qPCR for the presence of the LV transgene. Samples were assayed in triplicate as in 2.2.4.2.2. Each assay contained a sample for detecting the Gag sequence, and the house-keeping gene mouse-transferrin sequence that was normalised using a single copy sequence (see 2.1.6.2). DNA from an NIH3T3 derived cell line, and containing a single copy of the LV vector was used to provide an absolute standard for copy number as previously described[337].

Results expressed as ΔCt = Ct (transgene-gag) – Ct (mouse-transferrin)

ΔΔCt = Normalised to control standard (NIH3T3 cells)

Vector Copy Numbers = $2^{-\Delta\Delta Ct}$

2.2.7 Presence of Circulating Antibodies

Sera collected from mice were analysed for antibody presence using an ELISA assay. Uncoated EIA 96 well plates (Corning Scientific, USA) were coated with 50 μl/well (500 ng/ml) of either β-galactosidase from *E. coli* for antibodies to LacZ; recombinant Luc for antibodies to Luc and PDZK1 peptide for antibodies to the CFTR protein. For detection of antibodies to the LV envelope protein (VSV-G) plates were coated with either cell supernatant diluted in PBS or a LV vector containing an alternative transgene such as enhanced yellow fluorescent protein (EYFP) diluted in PBS (5 μl/ml). All incubations were performed for 1 hour at 37°C (unless stated otherwise) and 0.05% (v/v) PBS-Tween (PBST) washes performed 3 times. Coated plates were incubated at 37°C for 1-2 hours and then stored

overnight or up to 1 week at 4°C. Wells were washed with PBST and blocked with 100 μl of 5% (w/v) Skim milk PBST (SKMPBST). Following incubation and PBST washes, 50 μl of the primary antibody (mouse sera diluted 1/50 in PBS) was added to wells and serial dilutions were performed. Negative and positive controls were included in each assay. After incubation and PBST washes, 50 μl of secondary antibody (anti-mouse IgG HRP, derived from sheep) was then added to all wells and incubated. Wells were washed again and 50 μl of OPD substrate was added and incubated for 15 minutes in the dark at room temperature. Reaction was stopped with 25 μl of 2.5 M H_2SO_4 and absorbance read at 490 nm.

Linear dilution curves were constructed on a semi-log scale and the limit of detection (cut off) was calculated as the average of the optical density (OD at 490nm) plus 3 standard deviations above the negative controls and blank wells. The end point titre was determined by the last diluted specimen to give a positive result above the determined cut off. The level of circulating antibodies was expressed as the dilution to achieve the end point $\log_{10}$ titre.

2.3 METHODS - *IN VIVO*

2.3.1 Animal Care and Maintenance

All animal studies were conducted with dual approvals from both the Animal Ethics Committees at the Women's and Children's Hospital (WCH) and the University of Adelaide, Adelaide, SA, Australia. All mice were observed weekly by WCH animal house staff. All transgenic mice and LV transfected mice were held under physical containment 2 (PC2) conditions as directed by the Office of the Gene Technology Regulator (OGTR) guidelines. Female C57Bl/6 mice were used due to convenience of housing, but both genders were used due to low numbers of animals bred by the CF null colony. We have previously demonstrated that there is no difference between LV transduction in the nasal airways of both normal and CF mice of both genders (Cmielewski, unpublished). For CF knockout mice, the addition of a mild diarrhoeal agent (ColonLytely®, Dandy Pharmaceuticals, AUS) diluted in drinking water (0.045% w/v) prevents fatal gut obstructions. CF mice were fed a mixture of high (10%) and low (5%) fat mouse chow.

All LV experiments were performed in a biological safety cabinet in a PC2 laboratory. All mice were monitored and weighed daily for a week after any procedure involving an anaesthetic. For other procedures, such as submandibular blood sampling, mice were monitored and weighed daily for 2-3 days post procedure. For mice undergoing long term experimentation (6 months - 2 years) mice were examined twice weekly and weighed fortnightly. As the mice in this experimental group aged, monitoring was increased when necessary.

2.3.2 Lentiviral Nasal Instillation

2.3.2.1 Pre-treatment Preparation

PBS without calcium and without magnesium (MP Biomedicals, USA) was used as a non-treatment control. One tablet was dissolved in 100 ml of MiliQ water, sterile filtered and aliquots stored at -20°C until required. LPC (Sigma Aldrich, USA) was dissolved in sterile PBS via sonication to avoid excessive bubble formation. LPC was stored at 4°C for up to 1 month.

2.3.2.2 Lentiviral Vectors

The studies using the LV vector containing only one transgene were instilled at the maximum titre. In long term studies mice were instilled with a mixture of two different LV preparations. For these dual vectors – containing the Luc gene and either the LacZ gene for C57Bl/6 mice or the CFTR gene for CF mice – a 1:1 mixture of the LV vectors at similar titres was created. The "empty" LV vector i.e. containing no transgene present (MT) was diluted 1:1 in PBS.

2.3.2.3 Nasal Instillations

Female C57Bl/6 mice, 8-10 weeks of age and male and female knockout CF mice (*cftr*^{tm1unc}) aged 7-15 weeks of age, were anaesthetised with 10 µl/g body weight of medetomidine (domitor) (0.1 mg/ml, Orion Corporation, Finland) and ketamine (7.6 mg/ml, Parnell Laboratories, AUS) mixture, delivered by an intraperitoneal (i.p.) injection. Instillations were made into the right nostril *via* a micropipettor with a gel-loading tip (Eppendorf, USA), as described previously.[296-298] Pre-treatment with 0.3% LPC or control (PBS) was via a 4 µl aliquot one hour prior to a 20 µl bolus of the LV vector; the latter delivered in 2 x 10µl aliquots over a minute, via passive (inhalation-driven) fluid uptake. Anaesthesia was reversed with 2 µl/g i.p. injection of atipamazole (0.5 mg/ml, Orion Corporation, Finland). Mice were kept warm in a

small temperature controlled cabinet at 32°C between nasal instillations and during the recovery period after reversal of the anaesthetic.

2.3.3 Gene Transfer Assessment

2.3.3.1 Reporter Gene Transfer

2.3.3.1.1 LacZ expression via Xgal staining method

Mice treated with the LacZ reporter gene were humanely killed by CO_2 asphyxiation and blood was removed via cardiac puncture. Mice were decapitated; the fur removed from the head and the tip of the nasal cartilage was trimmed. Using a blunt needle attached to a syringe; at least 1 ml of fixation solution was flushed through the tracheal stump along the nasal passages and drained out from the nares. The entire head was then fixed in the 2% (w/v) paraformaldehyde, 0.5% (v/v) Glut in PBS fixation solution for 2 hours at 4°C, with the fixation solution flushed through the tracheal stump every 30 minutes. Heads were then rinsed twice in 1 mM $MgCl_2$ for 15 min each at 4°C, then stained with a 1:20 dilution of Xgal : Pre-Xgal solution (see 2.1.9) for 4 hours at 37°C, with hourly flushing through the tracheal stump. Tissues were rinsed in 0.9% NaCl for 15 min and fixed in 10% neutral buffered formalin for at least 22 hours at room temperature. Heads were then placed in Decal solution (see 2.1.9) for 22 hours and the solution rinsed off in running water for 15-30 min. Heads were then stored in 70% (v/v) ethanol ready for histological processing.

2.3.3.1.2 Histological processing for LacZ expression

The lower jaw of the mouse was removed to expose the upper palate. The treated side (right side) of the head was marked with Indian ink and fixed by brief dipping into a solution of Carnoy's fixative (see 2.1.9). Using a microtome blade, the palate was dissected at 4 designated locations originating from the

nasal tip in a caudal direction. Section 1: directly behind the front incisors; section 2: between the 2nd and 3rd ridge of the hard palate; section 3: before the last palate ridge; and section 4: level of the ears. The sections were placed into histological cassettes with foam squares to keep the sections in their correct orientation for embedding. Gross sections were examined under a dissecting microscope and *en face* photographs were recorded for LacZ gene expression.

Histological cassettes were then sent to the WCH Histopathology Department for embedding in paraffin wax. Tissue was sectioned and stained at either the WCH Histopathology Department or Adelaide Microscopy (University of Adelaide, SA). Two sections per sample were cut and one was stained with Haematoxylin and Eosin (H&E) for morphological analysis and the other with Safranin O (SafO) as a counterstain to reveal the blue staining of LacZ gene expression. The total number and type of positive blue stained cells from the SafO stained samples were counted in all sections under light microscopy at 400-1000 x magnification.

2.3.3.1.3 Luciferase gene expression via biophotonic imaging

Mice treated with the Luc reporter genes were transported under PC2 conditions according to OGTR guidelines, to the Biophotonic Imaging Facility at Adelaide Microscopy. Mice were anaesthetised with domitor:ketamine mixture (see 2.3.2.3) and a bolus of the substrate D-luciferin (see 2.1.8.2) was administered as either an i.p. injection or intranasally (i.n.). The dose of D-luciferin was either 10 μl /g body weight injected i.p. or more commonly as a 50 μl volume instilled or “sniffed” into both nostrils as a single bolus inhalation[267]. After a lag time of 10 min, mice were placed in a supine position on the warmed (34°C) platform in the IVIS-100 Bioluminescence Imaging Machine (Xenogen Corp, USA) and luminescence measured with medium binning, fully open f-stop, for 1-2 min exposure.

The ensuing graphic image of photon (Ph) flux was represented by colour intensity in units of Ph/sec/cm^2/sr. Luminescence was calculated by the Igor Pro 4.09A Living Image Software (Xenogen Corp, USA) as flux (Ph/sec) in an area defined by contour measurement parameters. Following imaging mice were allowed to recover after the reversal of the anaesthetic (see 2.3.2.3) and transported back to the WCH Animal Care Facility, again under OGTR PC2 guidelines.

2.3.3.2 CFTR Gene Expression

2.3.3.2.1 Nasal transepithelial potential difference measurements

In all CF mice (including non-treatment controls) CFTR gene expression was estimated using the nasal TPD technique as previously described[296, 300]. Mice were anaesthetised as described in 2.3.2.3 and suspended by their front incisors with their weight supported. Mice were kept warm using radiant heat during the procedure by the placement of a heated wheat bag adjacent to the body of the mouse. An agar filled bridge (see 2.1.11) was inserted subcutaneously via a 21G needle into the abdomen of the mouse with the tubing system connected to a tube containing 3 M KCl, as a reference electrode. A fine, heat pulled polyethylene (PE-10) cannula was inserted 2.5-3 mm into the right nostril of the mouse, the region of respiratory epithelia in the nasal cavity[338], while connected via PE tubing to a 1 ml gas tight glass syringe (Hamilton, USA) filled with krebs-ringers buffer (KRB) solution (see 2.1.11) that was attached to a perfusion pump SP200 series (WPI, USA). Another agar filled bridge connected the liquid tubing system to another tube containing 3 M KCl solution. Two calomel electrodes (Cole-Parmer Instrument Corp, USA) were suspended in the KCl solution and connected to an Iso millivolt meter (WPI, USA) completing the liquid/electrical circuit. The electrical output in millivolts (mV) was recorded using a data logger PasPort sensor (Pasco Scientific, USA) and nasal TPD recordings graphically

represented by computer software DataStudio, Version 1.9.8r2 (Pasco Scientific, USA). Diagrammatic representation of the nasal TPD set up is shown in Figure 2-1.

Basal KRB solution was infused at 1 μl/min establishing a baseline TPD until at least 1-2 min plateau was reached. The low perfusion rate prevented fluid overload in airways in long TPD studies. The solution was then replaced with basal KRB but with the addition of the sodium channel blocker amiloride, at a concentration of 0.1 mM (B + A), and perfused for at least 10 minutes until the recorded TPD value reached a new plateau. The infusion was then switched to low chloride KRB solution, NaCl replaced with Na gluconate, with 0.1 mM amiloride (Low Cl^- (LC) + A) and the TPD recorded until a new plateau was established.

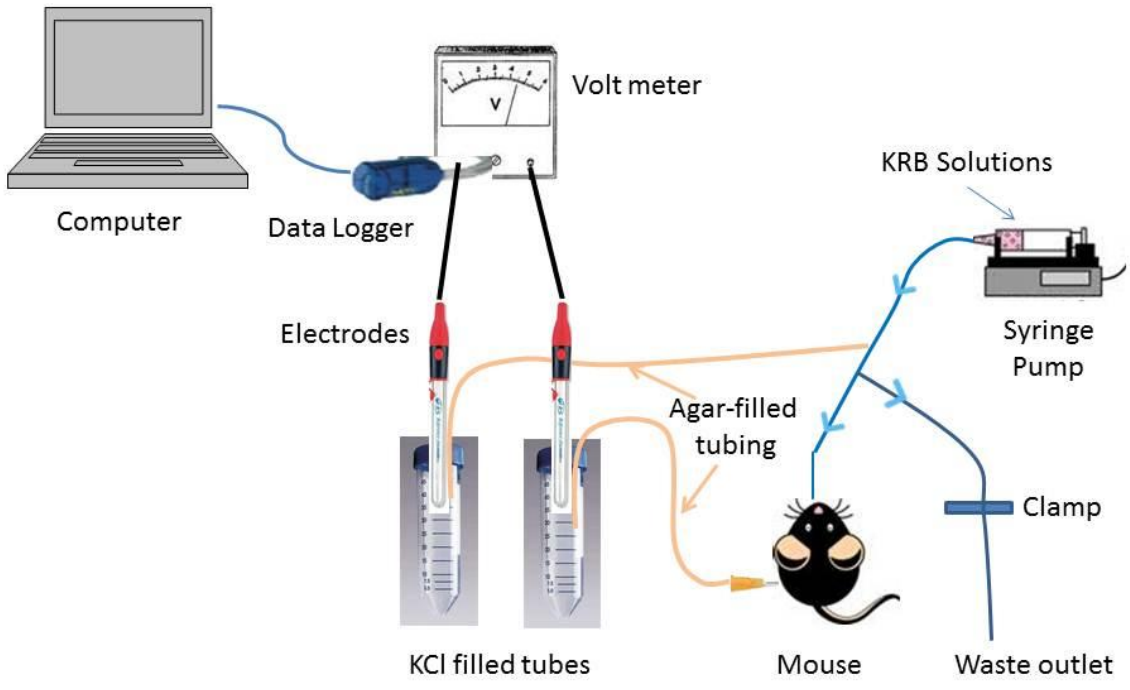


Figure 2-1 Diagrammatic Representation of the Nasal TPD Setup.
Nasal TPD set up as described in section 2.3.3.2.1

At the termination of recording the electrical drift was measured by changing the KRB back to basal and placing the nasal cannula and the needle reference electrode into a solution of 0.9% NaCl. If the difference in the electrical reading from the beginning of the recording to the end was greater than 2 mV the TPD measurement was rejected and the TPD was repeated at least one week later. This delay allowed for ample time for recovery of any damage to the nasal epithelial layer due to cannula insertion[339]. A cohort of Het (+/-) CF mice was also tested using the same nasal TPD protocol to provide a "normal" range for ion transport electrophysiology.

The mice were monitored continuously throughout the TPD procedure and any excess fluid from the nostril openings was removed by absorption with tissue wicks. When anaesthesia was reversed mice were allowed to recover in a chamber warmed to 32°C (see 2.3.2.3). As CF mice aged greater than 1 year, the dose of anaesthetic was reduced by 10-20% to reduce accidental anaesthetic overdoses. The treatment of the mice was blinded to the TPD operator for early time point assessments. However due to the natural attrition of CF mice as they age, as well as the repeated nasal TPD measurements on the same animal, blinded testing was no longer possible after the 9 month time assessment.

The values from the TPD recording were assessed by 2 other experienced TPD technicians who were also blinded to the treatment. If TPD values from all 3 assessors were in agreement the results were accepted. The change in the TPD values (ΔPD) was calculated as subtracting the values at the TPD plateau for the different KRB solutions (Figure 2-2).

ΔPD_{Na+} = TPD (Basal + Amiloride) – TPD (Basal)

ΔPD_{Cl-} = TPD (Low Cl + Amiloride) – TPD (Basal + Amiloride)

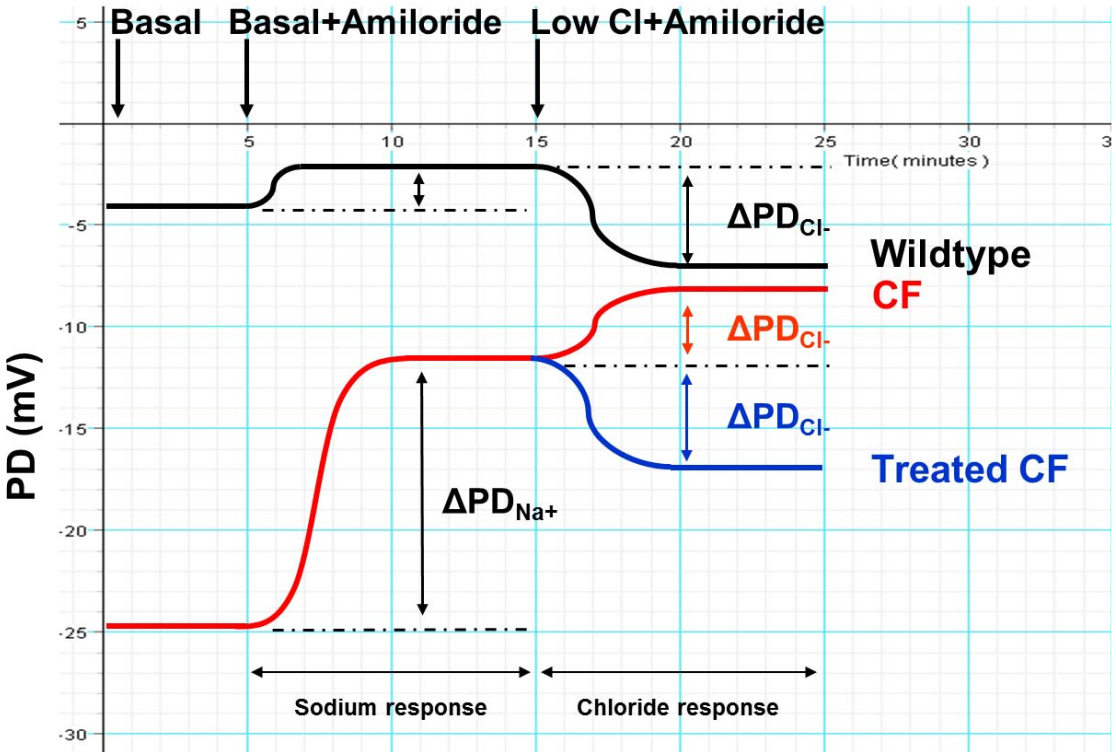


Figure 2-2 Schematic Representation of TPD Traces
Diagrammatic representation of nasal TPD traces for wildtype, CF (-/-) and LV CFTR treated CF mice.

2.3.4 Submandibular Blood Sampling

Blood sampling by submandibular puncture[340] was performed at various time points in both C57Bl/6 and CF mice. The veins that drain blood from various parts of the face meet in this area and form the jugular vein. This procedure was performed on both conscious and anaesthetized mice. For CF mice the procedure was predominantly performed after nasal TPD measurement, prior to reversal of the anaesthetic.

Mice were held by the scruff of the neck and their cheek pouch (area behind the hinges of the jawbone) was punctured using a goldenrod™ lancet (Medipoint International, USA). A 5 mm lancet was used for mice 6 months and under in age, and a 5.5 mm lancet was used for those mice greater than 6 months

of age. Blood was collected in an eppendorf tube (average of 200 µl, with a maximum volume of 300 µl in older mice, never greater than 1% of body weight) and bleeding stopped by gentle pressure with a tissue to the cheek. Mice recovered immediately with no side effects.

Blood samples were allowed to clot at room temperature for at least 30 min and then centrifuged at 13,000 rpm for 3 min. The supernatant was removed and the sera stored at -70°C until analyzed.

2.3.5 Nasal Septum Dissection

CF mice were humanely killed as described in section 2.3.3.1.1. After decapitation, fur and skin were removed and trimming of the nasal tip was conducted. Using fine iris scissors, cuts were made along the dorsal nasal bone suture lines, either side of the centre line of the nose. The top of the septum was carefully cut away from the nasal bridge, and the central bone flap was removed to expose the nasal septum. The septum was removed by fine dissection following the line of the base of the septum. Once detached from the nasal cavity, the septum was removed by fine forceps on the bony tissue portion, thus minimizing damage the respiratory epithelial area of the tissue. The nasal septum was then placed into an eppendorf tube and stored at -20°C until required.

2.3.6 Tissue Removal

Various tissues were removed for molecular analyses after mice were humanely killed (see 2.3.3.1.1), in particular lung, spleen and liver tissues. For CF mice that died unexpectedly only nasal tissue could be retrieved as full carcasses was sent to the Institute of Medical and Veterinary Science (IMVS) for necropsy. All tissue samples were stored at -20°C until required.

2.4 STATISTICAL ANALYSIS

Results in this thesis are represented as a mean and standard error of the mean ($\bar{X} \pm SEM$) where n=number of samples in each group. Statistical analyses were performed using SigmaPlot 11.0 and GraphPad Prism 5. Statistical significance was set at p = 0.05 and power = 0.80. When data failed normality, standard transformations or non-parametric methods were utilized. If power was below the 80%, caution in interpretation of data was noted.

Student t-tests were used for two group comparisons. Multiple treatment groups were analysed by one way analysis of variance (ANOVA) or repeated measures (RM) ANOVA, both with post-test multiple comparisons. For survival data Kaplan Meier plots were analysed by Mantel-Cox log-rank tests and linear regression performed by Spearman Correlation or Pearson's Correlation where appropriate.

Biostatistician Kate Dowling from the Public Health Research Unit (WCH) provided assistance with statistical analysis of long term data.

CHAPTER 3

LENTIVIRAL PRODUCTION & PRELIMINARY TESTING OF *IN VIVO* AIRWAY GENE EXPRESSION

CHAPTER 3 LENTIVIRAL PRODUCTION AND INITIAL *IN VIVO* TESTING

3.1 LENTIVIRAL PRODUCTION

3.1.1 Introduction

Gene delivery of HIV-1 derived LV vectors has been used in *in vitro*[341] and increasingly in *in vivo* model systems[342-344]. LV vectors have also been reported for use in clinical trials[283]. The effectiveness of LV vectors comes from their ability to transduce dividing and non-dividing cells, integrate into the host cell genome and because they possess a lower immunogenicity than other viral vectors[273, 345]. Our group has previously shown that persistent LV gene transfer, in conjunction with LPC as a pre-treatment, can be achieved[296, 297]. One of the main limiting steps in LV gene therapy is the ability to produce large quantities of vector at sufficiently high titres without affecting the safety profile. The optimization of the 5 plasmid LV vector system has enabled the production of consistent and large volumes of high quality vector and titres of 10-100 times higher than previously published[297]. This chapter describes the results of a large scale LV vector production system, *in vivo* pilot studies of the LV vector and the effects on gene transfer.

3.1.2 Aims

The aim of the studies described in this chapter was to produce large volumes of LV vector carrying reporter genes, no gene or the therapeutic CFTR gene without affecting concentration. Increasing titres of a LV vector may lead to improvements in the effectiveness of our gene transfer protocol, and allow for dilution of LV vectors. The mixing of LV vector batches containing different genes (reporter and/or

therapeutic) will enable assessments of gene transfer and expression in the same animal, without losing transduction efficacy of either gene.

3.1.3 Methods

3.1.3.1 Lentiviral Vector Titres

Each LV vector contained a single transgene as described in 2.2.3. LV vectors that would eventually be mixed and instilled as dual vectors were constructed with the same promoter. The promoter selected for these studies was the simian virus type-40 (SV-40), as used in earlier LacZ and CFTR studies[296, 297]. In single transgene studies using the reporter genes LacZ or Luc, the optimized promoter used was nuclear localized-codon optimized (MPSv-nls) and human elongation factor-α (EF1-α) respectively.

To obtain maximum titres from each virus batch, the final volumes from the medium scale LV preparation protocols ranged from 0.3 ml to 1 ml (from starting volumes of 800 ml – 1.1 L) depending on experimental needs, and a final volume of 2-5 ml was obtained for large scale preparations (from starting volumes of 2.2-5.1 L).

For LV-LacZ preparations the calculation of final LV titre was performed by two methods, Xgal staining as detailed in 2.2.4.1 and qPCR assay as described in 2.2.4.2. For the remaining LV preparations (LV-Luc, LV-MT and LV-CFTR) titre was calculated by qPCR only.

3.1.3.2 Replication Competent Virus

To test that the increase in titre did not produce an increase in replication competent virus, all LV vector batches were tested by the p24 ELISA assay as described in section 2.2.5.

3.1.4 Results

3.1.4.1 Lentiviral Vector Titres

Up-scaling of LV production resulted in consistent high titres compared to small scale preparations, as calculated by qPCR methods. For calculation of LV-LacZ preparations applying the visualisation method the final titre was under-estimated 15-50 fold compared to qPCR (Table 3-1).

LV PREPARATION	PROMOTER	FINAL VOLUME (ml)	Xgal Titre (tu/ml)	qPCR Titre (tu/ml)
LV-LacZ	SV-40	3.5	4.2×10^8	2.1×10^{10}
LV-LacZ $_{Co}$	MPSv-nls	5.0	1.0×10^9 †	1.6×10^{10}
LV-Luc	SV-40	0.5	-	1.8×10^{10}
LV-Luc $_{EF1\text{-}\alpha}$	EF1-α	1.0	-	0.9×10^{10}
LV-MT	SV-40	2.2	-	1.2×10^{10}
LV-CFTR	SV-40	0.3	-	4.9×10^{10}

Table 3-1 LV Preparations
Final titres of all medium and large scale LV vector preparations via Xgal or qPCR assays. Xgal assay performed on A549 cells or on †CHO cells. (Transducing units (tu) /ml, qPCR based on infectious unit equivalents).

3.1.4.2 Replication Competent Virus

After 6 days of LV transfection of cell cultures, low concentrations of p24 protein were present as expected (0.09-0.24 ng/ml). However by day 18, less than three weeks after transfection of cell cultures, no p24 protein was detected in any samples from any LV preparation.

3.1.5 Discussion

Regardless of the scale used for LV production, similar high titres of transducing units per ml were produced as determined by qPCR methods (Table 3-1). However, when calculating the titres of all the LV-LacZ preparations by the Xgal staining method on A549 cells, there was a 50 fold underestimation compared to qPCR. An optimized protocol using CHO-K1 cells instead of A549 cells (2.2.4.1) has greatly improved this assay, especially in relation to reducing unwanted and confounding background staining. There was an expected reduction in LV titre assayed by the Xgal method as it relies on the manual counting of clusters of cells that have been transduced and stained blue. The intensity of staining of cell clusters, the exclusion of single stained cells and background staining can all be contributing factors in this observed reduction in LV titre. This visualization method is not only influenced by operator bias, but also that the transgene itself may not be expressed at high enough levels to be counted and this effect may be dissimilar on different cell lines. That is, the Xgal staining assay has low sensitivity and low specificity. Quantitative PCR results are more reliable as the assay relies on a fluorogenic probe in addition to standard PCR primers. The advantage of the qPCR method is the specific hybridization between the probe and the target to generate a fluorescent signal, which reduces both background and false positives. As the DNA can be amplified and also quantified with this assay method, it achieves greater specificity as well as sensitivity compared to the visualization of the Xgal staining method.

The current need to apply prodigious numbers of gene vector particles to provide adequate levels of gene expression for potential therapeutic benefit *in vivo*[346] may also lead to an increase in constituents and contaminants in the vehicle that can induce strong host immune responses. All plasmid preparations were made with endotoxin free kits and all LV virus preparations were made in serum free media which helped reduce the potential of confounding contaminants in LV preparations. The up-

scaling of the LV preparations to produce larger batches without compromising titre efficacy – even increasing titre concentration by 10-100 times that previously reported[296, 297] – continues the development towards an effective and safe protocols for airway gene transfer within our group.

The finding that there was no detectable p24 levels (the 24 kilodalton protein that is the core component of HIV-1) after 3 weeks of transfecting cell cultures confirmed the safety profile of the LV vector preparations for absence of replication competent virus. The 5 plasmid system used in this thesis is based on second generation vectors. The later third generation vectors using a 3 plasmid system has more of the viral helper plasmids removed in an effort to increase safety. However the HIV-1 LTR may also provide an extra measure of safety as long as Tat is not transferred along with the vector[347]. Having a Tat-dependent self-inactivating vector reduces the probability of generating a functional replication-competent virus as this would require not only repair of the LTR, but also acquisition of the ability to express Tat.

3.2 TESTING OF VIRUS PREPARATIONS – *IN VIVO*

3.2.1 Introduction

To confirm and compare the transfection ability of the LV vector preparations, short term studies of LV gene expression using our nasal instillation protocol including LPC pre-treatment were conducted in mice. The standard dose of 4 μl of 0.3% LPC 1 hour prior to a 20 μl volume of the LV vector at maximum titre available is already established[297]. These studies were designed to determine the efficacy of the LV vector batches, which would occur with changes in our standard protocol in relation to volume, titre and the mixing of different LV preparations on gene transfer *in vivo*.

3.2.2 Methods

3.2.2.1 LacZ Gene Transfer

The pre-treatment dose and timing was the same in all studies, 4 μl of 0.3% LPC, this was given 1 hour prior to the LV vector (2.3.2.3). The right nostril of female C57Bl/6 mice (n=2) were instilled with either a volume of 20 μl (standard protocol) of LV-LacZ at the maximum titre or 1:1 mixture of LV-LacZ with either LV-MT or PBS. An increased volume of 40 μl LV-LacZ was also trialled. LV-LacZ reporter gene transfer was analysed after 7 days by Xgal staining, and for histological assessment blue stained cells were counted in 4 standard cross sections, as described in section 2.3.3.1.2.

3.2.2.2 Luciferase Gene Expression

Nasal instillations were performed as described in 2.3.2.3. LV vector carrying the Luc gene was diluted 1:1 in PBS delivered as a 20 μl bolus at 0.9 x 10^{10} tu/ml. The LV-MT vector was delivered in the same volume at maximum titre of 1.2 x 10^{10} tu/ml. The right nostril of anaesthetized female C57Bl/6 mice

(n=4) were instilled with either PBS (as a control) or 0.3% LPC 1 hour prior to receiving LV-Luc. Another group (n=4) of mice received 0.3% LPC 1 hour prior to the empty vector (LV-MT).

One week later Luc expression was monitored (see 2.3.3.1.3) and included a comparison of the effects of instillation of D-luciferin by either i.n. administration via sniffing[267] into both nostrils or by i.p. injection. Intranasal instillation of D-luciferin prior to imaging has been reported to increase sensitivity and allow for luminescence detection in the lung airways[267, 348, 349].

3.2.2.3 CFTR Gene Expression

In a pilot study to test the effectiveness of LV-CFTR gene delivery, nasal instillations were performed as described in 2.3.2.3 on two male CF knockout (-/-) mice aged 7 months. Mice received a bolus of 0.3% LPC an hour prior to a single bolus of 10 μl dose of LV-CFTR at 4.9 x 10^{10} tu/ml into the right nostril. This LV-CFTR dose was half the standard *volume* of our usual gene transfer treatments, but at the same final *titre* as used for all future studies. As demonstrated by LPC/LV-LacZ delivery a 10 μl volume at maximum titre produced the same gene expression as a 20μl volume of a 1:1 mix of maximum titre diluted in PBS. Similarly aged but untreated (UnRx) CF and Het (cftr +/-) male mice from the same colony were used as controls (n=2). Nasal TPD measurements were performed as described in 2.3.3.2.1. The same CF mice treated with LPC/LV-CFTR had repeated nasal TPD performed at 1 week to 2 month intervals.

3.2.3 Results

3.2.3.1 LacZ Gene Transfer

LacZ gene expression assessed 1 week following nasal delivery was restricted to the treated nostril (Figure 3-1a). When the LV-LacZ with the SV-40 promoter was used, the blue LacZ staining was limited

to the cytoplasm of the respiratory epithelia of the treated nostril (Figure 3-1b). However when the nuclear localised MPS promoter driven LV-LacZ$_{Co}$ was employed the resultant intense blue staining was observed not only in the nuclei but also in the cytoplasm of respiratory epithelia (Figure 3-1c).

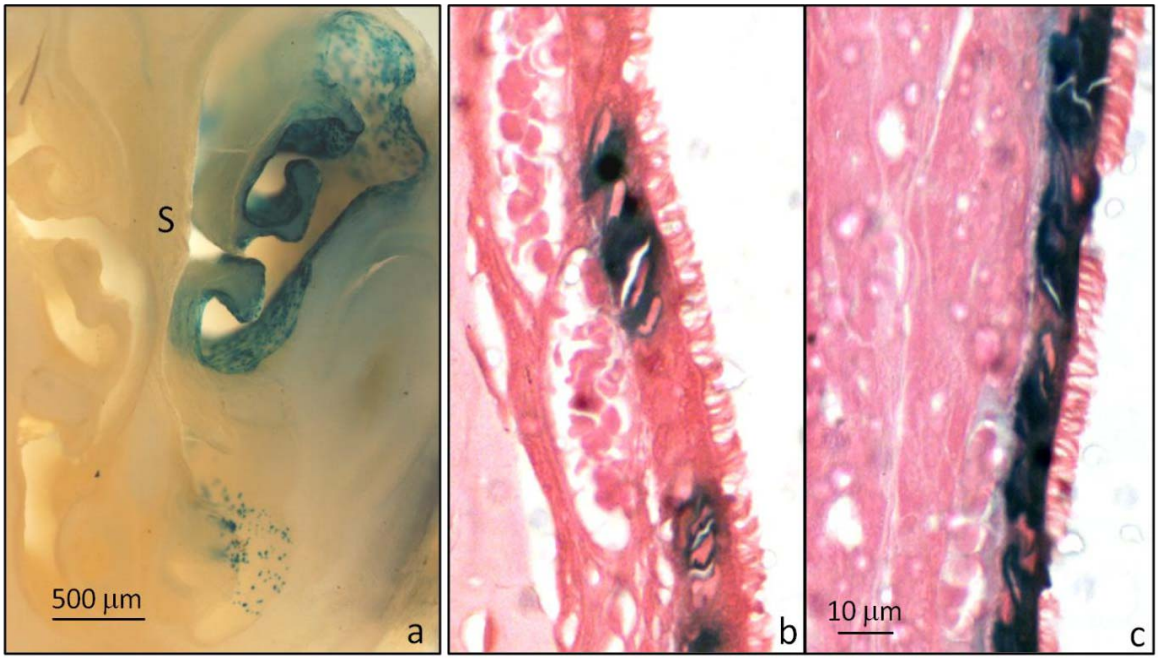


Figure 3-1 LacZ (blue staining) Transduction
a) En face image of LacZ staining (blue cells) on treated (right) side. S =nasal septum. Histological section of ciliated respiratory epithelia transduced with b) LV-LacZ SV-40 (cytoplasmic blue stain) and c) LV-LacZ$_{Co}$ (nuclear blue stain), SafO stain. Tissue cracking (b, c) is an effect of the SafO counter staining protocol.

Increases in LV titre resulted in an increase in LacZ gene transfer (Figure 3-2). Dilution 1:1 of the LV vector in PBS resulted in an approximate 2 fold reduction in LacZ expression. However with a 50 fold increase in LV titre, only a 2.4 times increase in the numbers of transduced cells was observed. There was a significant increase in LacZ gene transfer at titres of 2 x 10^{10} compared to 2 x 10^{8} tu/ml ($p<0.05$, ANOVA Tukey's post test). However due to the low sample size (n=2) in this pilot study, caution in interpretation of statistical analysis should be exercised.

The mixing of two different LV vector constructs in a 1:1 ratio reduced LacZ gene transfer 2 fold, which was similar to dilution with PBS and in accordance with a linear dose response at this titre. The presence of the different transgene had no interference in LacZ gene expression. The doubling in volume of the LV-LacZ vector at the same titre resulted in an approximate 1.2 fold increase in LacZ gene expression (Figure 3-3). Statistical difference between any of the three groups was not observed, however note the low sample size (n=2) used for this pilot study.

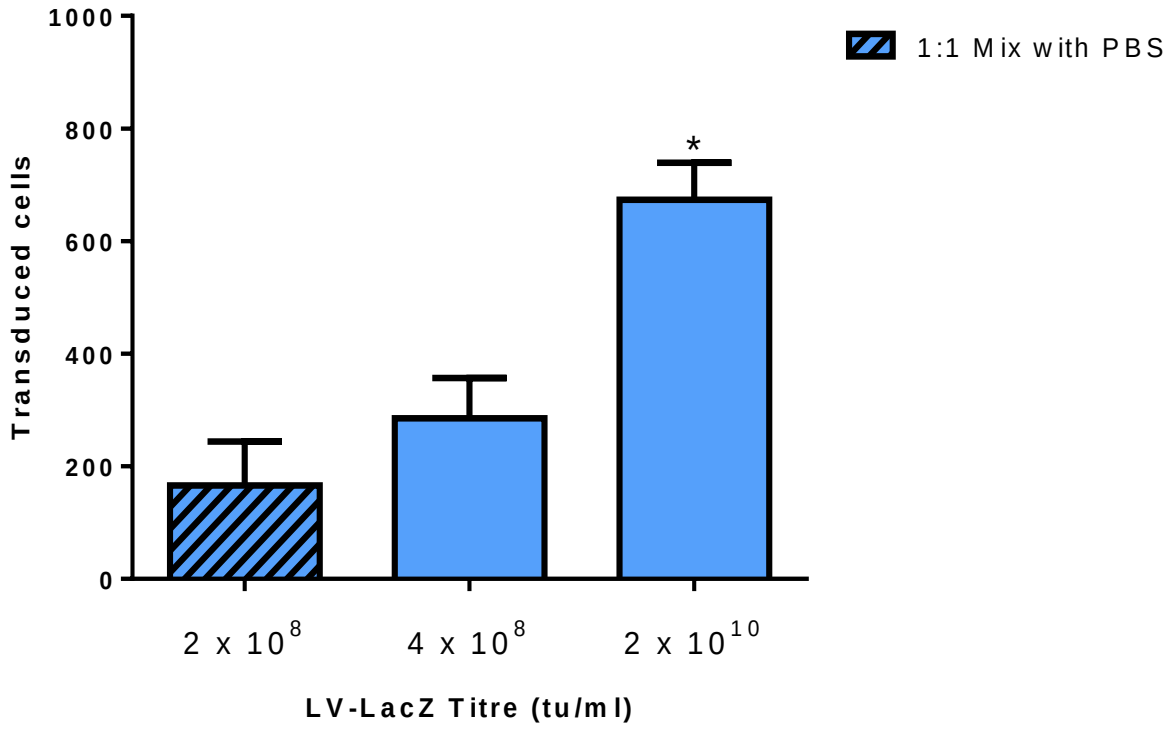


Figure 3-2 Titres LV-LacZ Gene Transfer – Dose Response
*LacZ gene transfer after 0.3% LPC pre-treatment of the standard 20 µl volume of LV-LacZ at different titres. The 1:1 mixture is a dilution of LV-LacZ in PBS. (*p<0.05, 2 x 10^8 vs 2 x 10^{10} tu/ml, Tukey's ANOVA, $\bar{X} \pm SEM$, n=2, low power p=0.5).*

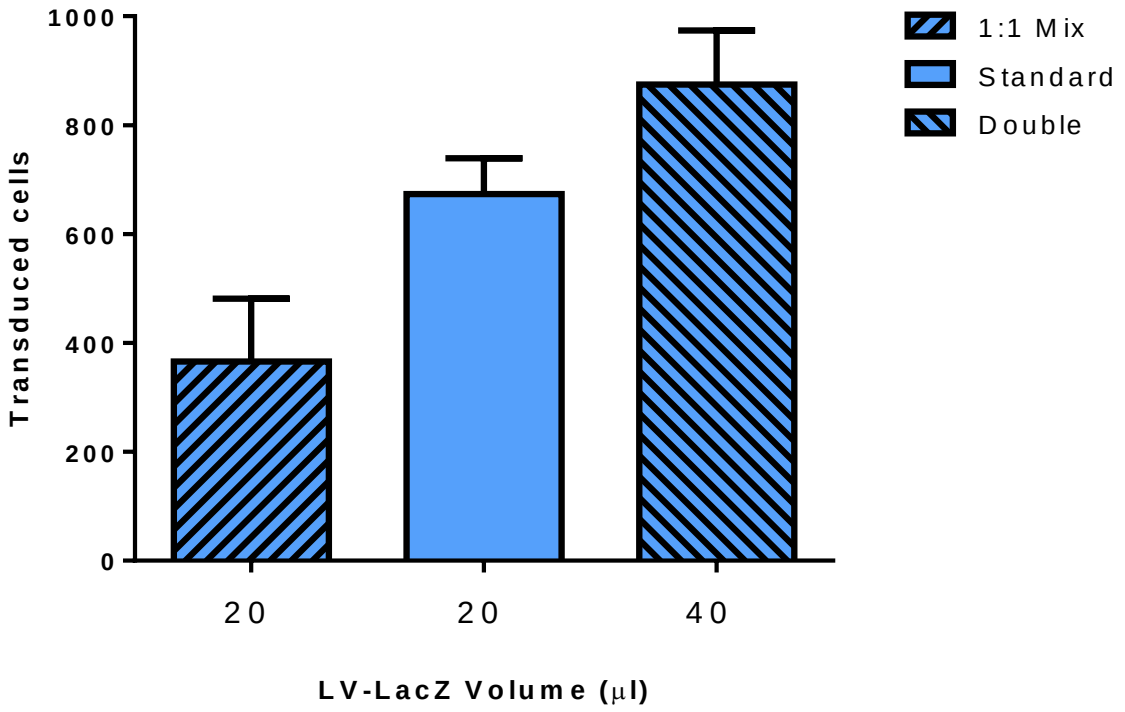


Figure 3-3 LV-LacZ Gene Transfer – Volumes
LacZ gene transfer after 0.3% LPC pre-treatment and different volumes of LV-LacZ (1.6 x10^{10} tu/ml). A 20 µl volume of a 1:1 mixture of LV-LacZ and LV-MT, a standard 20 µl volume of LV-LacZ versus a double dose of 40 µl (n.s., ANOVA, $\bar{X} \pm SEM$, n=2, power =0.44).

The proportion of respiratory cell types transduced was similar for all volumes tested. The majority of cells transduced with LacZ were ciliated and non-ciliated respiratory epithelial cells, with a small proportion of basal cells present (Figure 3-4).

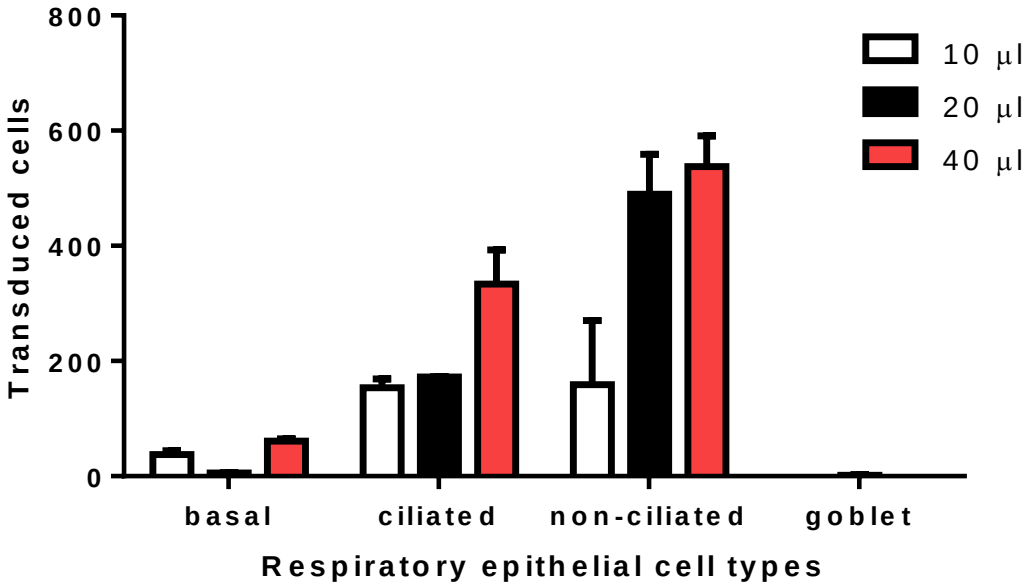


Figure 3-4 LacZ Transduced Cell Types
Proportion of respiratory cell types transduced with LacZ following LPC/LV-LacZ treatment ($\bar{X} \pm SEM$, n=2).

3.2.3.2 Luciferase Gene Expression

For those mice that received LPC prior to LV-MT, no Luc expression was observed (Figure 3-5a). Mice that received PBS as a pre-treatment prior to LV-Luc, expressed Luc in the lung airways (Figure 3-5b). However, luminescence was detected both in the nasal and the lung airways of mice that received LPC followed by LV-Luc (Figure 3-5c).

Luminescence was calculated as flux (Ph/sec) using either the contour region of interest (ROI) measurement or a defined square ROI (Igor Pro 4.09A, Living Image, Xenogen, USA) with the standard threshold setting including subtraction of the background image (Figure 3-6).

The effect of using these two flux parameters on Luc expression was tested as both have been published by various gene therapy groups[267, 348, 350]. Defined ROI square parameters were 1.5 cm^2 for nasal flux and a 2.2 cm height x 2.3 cm width for lung flux.

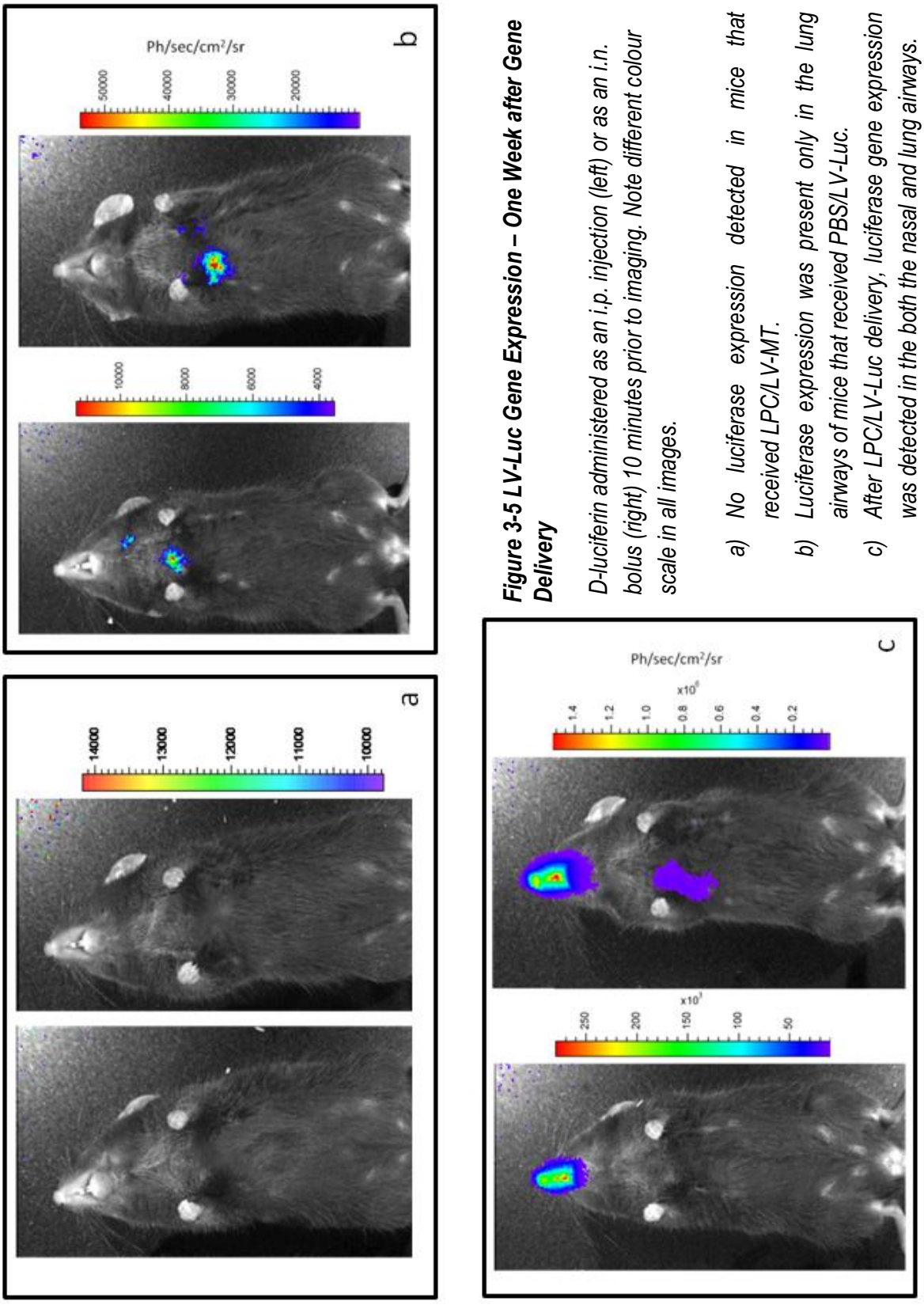


Figure 3-5 LV-Luc Gene Expression – One Week after Gene Delivery

D-luciferin administered as an i.p. injection (left) or as an i.n. bolus (right) 10 minutes prior to imaging. Note different colour scale in all images.

a) *No luciferase expression detected in mice that received LPC/LV-MT.*
b) *Luciferase expression was present only in the lung airways of mice that received PBS/LV-Luc.*
c) *After LPC/LV-Luc delivery, luciferase gene expression was detected in the both the nasal and lung airways.*

There was a difference in the luminescence flux in relation to the route of administration of D-luciferin 10 minutes prior to bioluminescence imaging (Figure 3-7). Nasal luminescence was significantly increased when D-luciferin was administered i.n. rather than by i.p. injection in the group of mice treated with LPC and LV-Luc ($p<0.01$, paired t-test, two tailed). Lung luminescence detected in the same mice was also significantly increased when D-luciferin was administered i.n., but only when flux was calculated as a defined square ROI ($p<0.05$, paired t-test, one tailed) and not by contour ROI parameters (Figure 3-8).

Due to the improvement in bioluminescence sensitivity in nasal airways, in subsequent Luc gene transfer experiments administration of D-luciferin was performed intranasally.

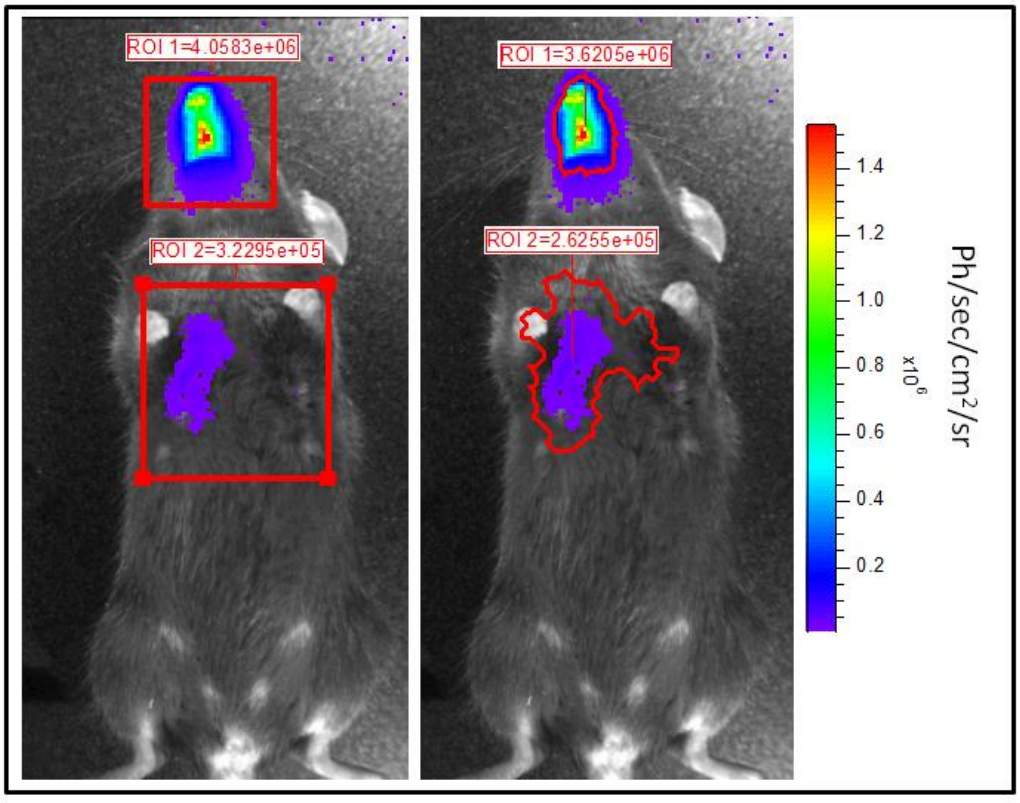


Figure 3-6 LV-Luc Gene Expression – ROI Calculations
Luciferase expression in the airways of the same mouse that received LPC/LV-Luc. Flux (Ph/sec) calculated as region of interest (ROI) as defined squared parameters (left) or by contour parameters (right).

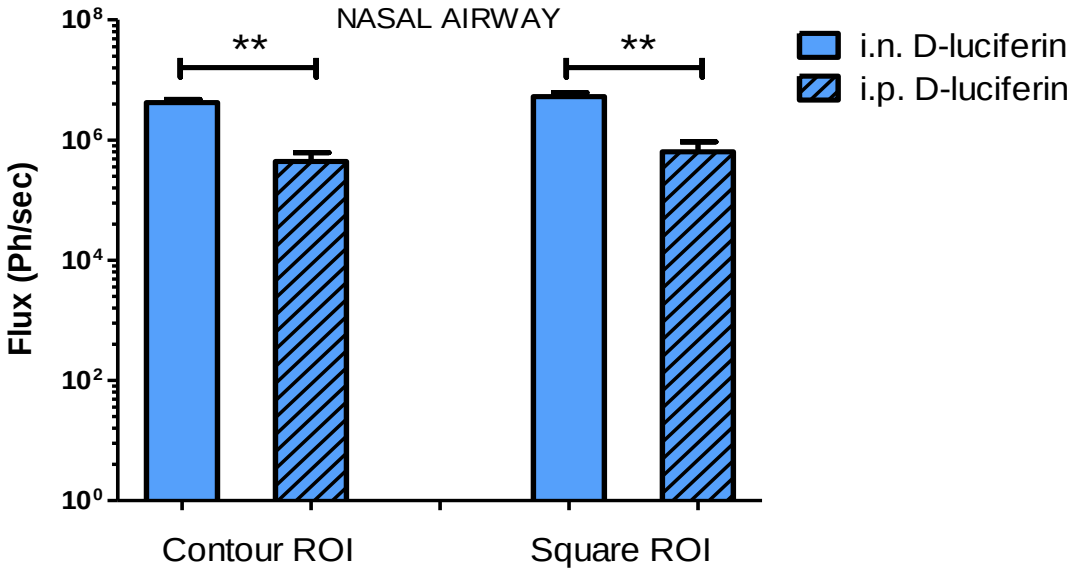


Figure 3-7 Nasal Luminescence – LPC/LV-Luc
*Nasal luciferase gene expression after mice treated with LPC/LV-Luc as calculated by ROI mesurements. Intranasal (i.n.) D-luciferin administration (open bars) vs interperitoneal (i.p.) injection (hatched bars) (**p<0.01, paired t-test, $\bar{X} \pm SEM$, n=4).*

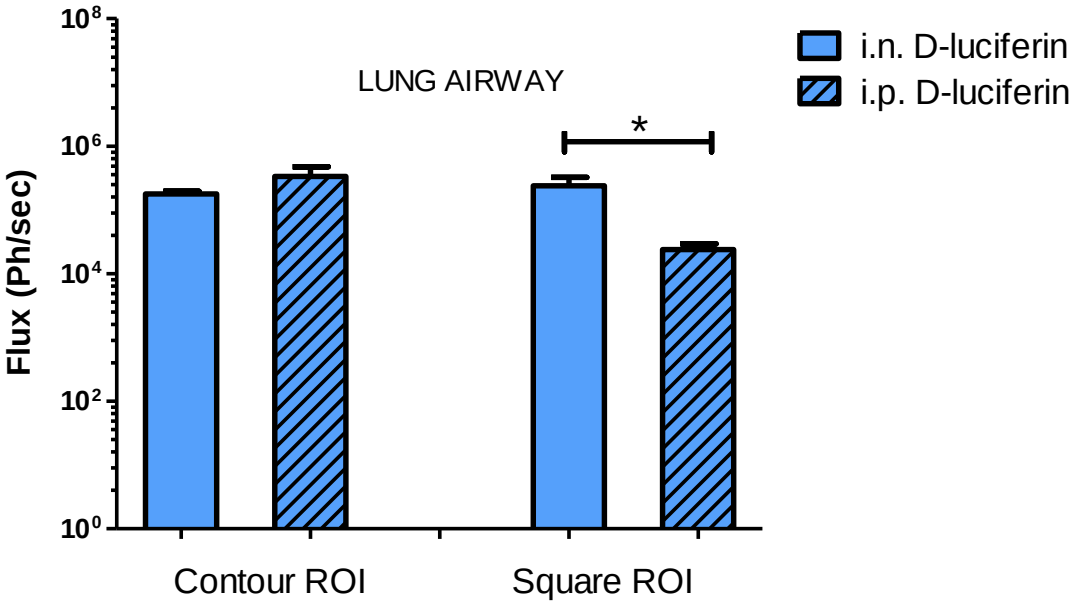


Figure 3-8 Lung Luminescence – LPC/LV-Luc
*Lung luciferase gene expression after mice treated with LPC/LV-Luc. Intranasal (i.n.) D-luciferin administration (open bars) vs intraperitoneal (i.p.) injection (hatched bars) (*p<0.05, paired t-test, one tailed, $\bar{X} \pm SEM$, n=4).*

The following findings determined which method of flux ROI measurement was a more accurate determinant of Luc expression.

Nasal luminescence was not detected at 1 week in mice that received either control treatments of LV-MT vector or LV-Luc pre-treated with PBS. When flux was calculated by the contour ROI method no Luc expression was detected (Figure 3-9). However, when the flux was calculated by the defined square ROI method, the adjusted flux was influenced by the background black colour measurement. This resulted in nasal luminescence measures that were significantly different between the two ROI methods in mice that received the control treatments ($p<0.01$, LPC/LV-MT and $p<0.05$, PBS/LV-Luc, paired t-test), but not in the LPC/LV-Luc treated group.

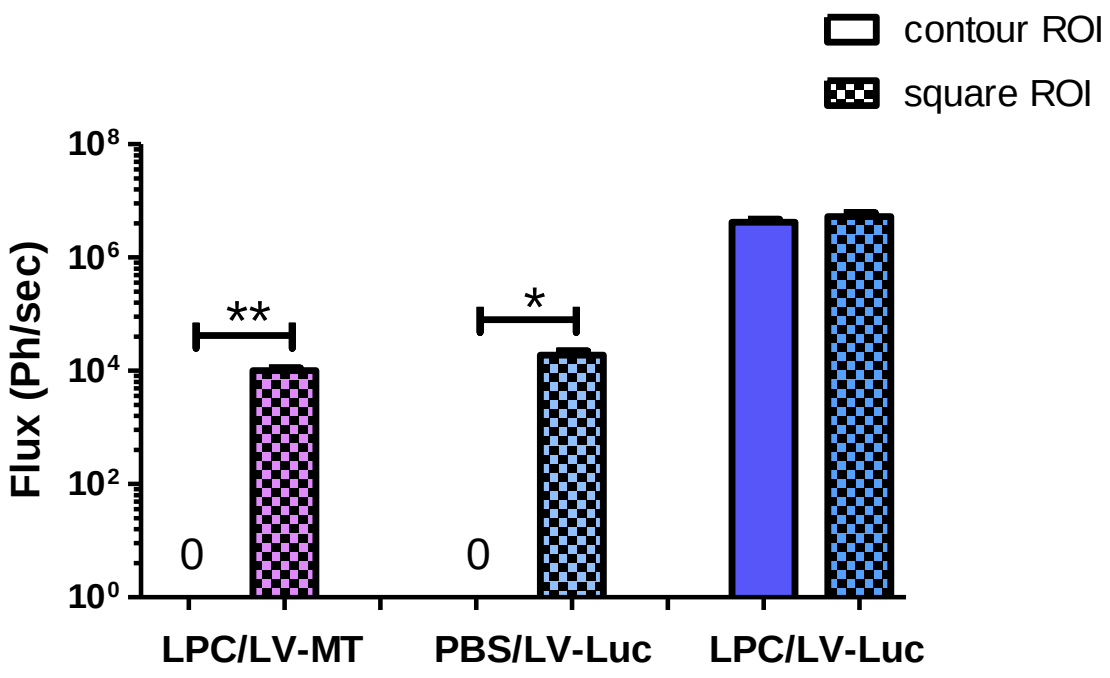


Figure 3-9 Nasal Luminescence – One Week
*Nasal luciferase gene expression following intranasal D-luciferin administration. Flux calculated by contour ROI (open bars) vs square ROI (chequered bars). Significant difference in flux for control groups only (**p<0.01, LPC/LV-MT and *p<0.05, PBS/LV-Luc, paired t-test, $\bar{X} \pm SEM$, n=4).*

Lung luminescence flux calculated by both contour and square ROI parameters, were similar to the nasal flux, with the exception of the PBS/LV-Luc control treatment. When measuring flux by contour parameters, luminescence was detected in the lung airways of mice that received PBS prior to LV-Luc. Lung luminescence was significantly different for both control groups ($p<0.05$, paired t-test) when comparing contour to square ROI measurement parameters (Figure 3-10).

The square ROI measurements created a "baseline" flux for both nasal and lung luminescence relative to the black background image that resulted in an artefactual increase in the detection of luminescence flux. Whereas when no luciferase was administered (e.g. LPC/LV-MT treated group) luminescence was not detected by contour ROI method and the resultant flux was calculated to be zero. For all future Luc experiments flux was calculated via contour ROI parameters after nasal administration of D-luciferin.

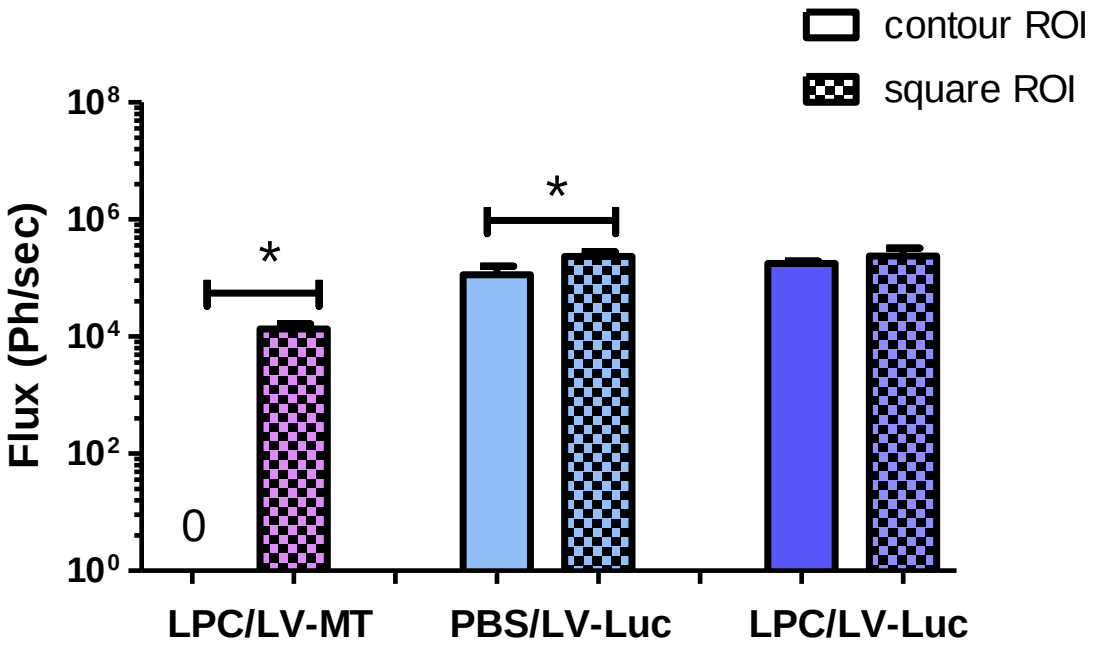


Figure 3-10 Lung Luminescence – One Week
*Lung luciferase gene expression following intranasal D-luciferin administration. Flux calculated by contour ROI (open bars) vs square ROI (chequered bars). Significant difference in flux for control groups only (*p<0.05, LPC/LV-MT and PBS/LV-Luc, paired t-test, $\bar{X} \pm SEM$, n=4).*

3.2.3.3 CFTR Gene Expression

Representative nasal TPD recordings are shown in Figure 3-11 for the three different treatments groups.

The nasal TPD measured in untreated (UnRx) CF and Het (normal) mice was different in relation to the initial basal TPD measurement and to the change in PD chloride response (ΔPD_{Cl}), as expected[339] (Figure 3-12). However there was an indication of a response in those mice that received LPC/LV-CFTR treatment (but note the small sample size (n=2)).

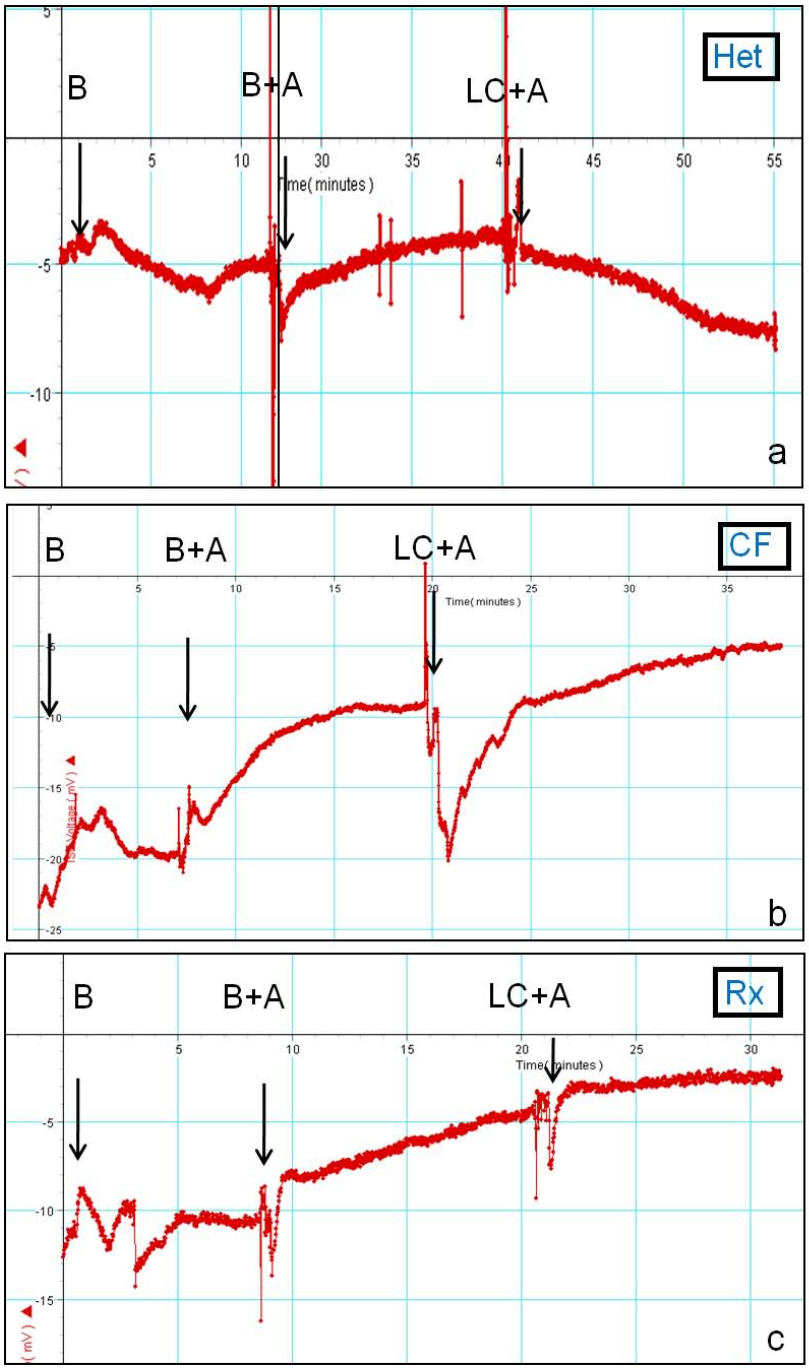


Figure 3-11 Nasal TPD Traces

Examples of nasal TPD recordings (mV) from CF mice during infusion of sequential KRB-based solutions: basal (B), basal + amiloride (B+A), and low chloride + amiloride (LC+A). Treatment groups are shown in panels a) normal Heteroyzgote (Het); b) untreated (UnRx) CF and c) CF treated with LPC/LV-CFTR (Rx). Images are screen shots of the actual recordings from the Data Studio software.

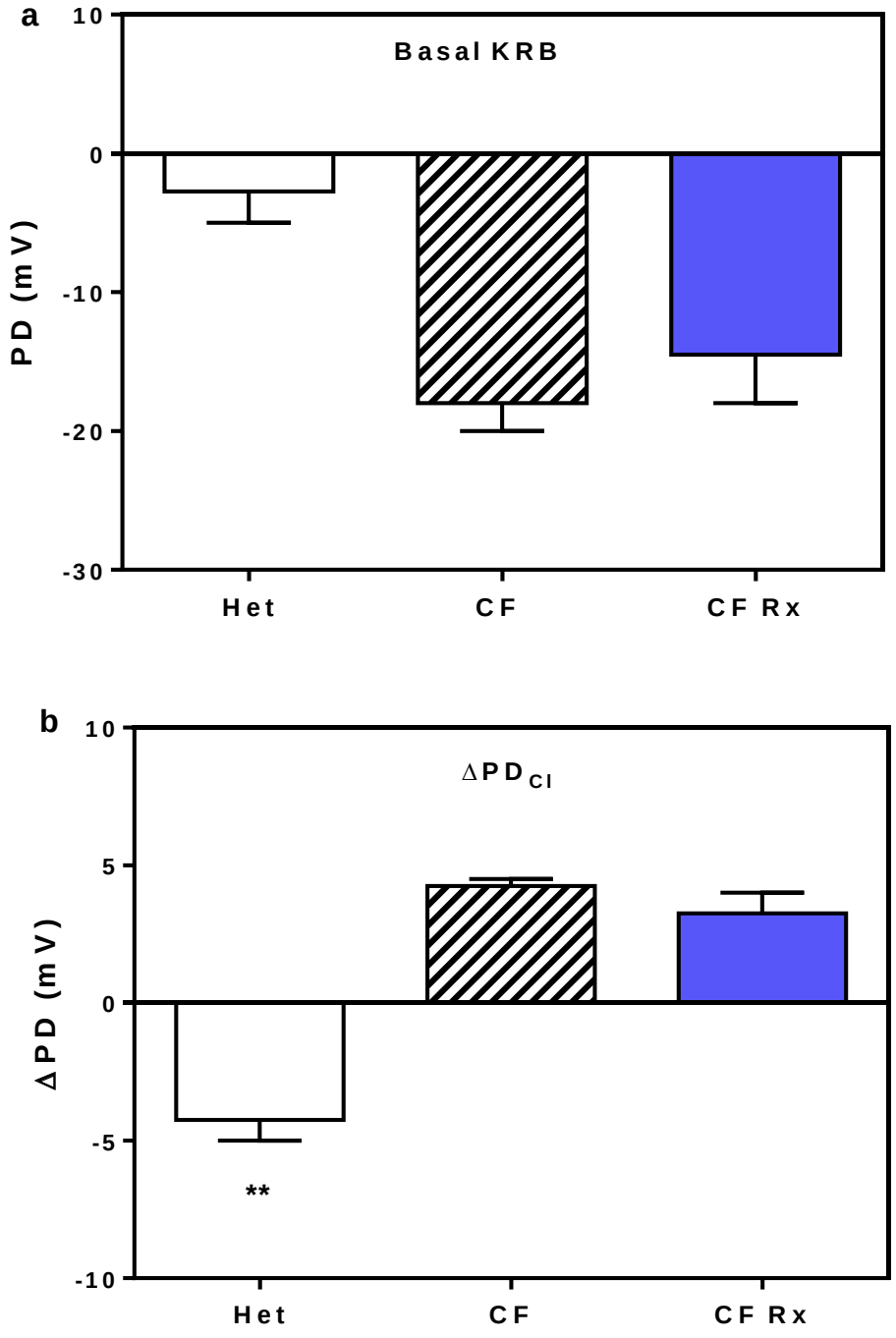


Figure 3-12 Nasal TPD Measurements

*a) Basal PD and b) the change in PD of chloride under amiloride (ΔPD_{Cl}) response (mV) for normal heterozygotes (Het), untreated CF and LPC/LV-CFTR treated CF (CF Rx) mice (**p<0.01, Tukey's ANOVA, $\bar{X} \pm SEM$, n=2). Despite small sample size (power = 0.67) these findings validated the expected differences between Het and CF mice.*

Nasal TPD's were repeated on the same CF mice after a 1-2 week time interval. This interval allows for the recovery of the nasal epithelial layer from any damage that may have occurred from the insertion and or removal of the nasal cannula[339]. One of the 2 CF mice treated with the LPC/LV-CFTR gene (CF Rx #2) appeared to show partial functional correction of the electrophysiological defect towards normal (Figure 3-13).

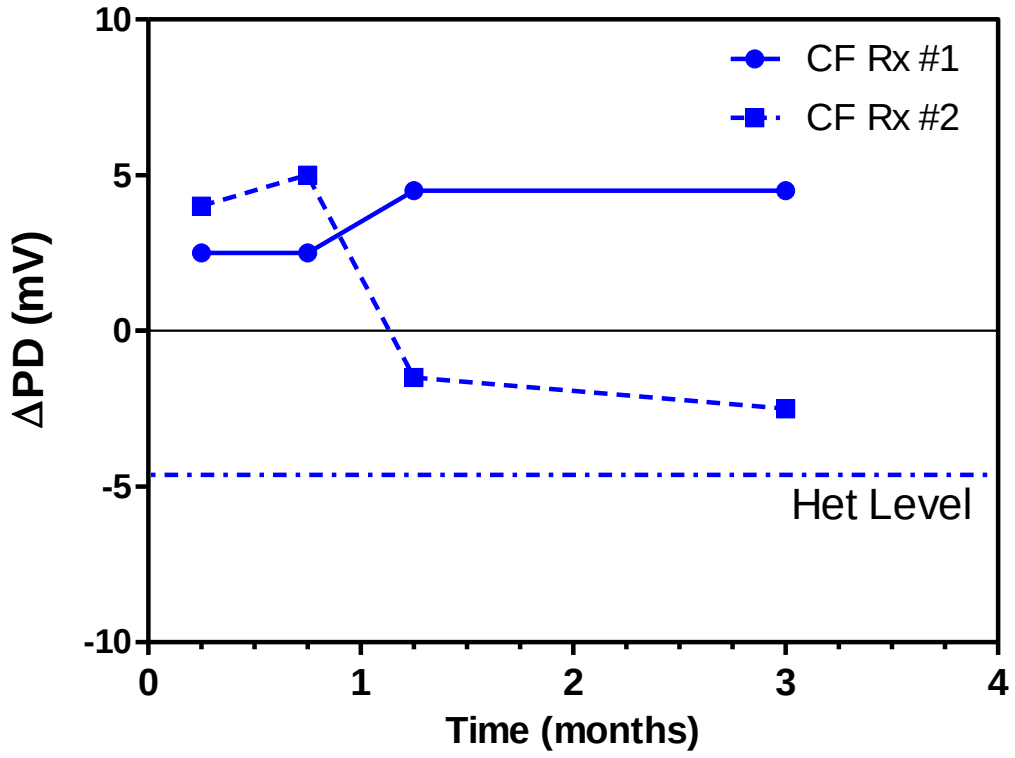


Figure 3-13 Repeated Nasal TPD's
Nasal TPD repeated over 3 months in the same LPC/LV-CFTR treated CF mice (CF Rx). Target level of correction (Het level).

3.2.4 Discussion

Results from these *in vivo* pilot studies confirmed the success of the two-step airway gene transfer protocol[296-298] using LPC as a pre-treatment followed by delivery of the LV vector. However a difference in response by nasal or lung airways was observed when different reporter genes were tested.

In determining the identity of the transduced cell types the LacZ reporter gene remains a valuable tool in gene transfer studies and also allows for the quantification of respiratory cell types transduced by a LV vector. This is not possible with the imaging of the gene expression of LV vectors containing the Luc gene: only regional expression is possible. One week after nasal administration of LPC/LV-LacZ, ciliated and non-ciliated respiratory cells expressed LacZ, with a small proportion of basal cells also transduced. It is proposed that a proportion of these basal cells are the progenitor cells for the respiratory epithelial layer[333, 351]. Consequently, not only were the correct cell types for CF gene therapy targeted by the two-step gene transfer protocol, but since basal cells (theoretically some of which may be progenitor cells) were also transduced, this method may enable the possibility for long term gene therapy. Of note, no olfactory cells were transduced with LacZ, confirming published findings in earlier studies[297].

When PBS was used as the pre-treatment (i.e. no LPC), LV LacZ gene transduction was absent in the nasal airway epithelia[297]. With the standard intranasal dose administration of both LPC pre-treatment and a LV vector carrying the LacZ reporter gene we have not observed gene transduction in the lung airways. This was an expected finding since dose protocols have been optimised over many years for nasal airway transduction only. However following intra-tracheal instillation with LPC and LV-LacZ we have observed LacZ transduction of lung airways in both mice and sheep[299]. It was interesting to note that transduction of the lung airways was possible when the Luc reporter gene was administered (Figure 3-10) with or without pre-treatment. This may be due, in part, to the increased LV vector titre compared

to previous studies[297, 298], but more likely due to the increased sensitivity of detection with the Luc imaging technique. Apical transduction of a VSV-G pseudotyped LV vector in the lower airways has also been demonstrated without the need for disruption of the tight junctions[352]. Therefore differences may exist between the upper and lower airways that are not just a result of immunological barriers or surface area, but could be due to differences in the position of available receptors for the VSV-G protein[352] or differential expression of cellular factors specific for LV vector transduction[353]. Administration of D-luciferin by the nasal route to reveal Luc expression, instead of intraperitoneally, also enhanced the sensitivity of this technique. To achieve comparable levels of luminescence in black furred mice to that in white or nude mice[293, 354] was gratifying since the majority of luminescence studies have been performed on the latter and dark fur may have limited the image quality. Based on these findings, to maximize sensitivity all subsequent studies presented in this thesis using the Luc reporter gene employed D-luciferin substrate administered as a nasal bolus 10 minutes prior to imaging.

The ultimate goal of gene therapy is to adjust the levels of deficient gene products to normal physiological levels. In the case of CF, a restoration of the ion transport in the airways to normal functioning levels resulting from correction of the defective CFTR gene is sought. CFTR is expressed in the submucosal glands and in the ciliated epithelial cells of the conducting airways[322] so these are the two main cell types targeted for CFTR gene replacement therapy, though most gene vectors preferentially target the ciliated surface cells. As little as 5-10% of CFTR function, has been shown to normalise ion transport[325].

In the nasal cavity olfactory cells account for ~50% of the epithelial lining and have been shown to influence the bioelectrics of nasal TPD measurements[312]. The nasal TPD method is currently the only measurement of functional correction in CF mice models however it can suffer from high intra-animal

and inter/ intra-operator variability[339]. This means that in some cases large animal numbers are required to achieve adequate statistical power[311].

Low sample numbers were used in this preliminary study simply to establish the nasal TPD technique using UnRx mice, and demonstrate the ability to repeat the nasal TPD measurement in CF mice treated with LPC/LV-CFTR. Nevertheless, there was a clear delineation apparent between normal mice (CF Het) and non-treated CF mice in relation to basal (normal krebs buffer) TPD, and the response to a low chloride solution containing the sodium channel blocker amiloride. These *in vivo* CFTR pilot studies were also encouraging as 1 out of the 2 CF mice treated with LPC/LV-CFTR showed some correction towards normal ion transport across the nasal epithelial layer.

Therefore, by increasing animal numbers in each treatment group and performing TPD's repeatedly on the same animal over its lifetime, validation of statistical analyses and reduction of the inherent variability in this technique may be achieved.

3.3 CHAPTER SUMMARY

The up-scaling of our LV vector production system was effective in producing large volumes of LV vector without affecting titre efficacy, which was necessary for the planned experimental studies. Increases in LV titre did not produce an increase in replication competent virus, confirming the safety profile of the LV preparations. Increases in the final titres of the LV vector led to significant increases in reporter gene transduction over 7 days. The mixing of LV vector batches with different genes did not appear to affect transduction efficacy of either gene. Transduction of both the nasal and lung airways (in black furred C57Bl/6 mice) with the reporter gene Luc confirmed the assay sensitivity for detection of *in vivo* gene expression. Pre-treatment with LPC remained necessary for nasal gene transfer but did not appear to be essential for lung transduction.

Nasal TPD measurements were shown to discern differences in the electrophysiological function of ion transport across the epithelial layer in normal, treated and untreated CF mice. Nasal TPD measurements could be repeated safely and ethically in the same CF animal, increasing the reproducibility and statistical power of this assay while minimising the number of animals required.

These initial studies confirmed the two-step gene therapy protocol employing the dose of 4 μl of 0.3% LPC 1 hour prior to a 20 μl bolus of a LV vector at titres to achieve optimum gene transduction in nasal airways.

CHAPTER 4

LONG TERM LENTIVIRAL AIRWAY GENE TRANSFER *IN VIVO*

CHAPTER 4 LONG TERM LENTIVIRAL GENE TRANSFER

4.1 LONG TERM GENE EXPRESSION IN NORMAL MICE

4.1.1 Introduction

Three major issues have emerged in the field of gene therapy for CF airway disease; they are the poor efficiency of gene transfer; low persistence of gene expression; and the abrogation of initial gene expression by host inflammatory and immune responses[202, 355, 356]. New generation viral vectors such as AAV, HD-AdV and LV are less immunogenic than the early AdV vectors, and persistent gene transfer after repeat dosing has been achieved[247, 293].

We have previously shown that persistent, LV reporter gene transfer using LPC as a pre-treatment can be achieved with only a single dose of a LV vector[297]. However, only partial correction of the CFTR defect in CF mice was detected and the persistence was inconsistent up to 12 months. After 3 months, a proportion of both normal and CF mice displayed little or no reporter gene transfer or CFTR expression respectively. In that particular study the delivered doses may not have been high enough for efficient gene transfer and expression, or an immune response may have reduced gene expression efficacy. The persistence of LV gene expression was measured in individual mice at one time point only and this would also account for the wide variability in outcome.

To accurately assess persistence of any gene therapy treatment, the ability to repeatedly monitor individual animals over their lifetime is a major advantage. Performing non-invasive repeated measures analyses on mice not only reduces within- and between-animal variability but provides a direct measurement of longevity of gene expression within each individual.

This chapter describes the *in vivo* testing of a single dose containing a mixture of different LV vector genes and the effects of gene transfer are quantified by repeated measure analyses.

4.1.2 Aims

The aim of the studies described in this chapter was to determine the longevity of LV gene expression in both normal C57Bl/6 and CF knockout mice. The inclusion of the reporter gene Luc allows for non-invasive monitoring of gene transduction (via bioluminescence flux) at regular intervals over a long time frame, potentially over the lifetime of each mouse. Bioluminescence can then be correlated with LacZ gene transduction at the final time point (i.e. at study termination or euthanasia) in normal mice and also with the measures of functional correction of the CFTR defect in CF mice (via TPD) at all corresponding time points over the lifetime of the CF mice.

4.1.3 Methods

4.1.3.1 Luciferase Gene Expression – Normal Mice

The right nostril of female C57Bl/6 mice was instilled with a 1:1 mixture of LV vectors containing the reporter genes Luc and LacZ (LV-Luc+LacZ). Nasal instillations were performed as described in section 2.3.2.3. Mice were randomly assigned to three treatment groups. The control group was divided into two cohorts with half receiving PBS as the pre-treatment prior to LV-Luc+LacZ (n=5) and the remainder receiving LPC prior to an empty vector (LV-MT) diluted 1:1 with PBS (n=5). This allowed for assessment of gene transduction of the nasal and lung airways as well as the effect of LPC alone. The treatment group received LPC 1 hour prior to LV-Luc+LacZ (n=10). The final concentrations in the 20 μl bolus for LV vectors were: LV-LacZ at 1 x 10^{10}, LV-Luc at 0.9 x 10^{10} and LV-MT at 0.6 x 10^{10} tu/ml.

Mice were weighed daily to monitor the post-operative effects of the instillation procedure and on day 7 they were imaged for detection of Luc expression (2.3.3.1.3) 10 minutes after a 50 μl intranasal bolus of D-luciferin (see section 3.2.3.2). Mice were imaged in a supine position using an exposure length of 1-2 minutes (IVIS 100, Xenogen, USA). The resultant bioluminescent flux was measured by contour parameters.

To test if the nasal luminescence signal interfered with the luminescence detected from the lung, a second image was taken with a strip of black cardboard placed over the heads of each mouse. Mice were imaged again at 1, 3, 6, 9, 12, 15, 18, 21, 24 months post LV vector administration in which the nasal and lung luminescence were detected in the same image, followed by lung luminescence detected in a separate image.

4.1.3.2 LacZ Gene Expression – Normal Mice

At the final time point (at euthanasia or at 24 months) mice were humanely killed and heads were processed for LacZ gene expression as described in 2.3.3.1.1 and 2.3.3.1.2.

4.1.3.3 Circulating Antibodies – Normal Mice

Blood was removed by submandibular puncture (2.3.4) at various time points during the long term study on either the same or following day as bioluminescent imaging and via cardiac puncture at study termination. Blood was centrifuged at 13,000 rpm for 3 minutes and the sera were stored at -70°C until analysis. The presence of circulating antibodies to the VSV-G envelope, and the reporter genes Luc and LacZ were then analysed by ELISA as described in 2.2.7.

4.1.3.3.1 Anti-VSV-G protein

To detect the presence of antibodies to the VSV-G protein found on the envelope of the LV vector, ELISA plates were coated with a LV vector carrying a different transgene (EYFP) diluted in PBS (5 μl/ml) and incubated at 37°C for 1 hour. ELISA plates were either used immediately or stored at 4°C overnight and processed as described in 2.2.7. All plates included one or all three negative controls of PBST, SKMPBST and UnRx mouse sera, as well as a positive control. The positive control sera were obtained from a previous immune response study in which mice received multiple doses of our LV-MT vector and were known to be positive for VSV-G antibodies[357]. No sera were taken at the earlier time points (0 - 6 months) as the submandibular bleeding technique was newly introduced and awaited approval by the WCH animal ethics committee, therefore UnRx normal Het mouse sera were used as the surrogate zero time point.

4.1.3.3.2 Anti-Luciferase

To detect the presence of sera antibodies to the protein of the transgene Luc, ELISA plates were coated with a 500 ng/ml solution of recombinant Luc diluted in PBS and assayed as detailed in 2.2.7. Sera used as the positive control were obtained from another experiment in which mice received two doses of Luc and were positive for circulating antibodies to Luc (see Chapter 5, Figure 5-6). Untreated Het mouse sera were used for surrogate zero time point as above.

4.1.3.3.3 Anti-β-galactosidase

The same sera that was analysed for circulating antibodies to both LV envelope and Luc was also analysed for serum antibodies to the other reporter transgene LacZ. ELISA plates were coated with 500 ng/ml of β-galactosidase from *E. coli* diluted in PBS as described in section 2.2.7. Sera for positive

controls were obtained from mice from previous experiments that were positive for circulating antibodies to LacZ (see Chapter 5, Figure 5-3).

4.1.3.4 Genomic DNA in Other Tissues – Normal mice

The presence of lung tissue transduction and off-target transduction in liver and spleen tissue samples taken from normal mice at study termination (for either Luc or LacZ genes) was determined by qPCR as described in Section 2.2.6.

4.1.4 Results

4.1.4.1 Animal Weights – Normal Mice

The body weight of each mouse increased with age, with loss of not more than 10% of body weight after each anaesthetic event. Body weight was restored without intervention, to pre-anaesthetic weight within 2-5 days (Figure 4-1).

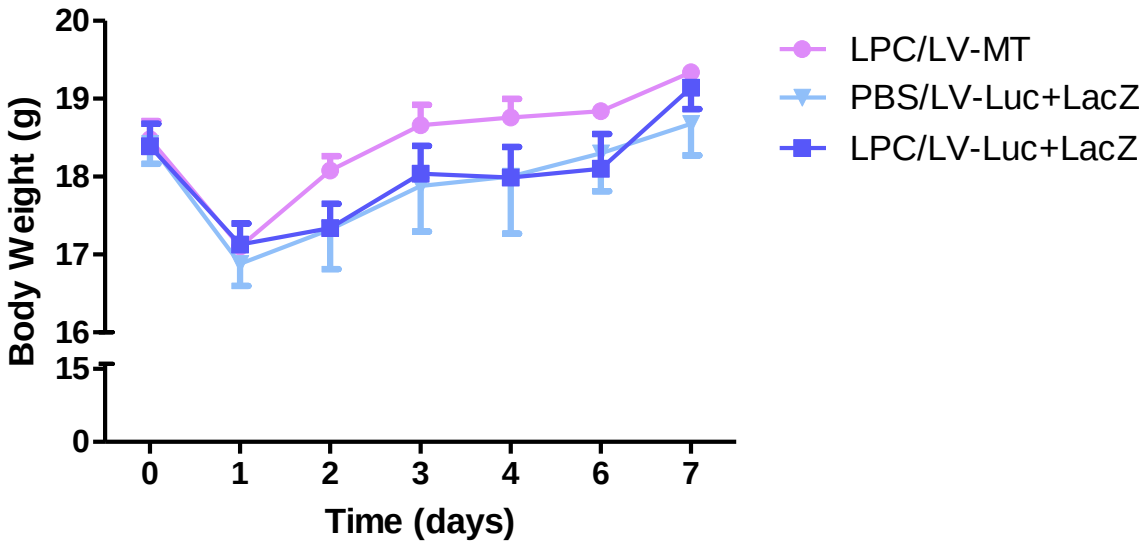


Figure 4-1 Body Weight – One week
A reduction in body weight of <10% occurred following an anaesthetic procedure after the first day ($\bar{X} \pm SEM$, n=5-10).

Regardless of treatment across the three groups, there was no difference in body weight between all three groups (Figure 4-2, n.s., ANOVA, n=2-10) over the 2 year study.

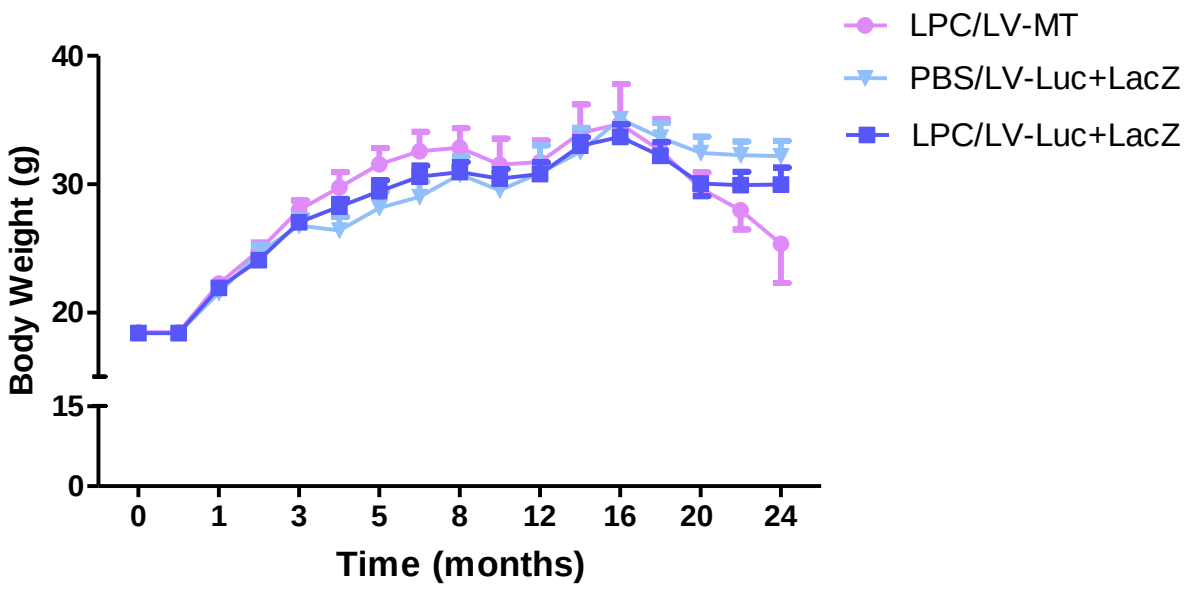


Figure 4-2 Body Weight over Time
Body weight was similar for all treatment groups over the 2 year study (n.s., RM ANOVA, $\bar{X} \pm SEM$, n=2-10).

4.1.4.2 Luciferase Gene Expression – Normal Mice

By placing a strip of black cardboard over the heads of the mice during the imaging procedure the resultant nasal luminescence was blocked out and only the lung luminescence was measured (Figure 4-3). This manipulation showed that with nasal administration of LPC/LV-Luc+LacZ the luminescent signal from the nasal airways did not interfere with the luminescent signal from the lung airways in the same mouse (Figure 4-4, n.s. paired t-test).

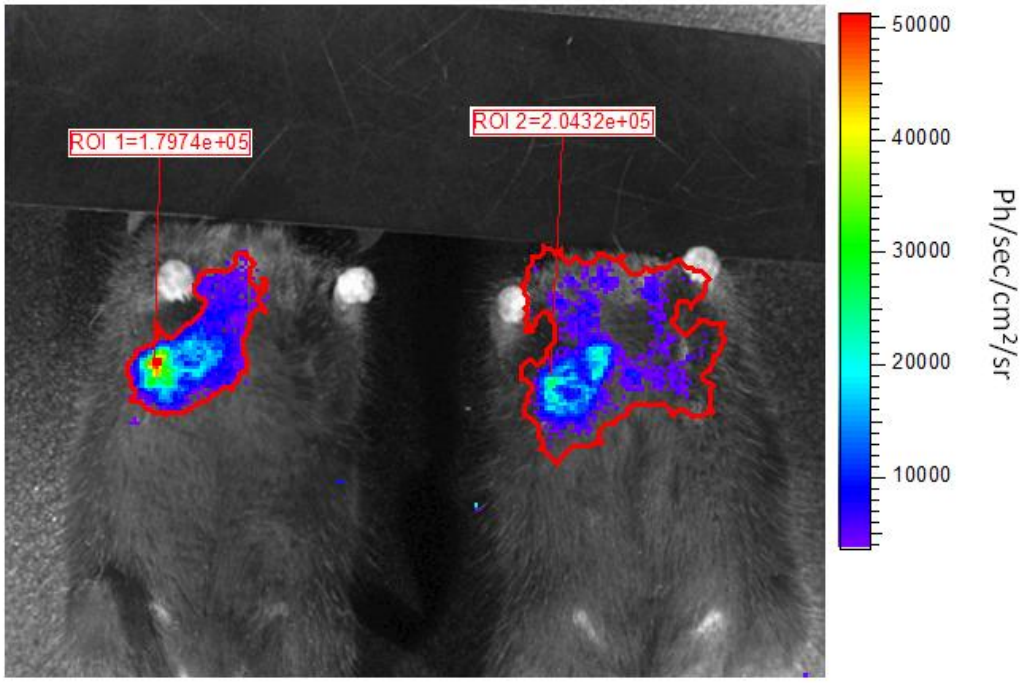


Figure 4-3 Lung Luciferase Imaging – LPC/LV-Luc+LacZ
Lung luminescence after 1 week nasal administration of LPC/LV-Luc+LacZ. Nasal luminescence signal blocked by black cardboard strip.

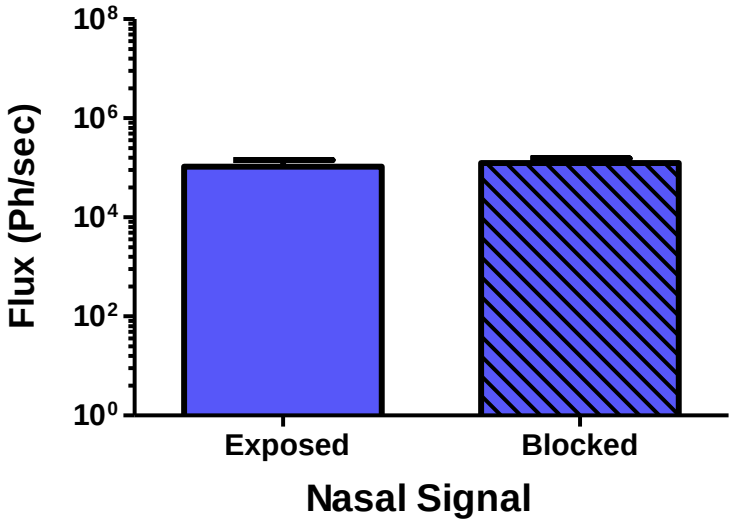


Figure 4-4 Lung Luminescence – LPC/LV-Luc+LacZ
Lung Luminescence detected 1 week after LPC/LV-Luc+LacZ nasal administration. Nasal luminescence signal not blocked (open bar) and nasal signal blocked (striped bar) with black cardboard (n.s., paired t-test, $\bar{X}$±SEM, n=10).

4.1.4.2.1 Nasal Luminescence

The limit of detection by the contour ROI parameters is approximately 10^3 Ph/sec. Only mice that had detectable contour ROI levels of luminescence (i.e. Flux >10^4 Ph/sec) were designated positive for Luc expression. Mice that received LPC/LV-MT showed no Luc gene expression at any time point. For the other control group, when PBS pre-treatment was used prior to LV-Luc+LacZ, 2 of 5 mice displayed nasal luminescence at some time points, however this was not significantly different to the other control group LV-MT (n.s., Dunn's RM ANOVA, n=2-5).

Mice that received LPC prior to LV containing the Luc gene showed a significant increase in nasal luminescence compared to both control groups (Figure 4-5a) at the time points tested ($p<0.05$, Dunn's RM ANOVA, n=2-10). This confirms the need for LPC prior to an active gene (Luc) to effectively transfect nasal epithelia.

4.1.4.2.2 Lung Luminescence

In contrast to the nasal luminescence, Luc gene expression detected in the lung airways was not different between the mice receiving PBS or LPC prior to LV-Luc+LacZ. Nevertheless, irrespective of the pre-treatment used, lung luminescence detected in mice that received the Luc gene was significantly different from those mice that received no active gene (LV-MT) (Figure 4-5b, $p<0.05$, Dunn's RM ANOVA, n=2-10).

4.1.4.2.3 Nasal Luciferase Expression – LPC vs PBS

In the cohort of mice that received LPC/LV-Luc+LacZ, the resultant nasal gene expression reduced over time and was significantly different after 3 months compared to luminescence measured at 1 week post instillation (Figure 4-6a, $p<0.05$, Dunnett's ANOVA, n=6-10). The reduction in nasal luminescence

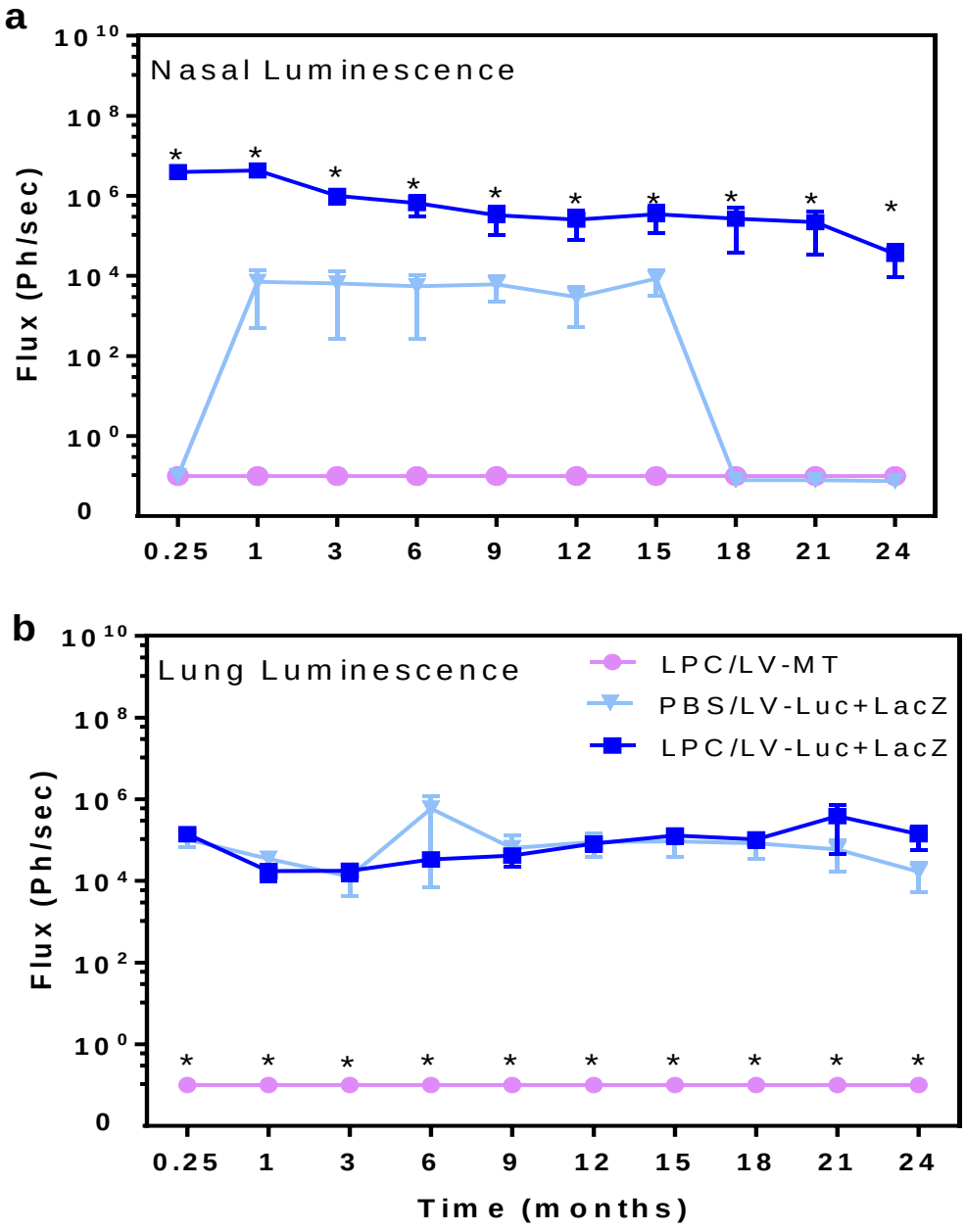
plateaued from 9-21 months, with another slight drop at the final 24 month assessment time point. An explanation for this reduction in mean luminescence could be that in a proportion (13-67%) of mice no nasal gene expression was present (i.e. was reduced to zero) from 6 months after LV instillation $p<0.001$, Spearman correlation $r = -0.90$ (refer Appendix 8.1-1).

The majority of mice (60-80%) that received PBS pre-treatment had no detectable nasal luminescence (Figure 4-6c).

4.1.4.2.4 Lung Luciferase Expression – LPC vs PBS

The lung luminescence in the Luc treated groups' demonstrated completely different profiles to nasal Luc expression. There was an initial drop in lung luminescence after 1 month and thereafter lung gene expression gradually increased over time in the LPC treated mice (Figure 4-6b) or stabilised as in PBS pre-treated mice (Figure 4-6d).

After LPC/LV-Luc+LacZ treatment, 90% of mice displayed Luc gene expression in the lung airways at 1 week, with the majority of lung gene expression disappeared at 1-3 months.



n=				
LPC/LV-MT	4	4	3	2
PBS/LV-Luc+LacZ				4
LPC/LV-Luc+LacZ	8	7	7	6

Figure 4-5 Nasal and Lung Luminescence

*Luciferase gene expression after nasal LV administration in the a) nasal and b) lung airways of normal mice (*p<0.05, Dunn's RM ANOVA, $\bar{X} \pm SEM$, n = 5 for LV-MT and PBS, n=10 for LPC, or as stated at later time points).*

Unexpectedly, luminescence in the LPC/LV-Luc+LacZ groups re-appeared in the lung airways, steadily increasing lung expression over the following 6 months and was then maintained for the remainder of the assessment period. At the final assessment (24 months) only one mouse still displayed zero lung gene expression (refer to Appendix 8.2-1 and Appendix 8.2-2).

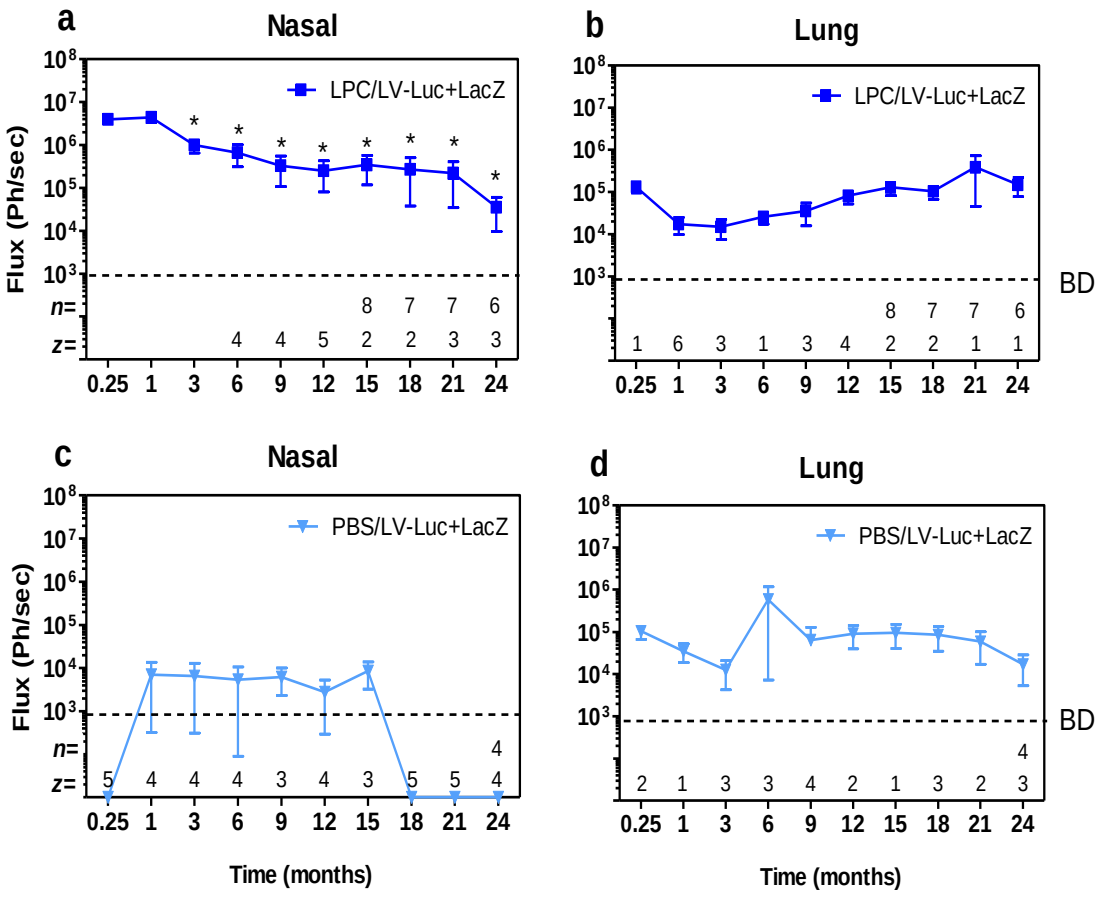


Figure 4-6 Average Luciferase Expression – LPC vs PBS

*Luminescence detected in a, c) nasal and b, d) lung airways in mice that received LV-Luc+LacZ (*p<0.05, Dunnett's ANOVA compared to 1 week, $\bar{X} \pm SEM$, n = 5 for PBS, n=10 for LPC, or as stated at later time points). Z = number of mice displaying zero gene expression; BD = below detection (10^3 Ph/sec).*

For mice that received PBS prior to the Luc gene, lung expression also dropped between 1-3 months due to complete loss of lung luminescence in the majority of mice. Re-emergence of lung expression occurred at 6 months and remained stable over the study period. However this stable expression was due to a combination of extended lung expression, re-emergence of lung expression or loss of lung expression (Appendix 8.2-3).

4.1.4.3 LacZ Gene Expression – Normal Mice

Photographs of LacZ staining were taken prior to histological processing (Figure 4-7) and show artefactual staining in the control groups and punctate LacZ staining in the LPC/LV-Luc+LacZ treated mice.

Histological cassettes were sent to Histopathology for embedding into paraffin blocks and then sectioned and stained with H&E and SafO (2.3.3.1.1 - 2.3.3.1.2). However, a change in the standard protocol and the use of a new embedding machine compromised processing of all samples from this study. No LacZ staining was evident in any of the paraffin tissue blocks despite several re-processing sectioning trials. Further investigation showed that the neutralization of the expected blue LacZ staining had resulted from an un-planned increase in the temperature of 10°C at the ethanol wash stages. Therefore LacZ cell counts and the type of cells transduced could not be determined at the final time point.

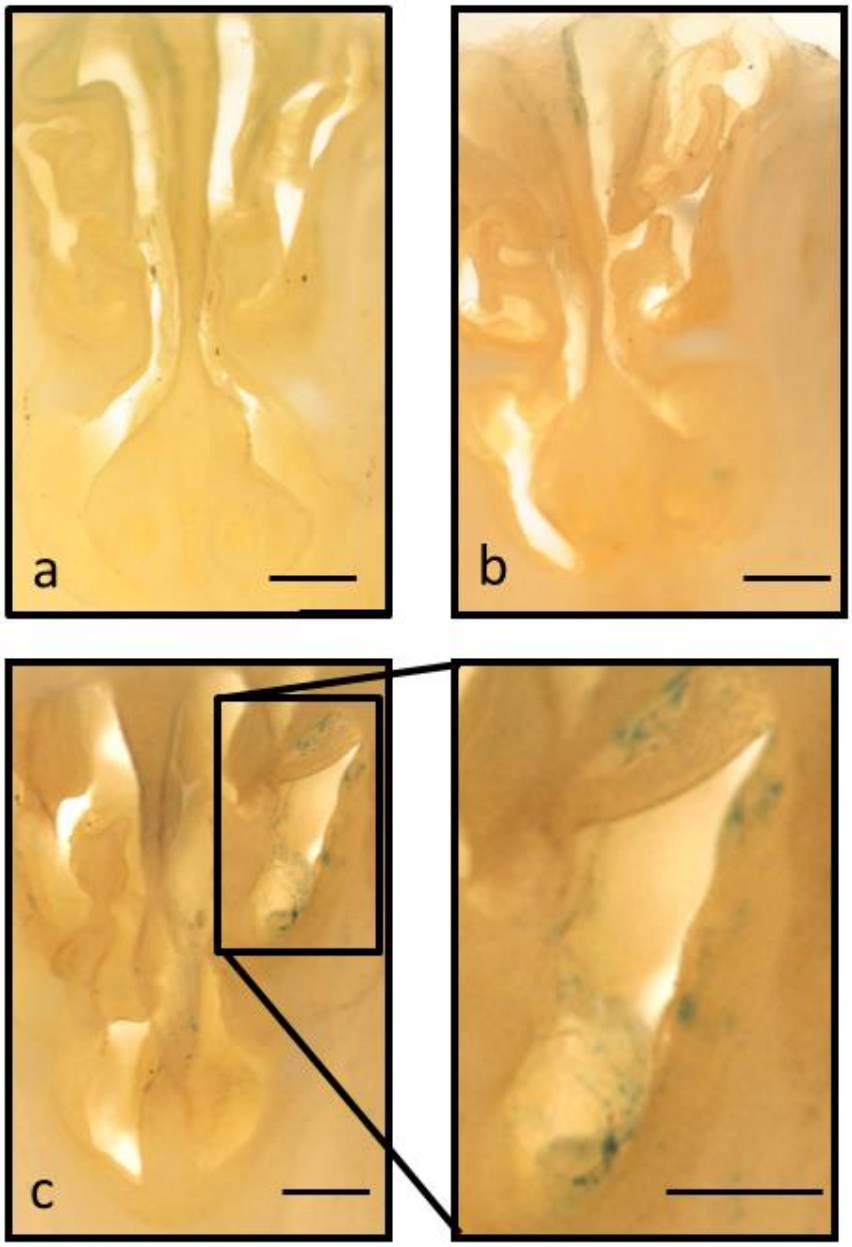


Figure 4-7 Long Term en face LacZ Transduction

En face LacZ transduction after Xgal staining in mice that received a) LPC/LV-MT, b) PBS/LV-Luc+LacZ and c) LPC/LV-Luc+LacZ after 24 months. Insert of punctate blue LacZ staining in right nostril only of mice administered with LPC/LV-Luc+LacZ. Artefact (non-punctate) smooth staining noticeable in upper portion of panel a and b. All scale bars are 500 µm. Cross sections were not available for this study (see explanation in text).

As shown on the example *en face* photographs (Figure 4-7) LacZ expression was not detected in the nasal epithelia from either control group; i.e. in any animals that received either LPC/LV-MT or PBS pre-treated Luc+LacZ. Approximately 72% of the mice that received LPC prior to LV-Luc+LacZ displayed evidence of LacZ expression via blue stained cells. This ranged from a few blue stained cells to extensive LacZ expression extending throughout the depth of the nasal passage. Unfortunately due to the previously described technical difficulties during the embedding process a correlation between LacZ score and Luc expression could not be explored.

4.1.4.4 Circulating Antibodies – Normal Mice

No sera were collected prior to and for the first 6 months after nasal LV vector delivery. This was unfortunate but unavoidable due to the timing of the regulatory authority committee meetings. The new sub-mandibular procedure for obtaining sufficient volume of sera for ELISA analyses without adverse effects on animal wellbeing was granted as a modification in time for the later time assessments. The expected time of the peak of circulating antibodies was therefore absent (1-6 months), but nevertheless sera from 9 months after LV delivery was tested for antibody presence to the LV vector envelope constituents (VSV-G) and the reporter gene proteins compared to UnRx control sera.

4.1.4.4.1 Anti-VSV-G protein

A statistical increase in circulating antibodies to the VSV-G protein was observed 12 months following nasal instillation of LPC/LV-Luc+LacZ, compared to sera analysed from UnRx mice (Figure 4-8a, $p<0.05$, Dunnett's ANOVA, n=2-10). For both control groups (PBS pre-treatment and LV-MT) there was no difference in circulating antibodies compared to sera of UnRx mice.

4.1.4.4.2 Anti-Luciferase

For the control group LV-MT there was no difference in circulating antibodies to the Luc transgene protein compared to sera of UnRx mice. An increase in circulating Luc antibodies was detected in sera from 9-12 months after nasal instillation in mice treated with LPC prior to LV-Luc+LacZ, and a sustained antibody response from 9-18 months in mice treated with PBS prior to LV-Luc+LacZ, compared to UnRx mouse sera (Figure 4-8b, $p<0.001$, Dunnett's ANOVA, n=2-10).

4.1.4.4.3 Anti-β-galactosidase

For both control groups (PBS pre-treatment and LV-MT) there was no difference in circulating antibodies to the Luc transgene protein compared to sera of UnRx mice. An increase in circulating antibodies to the protein β-galactosidase was detected in mice sera from 9-15 months after nasal instillation in mice treated with LPC/LV-Luc+LacZ compared to unRx mouse sera, (Figure 4-8c, $p<0.05$, Dunnett's ANOVA, n=2-10).

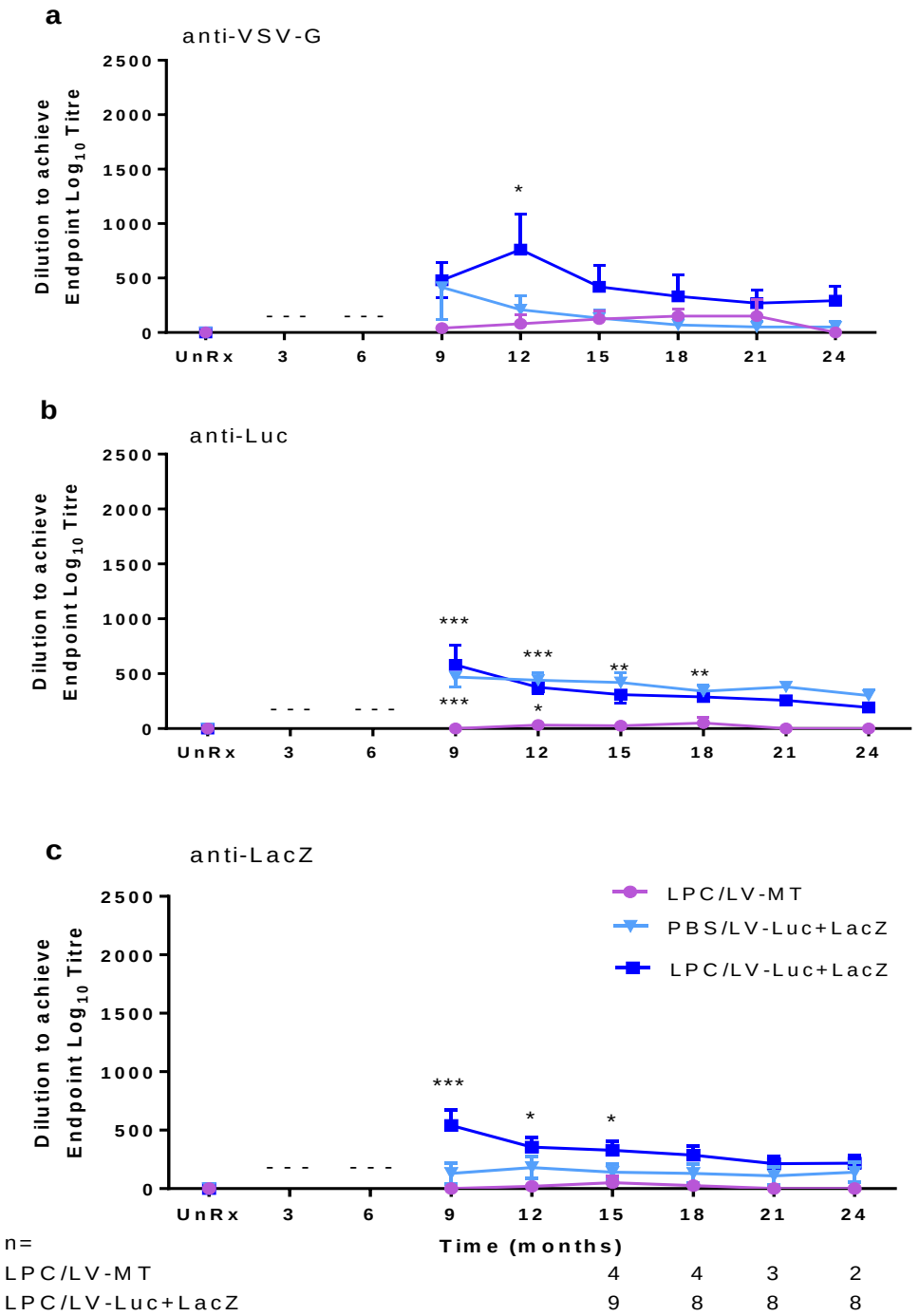


***Figure 4-8* Presence of Circulating Antibodies**

*Presence of circulating antibodies detected in sera to the a) LV envelope protein VSV-G, b) recombinant luciferase (Luc) protein and c) recombinant β-galactosidase (LacZ) protein over time. (***p<0.001, **p<0.01, *p<0.05., Dunnett's ANOVA compared to untreated, $\bar{X} \pm SEM$, n=5 for PBS and LV-MT, n=10 for LPC, unless stated at later time points) - = not done, UnRx = untreated.*

4.1.4.5 Presence of Genomic Reporter DNA in Tissues

No genomic DNA from the introduced reporter transgenes Luc and LacZ was detected in off target tissues such as liver and spleen.

Extraction of the lung tissue via the tail lysis method (Section 2.2.6.1.2) had a low success rate in producing sufficient DNA to be analysed by qPCR. From the available lung tissue DNA (n=7), only those animals treated with the active transgenes had just above detectable levels of the introduced genomic DNA (Vector copy numbers/cell = 0.001 +/- 0.0002, $\bar{X} \pm SEM$, n=3).

4.1.4.6 Survival Demographics of Normal Mice

Repeated anaesthetics, imaging and blood sampling have not previously been routinely performed in our laboratory. The factor of natural attrition due to age related illnesses becomes evident in mice aged over 18 months of age. A survival curve (Figure 4-9) was constructed to ascertain if there were any negative impact on animal welfare due to treatment or repetitive anaesthesia, procedures and assessment measures. The type of treatment had no effect on animal survival over the 2 year study period (n.s., Mantel Cox log rank test, n=2-10).

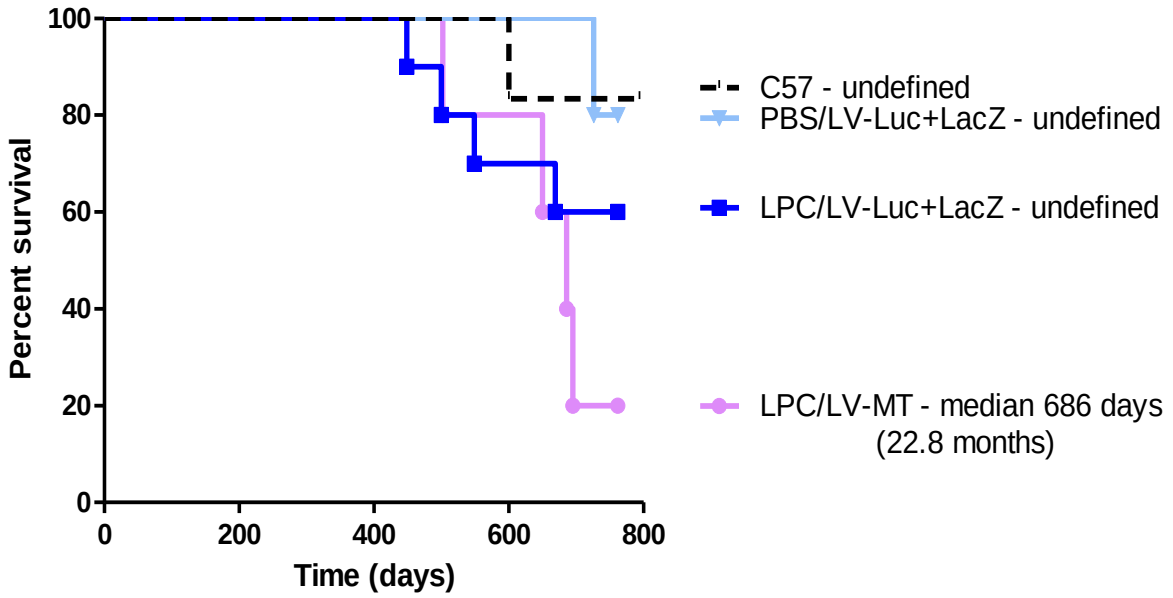


Figure 4-9 Survival Curves

Treatment had no significant effect on animal survival over the 2 year study period (n.s. Mantel-Cox log rank test, n=2-10). At the 24 month time point only n=2 for LPC/LV-MT. Mice that received LPC/LV-MT had a defined median survival of 22.8 months. Untreated C57 mouse data obtained from historical literature had an 82% survival of 692 days (23 months)[358, 359].

4.1.5 Discussion

In developing a gene therapy for CF the goal is to produce adequate gene expression to correct the bioelectrical defect that results from mutations in the CFTR gene[43, 360]. This requires effective levels of gene expression soon after dosing[325], however, the long term sustained presence of gene expression is the ultimate goal. Using a LV vector containing two reporter genes this study first investigated the effectiveness and longevity of airway gene transfer in normal C57Bl/6 mice *in vivo*.

The repeated-measure experimental procedures of these long term studies had no detrimental effect on animal health and survival of normal C57Bl/6 mice, a finding consistent with other long term gene transfer studies using normal mice[361].

Lifetime reporter gene expression was observed in the nasal airways of normal C57Bl/6 mice after only a single nasal dose instillation (of 0.3% LPC then the LV vector containing the transgene Luc). Without LPC pre-treatment (PBS as a control) nasal gene transfer was no different to those mice treated with an empty vector (LV-MT). This implies that LPC may be necessary to transfect nasal epithelia by transiently opening the tight junctions between cells[300] to allow access to the basolateral membrane where the receptors for our VSV-G pseudotyped LV vector reside.

The ability to repeatedly image the mice over their lifetime to assess bioluminescence allows for direct and non-invasive assessment of gene expression persistence in the same animal. Persistent reporter gene expression for up to 1 year in mouse nasal airways using a FIV-based LV vector has previously been reported[275]. More recently, long term (2 years) reporter gene expression employing a SIV based LV vector pseudotyped with the SeV derived F and HN envelope proteins, was demonstrated in the airways of normal mice but using a nasal LV vector instillation volume 5 times greater than that described in this thesis[361]. The large 100 µl volume would flood the nasal airways with fluid, increasing

the permeability of cells and effectively disrupting the tight junctions between the cells, explaining the longevity of airway expression seen in that study similar to using the pre-conditioning agent LPC reported here. This is the first time that 2 year sustained HIV-1 based LV reporter nasal and lung gene expression in the same mice over their lifetime has been shown after a single 20 μl vector volume dose.

The level of nasal flux in mice that received LPC/LV-Luc+LacZ did, however, reduce over time compared to the level of Luc gene expression detected at 1 week. The reduction in nasal luminescence in this cohort may be explained by the loss of the original gene expression in a proportion of mice, 6 months after LV administration. The loss of Luc gene expression in these mice could be due to the normal turnover of epithelial cells[328].

Luciferase luminescence was also detected in the *lung* airways of mice after the single *nasal* instillation of the LV vector containing the Luc transgene. In contrast to nasal gene expression, lung luminescence was detected in the mice that received LV-Luc, regardless of pre-treatment. The administration of D-Luciferin via the intranasal route instead of the intraperitoneal route has been shown to improve detection of luciferase expression in lung airways[348, 349, 361]. Direct intra-tracheal administration of LV vectors has been shown to transiently transduce lung airways without the need for tight junction disruption pre-treatments (such as LPC) in foetal animals such as mice[348], lambs[362] and rhesus monkeys[363] as well as in adult mice[348, 364].

Nasal instillation of a 100μl volume of a LV vector to specifically target lung airways has been demonstrated to transfect the nasal and lung airways in white mice after 48 hours[267] and more recently in black C57Bl/6 mice for 2 years[361]. The detection of long term lung luminescence after administration of a single 20 μl bolus of a LV vector, directed specifically towards targeting nasal airways, suggests that pre-treatment may not be necessary for effective lung transduction. Using synchrotron phase-contrast X-ray imaging studies the 4μl pre-treatment volume has been shown to remain in the nasal

region exclusively, whereas the 20 μl volume of LV vector used in these nasal delivery experiments reaches the lung[365]. Therefore, the pre-treatment volume of LPC essentially targets only nasal epithelia and its effect is negated in the detection of luminescence in the lung airways. To ascertain if LPC pre-treatment may potentially enhance lung transduction, instillation of LPC directly to the lung airways needs to be examined.

In the LPC/LV-Luc+LacZ treated mice the level of lung luminescence reduced after 1 month and then gradually increased over time. This reduction in lung luminescence at 1 month was due to a large proportion (60%) of mice that had no Luc gene expression. However, lung luminescence re-emerged after 6 months and remained for a further 18 months. The longevity of gene expression could be due to transduction of the animals own progenitor cells, a proportion of which are thought to reside as basal cells[333] or transduction of transient amplifying cells with proliferative capacity (e.g. a sub-population of Clara cells)[334].

In the mice that received PBS prior to LV-Luc+LacZ, lung expression also was detected at the early time points with a reduction of lung luminescence at 1-3 months due to complete loss of expression in some mice. Re-emergence of lung expression occurred at 6 months and eventually stabilised over the remaining assessment period. The stabilisation of lung expression could be due to a combination of long term expression, loss, and re-emergence of expression from the lung airways. This is likely due to transduction of long lived terminally differentiated epithelia[328] or progenitor cell transduction. From the reduction and then the re-emergence of luminescence in the lung airways of mice that received LV-Luc+LacZ, it is likely that our LV vector gene transfer targeted the animals' own stem cells in the lung airways without the need for LPC pre-treatment.

To ascertain which airway epithelial cell types were transduced and a possible explanation for the longevity of the reporter gene expression, the reporter gene LacZ was mixed in a 1:1 ratio instilled into

the treated nostril. The disadvantage of this technique is that the animal must be humanely killed to assess gene transduction. Therefore only the final time point can be analysed for gene expression for transduction of particular cell types. We have previously shown long term LacZ gene transduction, but in different cohorts of mice[297] that required timed culls of many different groups of mice. The strength of the repeated measures design allows the tracking of gene expression in individual animals and therefore the use of smaller numbers of mice.

Due to the increase in temperature of the ethanol washes during histological processing any LacZ transduction that may have been present was neutralized and no transduced cell types could be analysed. The loss of LacZ data means that the types of epithelial cells transduced could not be determined nor which nasal epithelial cells may be the possible stem/progenitor cells of the airway. The potential use of immunohistochemical techniques for determination of lung airway stem/progenitors cells currently has not be successfully translated to the nasal airways due to the necessary decalcification process for paraffin embedding. Future experiments with newer clonal strategies employing flow cytometry techniques analysis[366] and therefore a different reporter gene (e.g. green fluorescent protein) may elucidate not only the stem cells of the nasal airway but if our LV gene transfer techniques has transduced a proportion of these cell types.

An increase in circulating antibodies to both the LV vector envelope VSV-G protein and the transgene Luc was also detected from the 9 month time point up to 18 months (as no sera were analysed at the earlier time points). Another reason for the reduction in nasal luminescence could be due to an immune response to either the LV envelope protein or to the expressed transgene protein. Encouragingly, the immune response was not sufficient to completely block gene expression, and sustained Luc gene expression was present after a single LV vector dose. The presence of LacZ transduction as visualised on *en face* sections confirmed that LacZ gene expression was evident at the final 24 month assessment

time point. Even though LacZ gene expression could not be quantified histologically at the 24 month time point, circulating antibodies to the β-galactosidase protein were detected in the sera of those mice that received LPC/LV-Luc+LacZ. The presence of circulating antibodies suggests that the reporter gene LacZ was expressed after nasal instillation of LPC/LV-Luc+LacZ.

These repeat-measure design experiments performed on normal C57Bl/6 mice demonstrate the ability of a single LV vector nasal delivery treatment to produce sustained reporter gene expression. There was no detriment to animal survival due to either multiple assessments of reporter Luc gene expression, repeated anaesthetics or several blood removal procedures. The level of circulating antibodies detected in the sera of mice was not sufficient to block reporter gene expression.

This proof of principle study – employing a repeat-measure design with a two-step regime using a pre-treatment and a single dose of a LV vector for reporter gene expression – could now be trialled in CF mice using the therapeutic gene CFTR to test functional correction of the bioelectrical defect in murine nasal airways.

4.2 LONG TERM GENE EXPRESSION IN CYSTIC FIBROSIS MICE

4.2.1 Introduction

Reporter gene expression can give valuable insight into the potential effectiveness of LV gene therapy in the airways of mice. By using the Luc reporter gene, non-invasive measurement of *in vivo* airway gene expression in individual mice of any strain can be performed over their lifetime. However, the ultimate objective of a gene therapy treatment for CF is to be able to correct the CFTR gene defect in airway epithelia. The only functional method to test if CFTR has been corrected by gene therapy is the nasal TPD technique in CF mice. The bioelectrical defect is measureable in the nasal airways of transgenic CF mice and repeated nasal TPD measures can be performed on the same CF mouse at many time intervals to assess the success and longevity of a LV-CFTR gene therapy.

4.2.2 Aims

The studies described in this section were designed to extend the proof-of-principle repeat-measure design for reporter gene expression, to use in CF mice using the therapeutic CFTR gene.

4.2.3 Methods

4.2.3.1 Luciferase Gene Expression – CF Mice

The right nostrils of male and female CF transgenic mice (*cftr*^{tm1unc}) were instilled with a 1:1 mixture of LV vectors containing the Luc reporter gene and the therapeutic CFTR gene. Nasal instillations were performed as described in 2.3.2. Male and female CF mice were randomly assigned to three different treatment groups. CF mice are less robust so the number of animals in each treatment group was increased. The control groups received either a nasal delivery of PBS 1 hour prior to the mixture of LV-Luc+CFTR (n=6) or LPC 1 hour prior to LV-MT diluted 1:1 in PBS (n=6). For the treatment group CF

mice received LPC 1 hour prior to LV-Luc+CFTR (n=12). The final concentration of the individual transgene within the 20 μl LV vector nasal bolus was LV-Luc at 0.9 x 10^{10} tu/ml, LV-MT at 0.6 x 10^{10} tu/ml and LV-CFTR at 2.5 x10^{10} tu/ml.

Following the procedures used for normal C57Bl/6 mice, CF mice were weighed daily, and on day 7 imaged for detection of Luc expression (2.3.3.1.3) 10 minutes after a 50 μl intranasal bolus of D-luciferin. Mice were imaged in a supine position with an exposure length of 1-2 minutes (IVIS 100, Xenogen, USA). The resultant luminescence was measured by contour parameters as previously described in section 2.3.3.1.3. CF mice were re-imaged at 1, 3, 6, 9, 12, 15, 18 and 21 months after LV nasal instillation.

Even though cages of littermates were randomly assigned to treatment groups, the control groups LV-MT and PBS pre-treatment consisted of only male CF mice. The LPC/LV-Luc+CFTR treatment group consisted of 2 male and 10 female mice.

4.2.3.2 Therapeutic CFTR Gene Expression – CF Mice

Between 1-5 days following biophotonic imaging, the CF mice were examined for functional CFTR expression via nasal TPD as described in section 2.3.3.2.1. Nasal TPD's were also performed on both age-matched UnRx CF mice and age-matched Het colony controls.

4.2.3.3 Circulating Antibodies – CF mice

Blood from CF mice was removed following nasal TPD measurements by submandibular puncture (2.3.4) prior to LV administration (baseline), at each time an anaesthetic was administered during the long term study, and via cardiac puncture at study termination. The presence of circulating antibodies to the VSV-G envelope, the Luc protein and for the therapeutic CFTR protein were then analysed by

ELISA (2.2.7). All plates included negative controls of PBST, SKMPBST and UnRx CF mouse sera, as well as a positive control.

4.2.3.3.1 Anti-VSV-G protein

To detect the presence of antibodies to the VSV-G protein present on the envelope of the LV vector, ELISA plates were coated with a VSV-G pseudotype LV vector carrying a different transgene (EYFP) and incubated at 37°C for 1 hour. The positive control serum was obtained from a previous immune response study in which mice received multiple doses of our LV-MT vector and had been shown to be positive for VSV-G antibodies[357].

4.2.3.3.2 Anti-Luciferase

To detect the presence of serum antibodies to the protein of the transgene Luc ELISA plates were coated with recombinant Luc and assayed as detailed in 2.2.7. Sera used as the positive control were obtained from another experiment in which mice received two doses of Luc and were positive for circulating antibodies to Luc (see Chapter 5, Figure 5-6).

4.2.3.3.3 Anti-PDZK1 peptide for presence of the CFTR protein

To detect the presence of circulating antibodies to the CFTR protein ELISA plates were coated with a solution of peptide PDZK1 diluted in PBS and assayed as described in 2.2.7. Since there is no recombinant CFTR protein that could be used to coat the ELISA plate the PDZK1 peptide was used as a surrogate for the CFTR protein. The PDZK1 peptide is a scaffold protein that connects plasma membrane proteins and regulatory components, regulating their surface expression in epithelial cells apical domains. PDZK1 peptide's interaction with the C-terminus of the CFTR protein may potentiate

CFTR chloride channel function. Positive control sera were obtained from a CF mouse that was previously positive for the PDZK1 peptide.

4.2.3.4 Presence of Genomic CFTR

To determine if the CFTR gene had integrated into murine DNA, nasal septa tissue were collected at termination and were processed for the presence of genomic DNA using the direct tail lysis method as described in section 2.2.6.1.2. The crude lysates were then analysed for detection by qPCR (2.2.6.2) for the presence of the CFTR gene. All samples were performed in triplicate, included a negative template control and both gag sequence and mouse transferrin (house-keeping gene) were used.

4.2.3.4.1 Presence of genomic transgenes in other tissues

The presence of genomic DNA from the introduced genes in lung, liver and spleen tissue samples was determined by qPCR as described in Section 2.2.6.

4.2.4 Results

4.2.4.1 Body Weights – CF Mice

Body weight did not reduce by more than 10% after each anaesthetic event (Figure 4-10), and returned to pre-anaesthetic levels by 5-7 days. However due to the reduced production of *cftr (-/-)* null mice from the *cftr*^{tm1unc} colony, equal number of female and male mice were not available over the designated time frame of the study. Randomisation of mice into treatment groups was also limited to cages of littermates as mice could not ethically be single housed for the duration of the 2 year study and male mice could not be re-caged due to the potential of fighting to re-establish dominance.

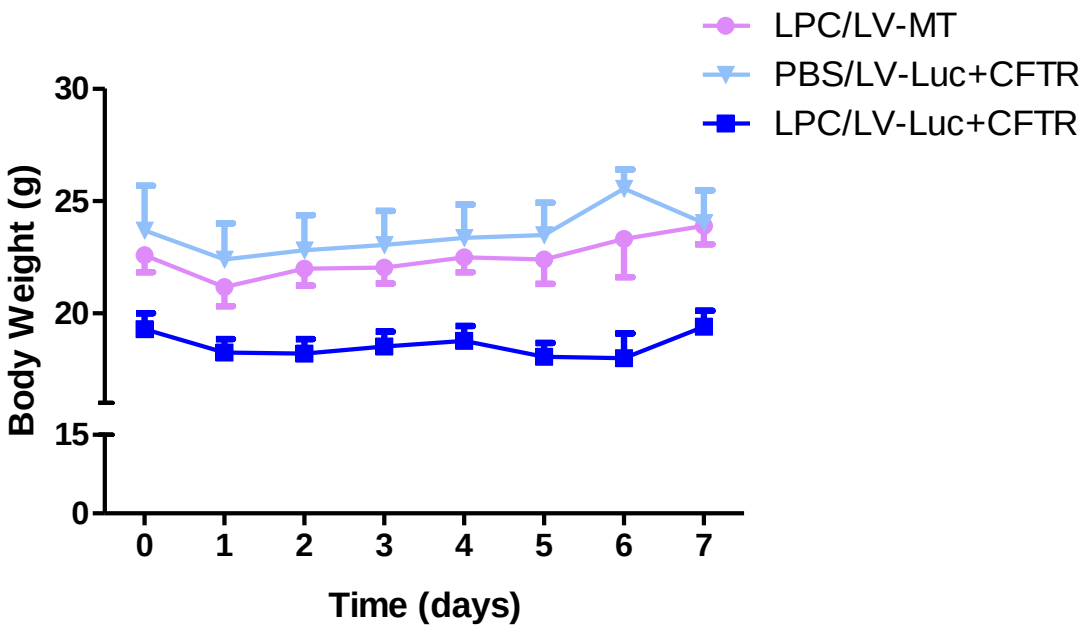


Figure 4-10 CF Mice Weights – One week
A reduction in body weight of <10% occurred on the first day after an anaesthetic event ($\bar{X} \pm SEM$, n=6-12).

Body weight increased over time, however the significant difference in body weight in the LPC-LV-Luc+CFTR treated mice was due to the cohort consisting primarily of females compared to the males in the control and treated groups (Figure 4-11, $p<0.001$, RM ANOVA, n=2-12).

Female CF mice had significantly lower body weight compared to CF males at the same age. There was a decrease in body weight in the male CF mice cohort that received LPC/LV-Luc+CFTR after the 6 month time assessment, but this was due in part to the death of one of these two mice.

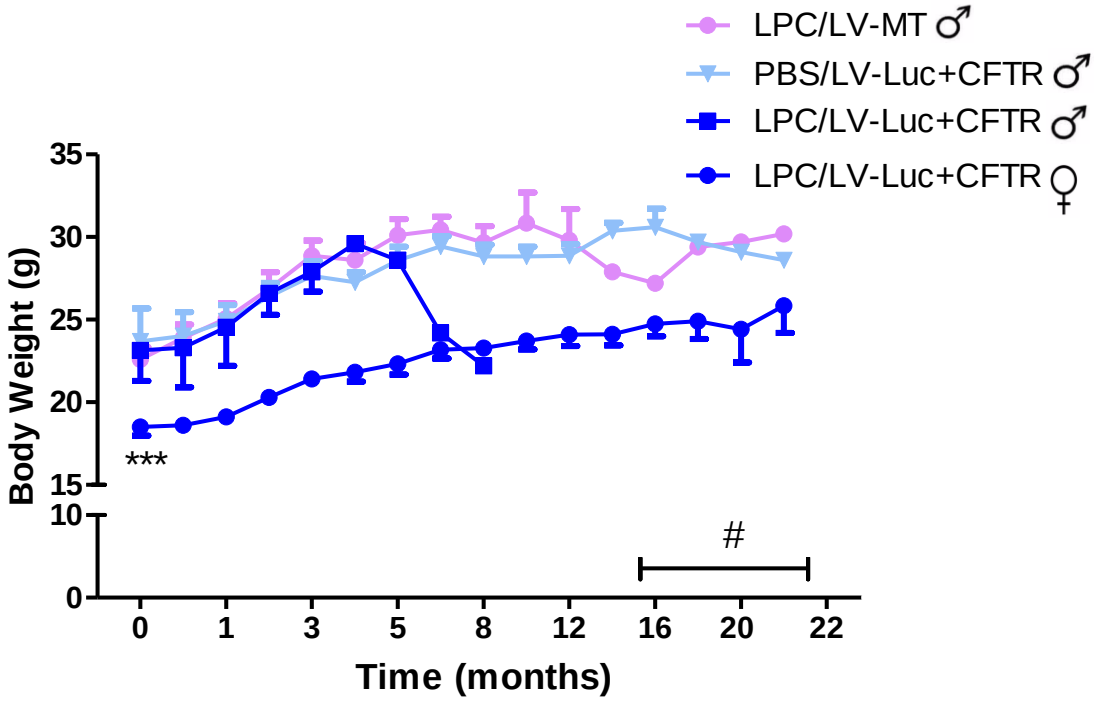


Figure 4-11 Body Weight of CF Mice over Time
*Body weight of LPC/LV-Luc+CFTR female (♀) CF mice was significantly different from all male (♂) CF mice (***p<0.001, RM ANOVA, $\bar{X} \pm SEM$, n=2-10). # numbers too low for analysis.*

4.2.4.2 Luciferase Gene Expression - CF Mice

After nasal LV gene treatment the airway luminescence detected in CF mice (Figure 4-12) was similar to that observed in normal mice (Figure 4-5). However, due to the natural high attrition of CF mice statistical analyses at the later time points (15-21 months) were unreliable because mouse numbers in the two control groups had become too low.

4.2.4.2.1 Nasal Luminescence

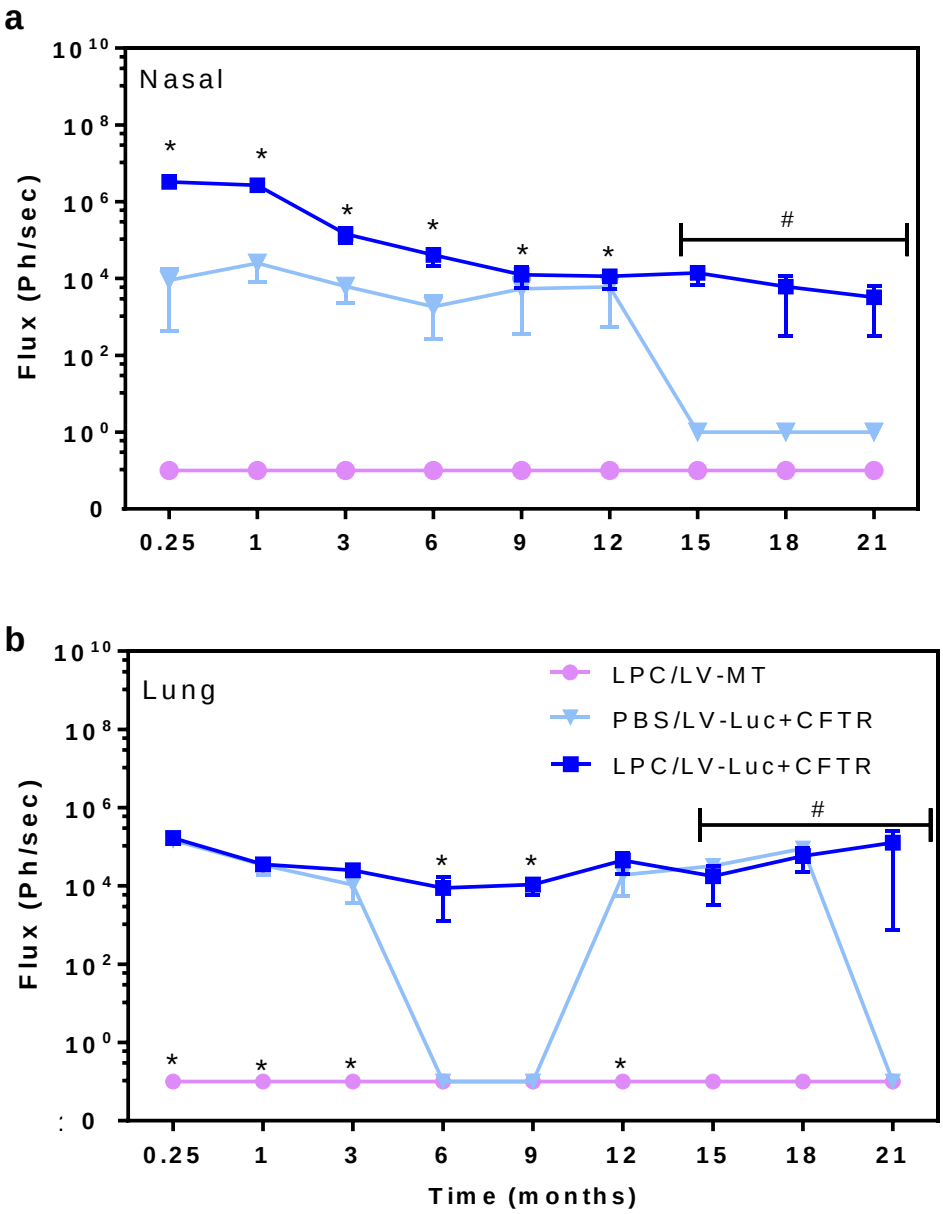
Figure 4-12a shows that no nasal luminescence was detected in CF mice that received the control treatment LPC/LV-MT, indicating that an active relevant transgene was required. Nasal luminescence was detected at the early time points in CF mice that received PBS prior to the Luc gene, however nasal

Luc gene expression was not significantly different from the other control LPC/LV-MT cohort (n.s. Dunn's RM ANOVA, n=2-6). For CF mice that were treated with LPC prior to the Luc gene, nasal luminescence was statistically significantly different compared to the controls for at least 12 months (Figure 4-12a, $p<0.05$, Dunn's RM ANOVA, n=2-12).

4.2.4.2.2 Lung Luminescence

Lung luminescence was also detected in CF mice that received the Luc gene, irrespective of pre-treatment, and was significantly greater than in CF mice administered nasally with the empty vector (LV-MT) (Figure 4-12b, $p<0.05$, Dunn's RM ANOVA, n=2-12). At the 6 and 9 month assessment, lung luminescence in the mice treated with PBS/LV-Luc+CFTR had disappeared, but re-emerged at the 12-18 month time points. This similar trend of re-emergence of lung luminescence was also observed in the LPC/LV-Luc+CFTR treated group, but was not a statistically significant effect.

In the Luc treated mice the disappearance and re-emergence of lung luminescence was not an artefact of the imaging technique or problems with i.n. D-luciferin administration, since in the LPC/LV-Luc+CFTR treated cohort nasal luminescence was also detected in these animals (refer to bioluminescence images over time in a LPC/LV-Luc+CFTR mice (Figure 4-13).



n=						
LPC/LV-MT		2	2	1	1	1
PBS/LV-Luc+CFTR	5	4	3	3	1	1
LPC/LV-Luc+CFTR	11	10	9	7	4	4

Figure 4-12 Nasal and Lung Luminescence in CF mice

*Luciferase gene expression after nasal LV administration in the a) nasal and b) lung airways of CF mice (*p<0.05, Dunn's RM ANOVA, $\bar{X} \pm SEM$, n=6 for LV-MT and PBS, n=12 for LPC unless stated at later time points). # number of animals too low for analysis.*

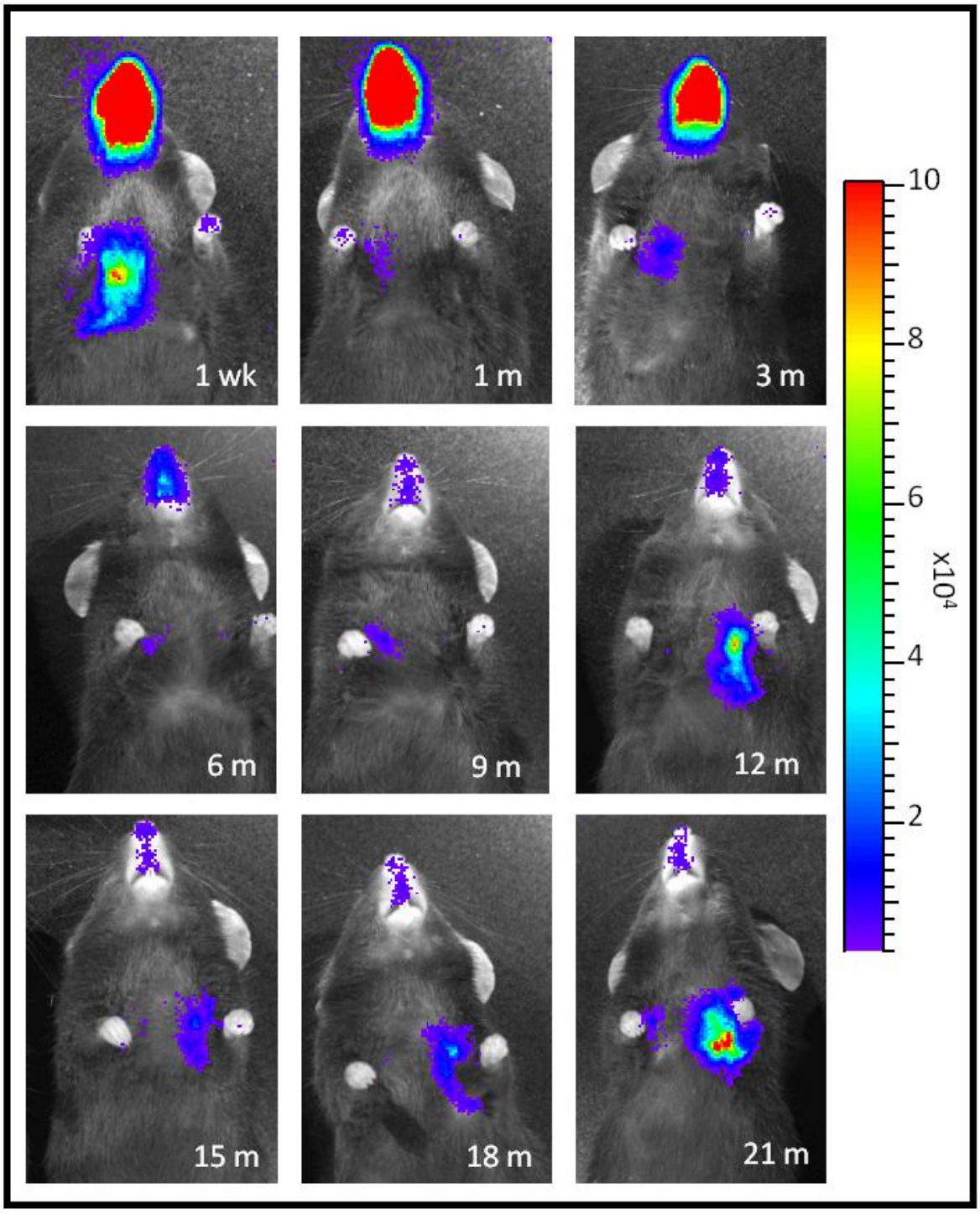


Figure 4-13 Bioluminescence over Time in a LPC/LV-Luc+CFTR treated CF mouse
Re-emergence of lung luminescence from 12 months with persistent and increasing levels of Luc flux following a single LPC/ LV-Luc+CFTR dose. Lung luminescence was below detection at 1-9 months, and nasal luminescence below detection at the 21 month time point only (via contour parameters). Colour intensity scale in Ph/sec/cm^2/sr.

4.2.4.2.3 Nasal Luciferase Expression – LPC vs PBS

Luminescence was detected in the nasal airways of all 12 CF mice that received the Luc gene with LPC as a pre-treatment, at the early time points (Figure 4-14a). Three months after LPC/LV-Luc+CFTR instillation, nasal luminescence slowly reduced over time ($p<0.05$, Dunnett's ANOVA compared to 1 week, n=3-12). This reduction in nasal luminescence corresponded to the increase in numbers of mice that displayed no nasal luminescence ($p<0.05$, Spearman correlation r = -0.71, range of zero luminescence from 3 months 16%-75%, see Appendix 8.3-1).

Nasal luminescence was detected in 16-50% of the CF mice that received PBS prior to Luc as a control treatment (Figure 4-14c), but the resultant flux was significantly (10^2-10^3 Ph/sec) lower than those mice that received LPC as a pre-treatment.

4.2.4.2.4 Lung Luciferase Expression – LPC vs PBS

Mice that received LPC prior to the Luc gene displayed sustained lung gene expression throughout the study period, with a slight reduction in lung luminescence detected at the 6-9 month assessments (Figure 4-14b). The non-significant reduction in lung luminescence was due to the loss of lung expression and the re-emergence of expression in those individual animals (Figure 4-13 and refer to Appendix 8.4-1 and Appendix 8.4-2 for individual lung expression in CF mice).

Lung luminescence completely disappeared in the group of mice that received PBS/LV-Luc+CFTR ($p<0.05$, Dunnett's ANOVA, n=2-6) and re-emerged 12 months after the initial nasal instillation (Figure 4-14d).

This re-emergence of lung Luc expression in CF mice was not maintained for the whole assessment time period. However due to low numbers of CF mice at the later time points, no statistical analysis

could be performed at the final two time point assessments (refer to Appendix 8.4-3 for individual lung expression in CF mice).

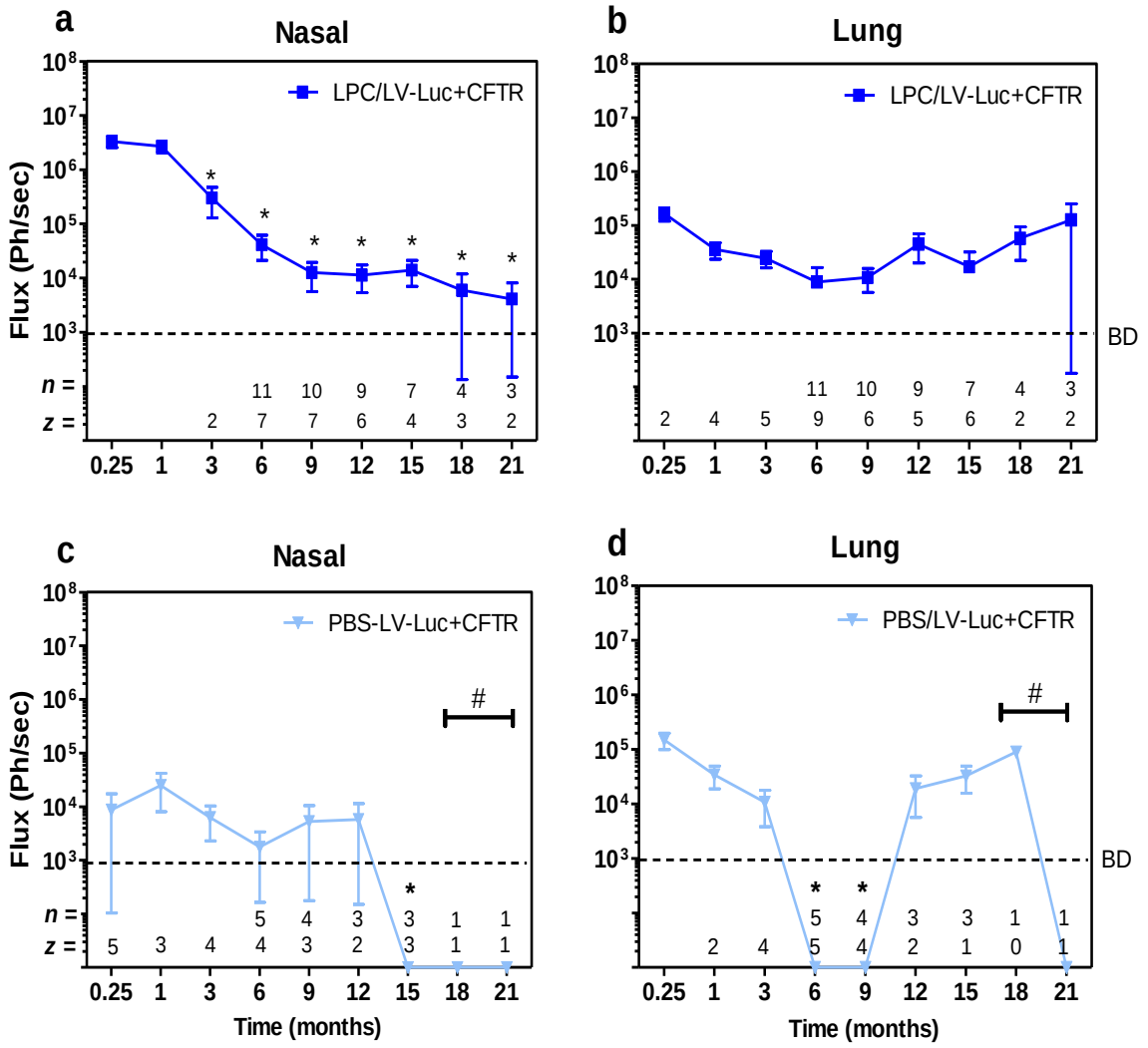
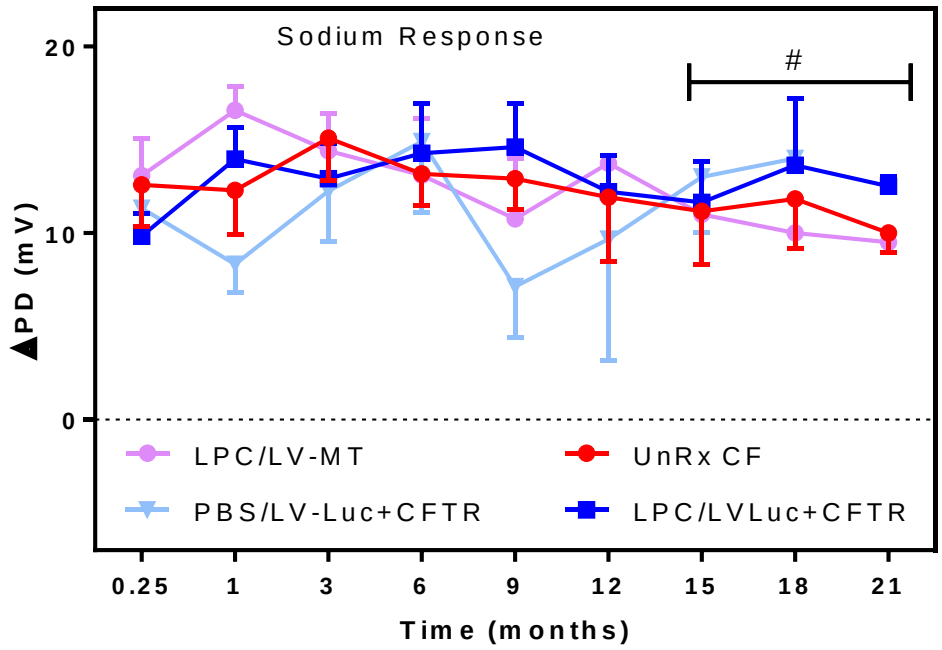


Figure 4-14 Average Luciferase Expression in CF mice – LPC vs PBS
*Luciferase gene expression after nasal LV administration in a, c) nasal and b, d) lung airways of CF mice (*p<0.05, Dunnetts ANOVA compared to 1 week, $\bar{X} \pm SEM$, n= 6 for PBS, n=12 for LPC, or as stated in later time points). Z= number of mice displaying zero Luc expression; BD= below detection (10^3 Ph/sec), # number of animals too low for analysis.*

4.2.4.3 Therapeutic CFTR Gene Expression - CF Mice

There was no difference in the change in nasal TPD caused by the addition of the sodium channel blocker amiloride to basal KRB infusion (ΔPD_{Na+}) across all treatment groups, and ΔPD_{Na+} was also no different to UnRx CF mice at the same time points (Figure 4-15).



n =							
LPC/LV-MT	5	5	2	2	1	1	1
PBS/LV-Luc+CFTR		5	4	3	2	1	0
LPC/LV-Luc+CFTR	11	11	10	9	7	4	3

Figure 4-15 Nasal ΔPD_{Na+} of the Sodium Channel Response in CF mice
Response of the nasal ΔPD_{Na+} to the sodium channel blocker amiloride (n.s.,RM ANOVA, $\bar{X} \pm SEM$, n=6 for PBS, LV-MT and UnRx, n=12 for LPC, unless stated at later time points). Untreated (UnRx), # number of animals too low for analysis.

To test if the CFTR defect in CF mice had been corrected by the administration of the CFTR gene into nasal airways, the change in response to chloride ions being transported across the nasal epithelia was examined. There was no change in the chloride channel response (ΔPD_{Cl-}) in the two control groups of mice (LPC/LV-MT and PBS/LV-Luc+CFTR) compared to age-matched UnRx CF mice (Figure 4-16a, n.s., RM ANOVA compared to UnRx CF, n=2-6).

In contrast, in the nasal airways of CF mice that received the therapeutic gene CFTR in conjunction with LPC pre-treatment, there was a sustained partial correction of the bioelectrical defect that persisted continually for up to 12 months (Figure 4-16b, $p<0.05$, RM ANOVA, n=3-12).

Due to the low numbers of mice that remained at the later time points in the control groups, statistical analysis was not reliable from 15 months to 21 months. However a continuous partial correction of the chloride response (functional CFTR correction towards normal of 33 +/- 5%, range 12-54%, $\bar{X} \pm SEM$, n=8-12) was seen in mice that received LPC and LV-Luc+CFTR. This correction persisted for 15 months, a time that equates to approximately ¾ of the lifetime for a CF mouse.

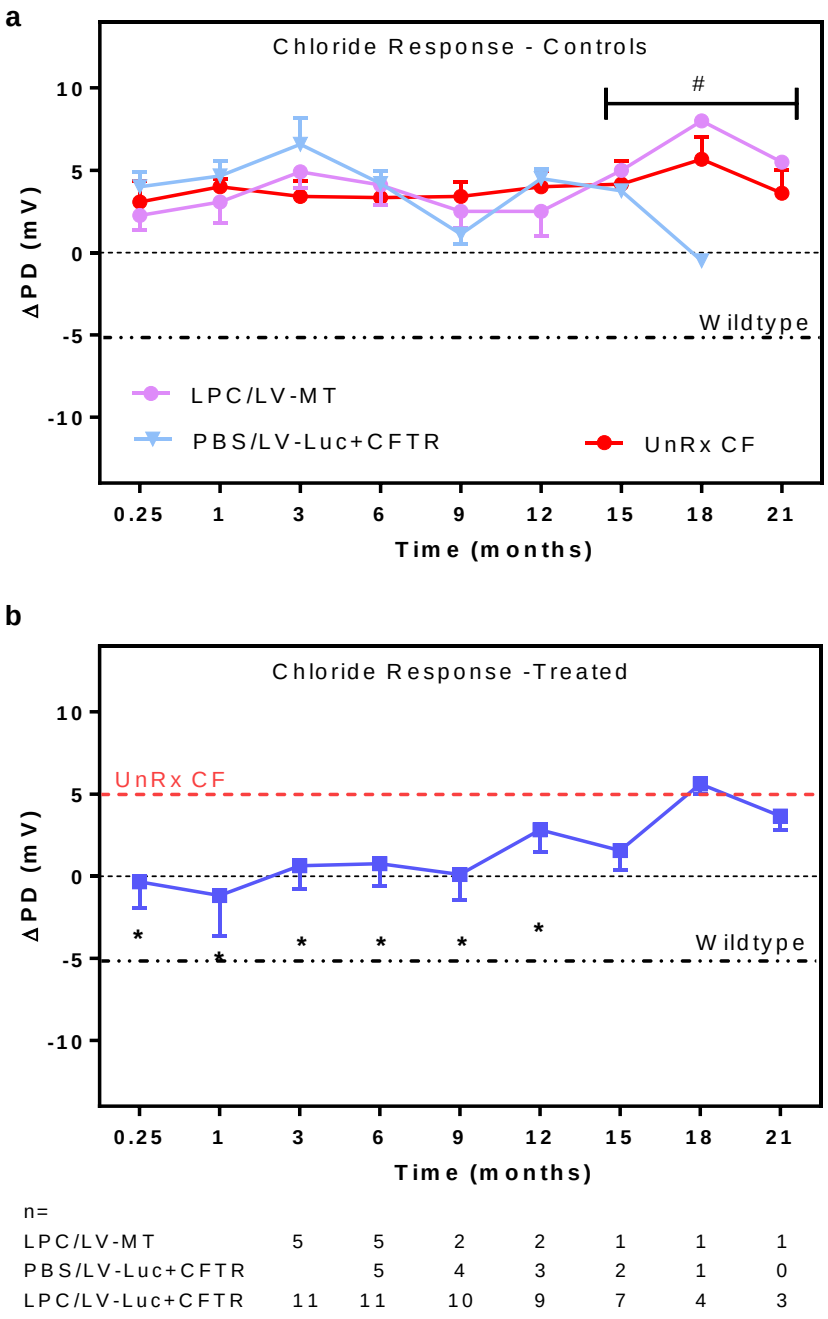


Figure 4-16 Nasal ΔPD_{Cl-} of the Chloride Channel Response in CF mice

*Response of the nasal ΔPD_{Cl-} to the chloride channel for a) control groups and b) CFTR Treated CF mice (*p<0.05, RM ANOVA vs untreated (UnRx) CF, $\bar{X} \pm SEM$, n= n=6 for LV-MT, PBS and UnRx; n=12 for LPC, unless stated at later time points). Red dotted line = average of untreated CF mice ΔPD_{Cl-} (n=6) and black dotted line = target level of wildtype mice ΔPD_{Cl-} response (n=6). # number of animals too low for analysis.*

Correction of the CF defect (ΔPD_{Cl-}) was also significantly and strongly correlated to the mean nasal Luc reporter gene expression in this LPC/Luc+CFTR treated cohort (Figure 4-17, $p<0.01$, Spearman $r = -0.92$).

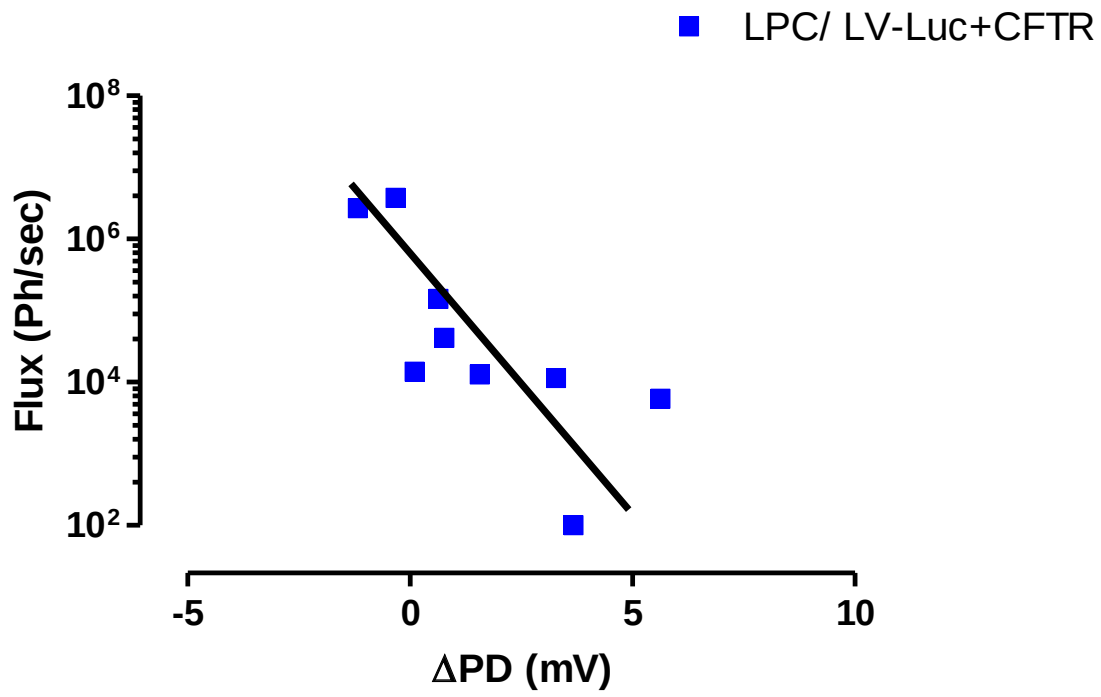


Figure 4-17 Correlation of Luminescence vs ΔPD in CF mice
Increase in luciferase gene expression was significantly correlated to the correction towards normal of the CF electrophysiological defect (more negative ΔPD_{Cl-}) in CF mice treated with both LPC, the reporter Luc gene and the therapeutic CFTR gene ($p<0.01$, Spearman $r = -0.92$).

4.2.4.4 Circulating Antibodies – CF Mice

4.2.4.4.1 Anti-VSV-G protein

Circulating antibodies to the VSV-G protein were significantly increased at time points from 1-9 months following nasal instillation of either PBS or LPC prior to LV-Luc+CFTR, compared to sera analysed from

the same mice prior to LV instillation (Figure 4-18a, $p<0.001$, Dunnett's ANOVA compared to time zero, n=2-12). The peak of antibody production to the LV envelope occurred at 1 month after LV delivery. However, the increase in circulating antibodies to the VSV-G protein was not greater than the level detected for the positive control (4 doses of LV vector).

For the control group LPC/LV-MT there was no difference in circulating antibodies to the VSV-G protein at any post-treatment time points compared to baseline (again designated as time zero).

4.2.4.4.2 Anti-Luciferase

At all post-treatment times there was no difference in circulating antibodies to the transgene protein Luc in CF mice that received either of the two control treatments LPC/LV-MT and PBS/LV-Luc+CFTR, compared to baseline. A significant increase in circulating antibodies to the transgene Luc was detected at 3 - 9 months, compared to baseline, only in CF mice that received LPC prior to LV-Luc+CFTR (Figure 4-18b, $p<0.001$ Dunnett's ANOVA, n=2-12). In mice that received LPC prior to Luc the peak of antibody detection occurred 2 months later than the peak antibody response to the LV vector envelope in the same mice.

4.2.4.4.3 Anti-PDZK1 peptide for presence of the CFTR protein

There was no significant increase in circulating antibodies to the CFTR protein in mice that received the two control treatments (LPC/LV-MT and PBS prior to CFTR) or the CF mice treated with LPC prior to LV-Luc+CFTR, compared to baseline (Figure 4-18c, n.s., Dunnett's ANOVA, n=2-12). Mice that received LPC prior to the functional CFTR gene had a slight increase in circulating antibodies, but the response was at least 3 times lower than that detected to both the VSV-G protein constituents and the Luc protein.

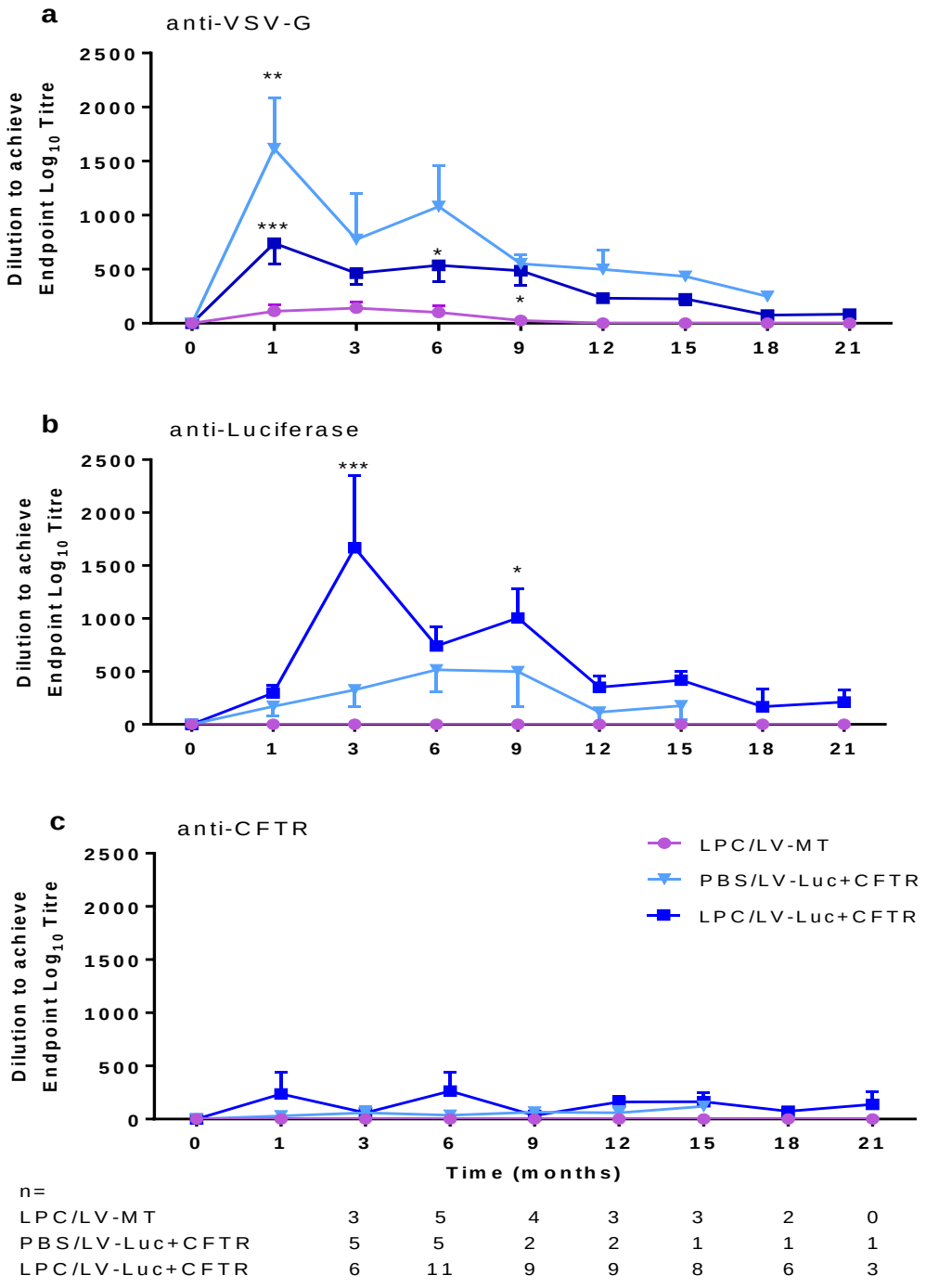


Figure 4-18 Circulating Antibodies – CF Sera

*Presence of circulating antibodies detected in sera to the a) LV envelope protein VSV-G, b) recombinant luciferase (Luc) protein and c) PDZK1 peptide for CFTR protein over time. (***p<0.001, **p<0.01, *p<0.05., Dunnett's ANOVA compared to zero time point (baseline), $\bar{X} \pm SEM$, n= n=6 for PBS and LV-MT, n=12 for LPC, unless stated at later time points).*

4.2.4.5 Presence of Genomic CFTR

The CFTR gene was not detected by qPCR in any nasal septum samples from both of the control groups PBS/LV-Luc+CFTR and LPC/LV-MT. A significant presence of the CFTR gene that successfully integrated into the genome was detected in those mice that received LPC prior to the CFTR gene compared to the two control groups (Figure 4-19, $p<0.001$, Tukey's ANOVA, n=5-10).

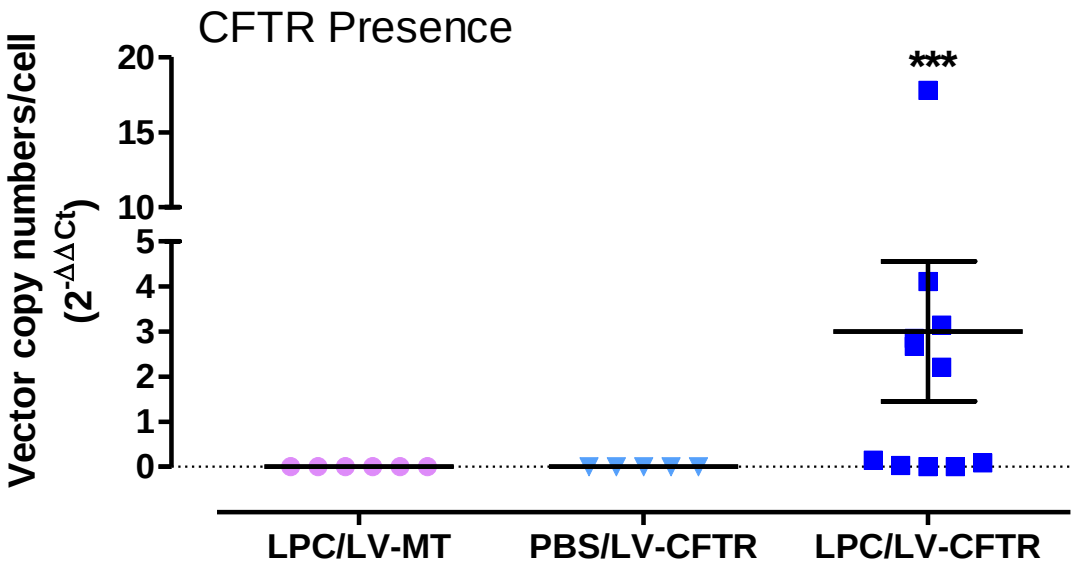


Figure 4-19 Presence of Genomic CFTR DNA from Nasal Septa
*Genomic CFTR detected in nasal septa via qPCR in LPC/LV-Luc+CFTR treated CF mice (***p<0.001, Tukey's ANOVA, n=5-11). Ct = Cycle threshold, bar represents data as* $\bar{X} \pm SEM$.

4.2.4.5.1 Presence of genomic transgenes in other tissues

Very few samples of lung, liver and spleen were available for qPCR analysis because the majority of animals in this long term study either died unexpectedly or were ethically euthanized and the carcasses were assessed by a pathologist for necropsy examination.

For the remaining six animals examined, the transgene was not detected in liver or spleen tissues. For CF mice treated with LV-MT no transgene was detected in lung tissue. Low copy numbers of genomic DNA was detected in the lung tissue of the CF animals treated with LV-Luc+CFTR (Vector Copy numbers/cell = 0.02 +/- 0.009, $\bar{X} \pm SEM$, n=4).

4.2.4.6 Survival Demographics of CF Mice

This was the first study worldwide to employ the repeated-measure design using both assessment of expression of the reporter Luc gene and the therapeutic CFTR gene in the same individual CF mice over their lifetime. Previous long term gene therapy studies by the Adelaide group have examined mice at all ages, however gene expression assessment was performed only once per animal[297]. As with normal C57Bl/6 mice, the impact on animal health of CF mice from repeated anaesthetics, imaging, nasal TPD measurements and submandibular bleeding was closely monitored according to Animal Ethics Committees' guidelines.

A survival curve (Figure 4-20) was constructed from survival statistics of the CF mice involved in the long term study. All treatment groups had a defined median survival due to either natural attrition from old age or required euthanasia due to injury or poor health from possible treatment effects. The LPC and CFTR treated group had a statistically longer median survival (20 months) than the control groups that received either PBS prior to CFTR (14.5 months) or an empty vector (9 months) ($p<0.05$ Mantel Cox log-rank test, n=6-12).

There was no significant difference in survival when the LPC/LV-Luc+CFTR treated CF mice were compared to untreated normal C57Bl/6 mice (n.s., Mantel Cox log-rank test, n=6-12).

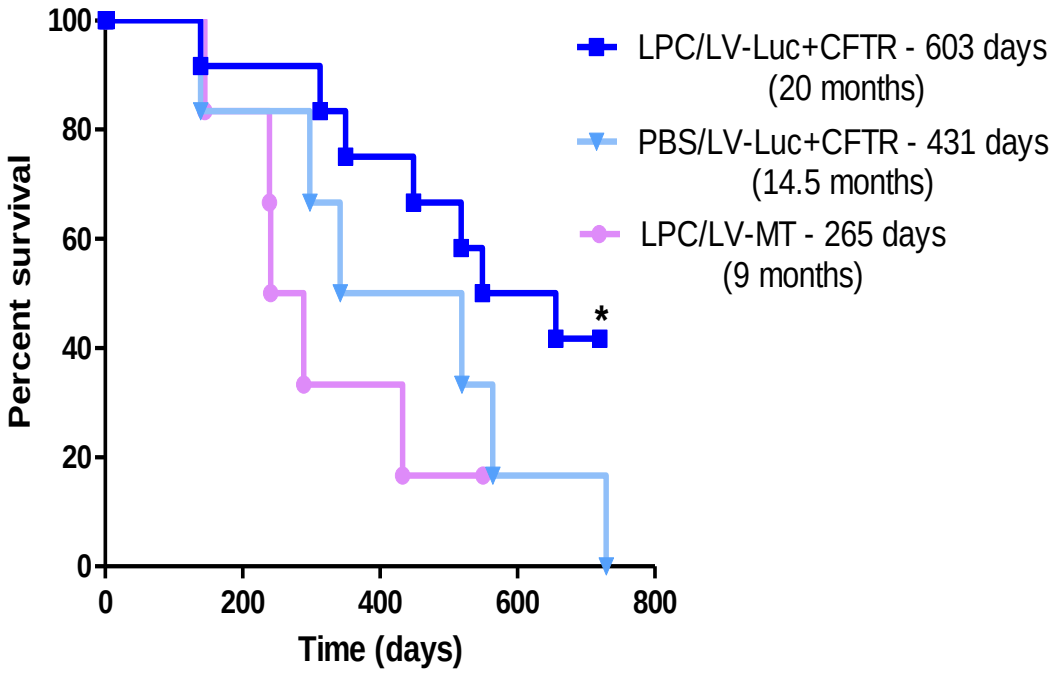


Figure 4-20 Survival Curve – CF Mice
*LPC/LV-Luc+CFTR treated mice had a longer median survival than the two control groups (*p<0.05, Mantel Cox log-rank test, n=6-12)*

CF (*cftr* ^{tm1unc}) mice do not develop lung disease and death is from intestinal obstruction from 40-60 days[301, 306] if no preventative dietary supplement (e.g. ColonLyetly®) is added to the animal's drinking water. Therefore, the administration of the therapeutic gene CFTR to the nasal airways would not be expected to increase animals' survival relative to the intestinal disease caused by CFTR dysfunction. To ascertain whether the difference in CF animal survival was due to a treatment effect, survival data was analysed for any correlating effects.

There was no correlation between the total number of anaesthetic treatments used for all procedures (such as nasal instillations, bio-photonic imaging, nasal TPD measurements and submandibular bleeding) and the number of animal deaths (Figure 4-21, n.s., r^2 =0.08, Pearson's Correlation). With increasing number of treatments there was no negative impact in animal survival. Five animals had over

20 anaesthetic events over 15-21 months without incident. Out of the 24 CF mice utilised in the 2 year study only 2 deaths were directly attributed to a known anaesthetic issue, but in both cases these were due to an overdose (Table 4-1).

All carcasses from unexpected animal deaths and earlier than expected deaths by required euthanasia, were sent to a veterinary pathologist for necropsy reports. Three mice were found to have lymphomas, but this was no different to the incidence of carcinomas in untreated mice[367, 368]. There was also no correlation between the time following the last procedure and mouse death (Figure 4-22, n.s, r^2=0.03, Pearson's Correlation).

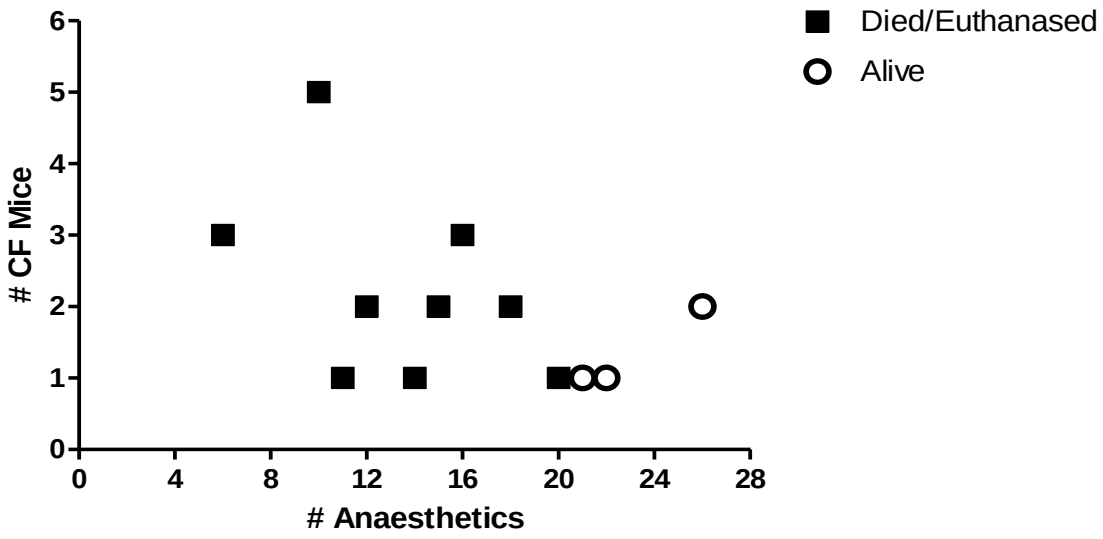


Figure 4-21 Number of Anaesthetics
There was no correlation between the number of anaesthetic treatments on CF animal survival (n.s., Pearsons correlation r^2=0.08).

TREATMENT	AGE	DEATH	EUTHANASED
LPC/LV-MT	3 m	Lymphoma	
LPC/LV-MT	9 m	Anaesthetic	
PBS/LV-Luc+CFTR	1 m	Intestinal Obstruction	
PBS/LV-Luc+CFTR	6 m	Pulmonary Odema	
LPC/LV-Luc+CFTR	6 m		Spinal Injury
LPC/LV-Luc+CFTR	9 m	Anaesthetic	
LPC/LV-Luc+CFTR	12 m		Eye Infection
LPC/LV-Luc+CFTR	15 m		Lymphoma
LPC/LV-Luc+CFTR	18 m		Lymphoma

Table 4-1 Necropsy Results
Known aetiology from necropsy results of CF mice fatalities (data; Dr. John Finnie, Senior Veterinarian Pathologist, IMVS, Adelaide, SA).

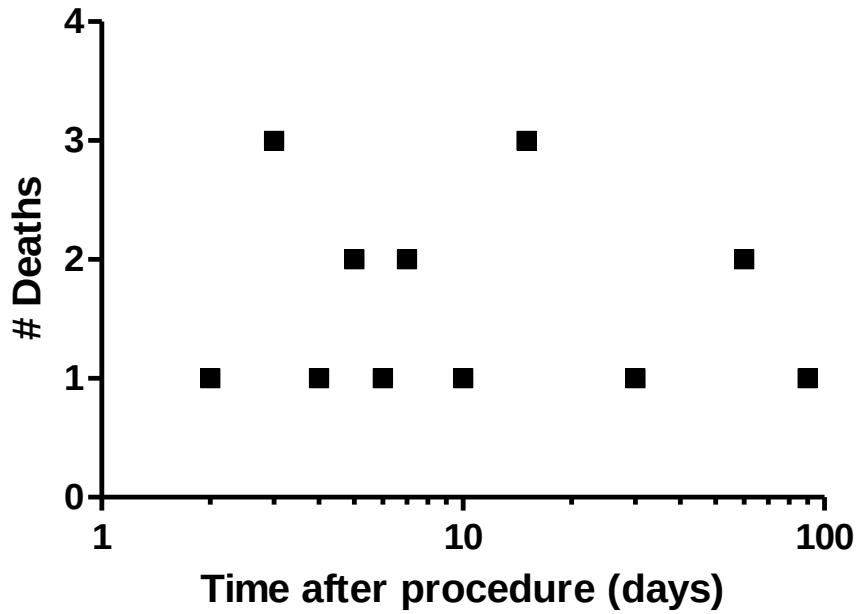


Figure 4-22 Time after Procedure
Deaths of CF mice following last procedure in days (r^2=0.03, Pearson's Correlation). Mice that died directly due to anaesthetic overdose not included.

For CF mice that died unexpectedly and where necropsy results also revealed no significant morphological changes, the age of the mice were analysed against treatment type (Figure 4-23a) and gender (Figure 4-23b). The age at which the mice died was not related to the type of treatment (n.s., r^2=0.38, Pearson's Correlation for LPC/LV-Luc+CFTR) nor for the sex of the animal (n.s., r^2=0.11, Pearson's Correlation for male).

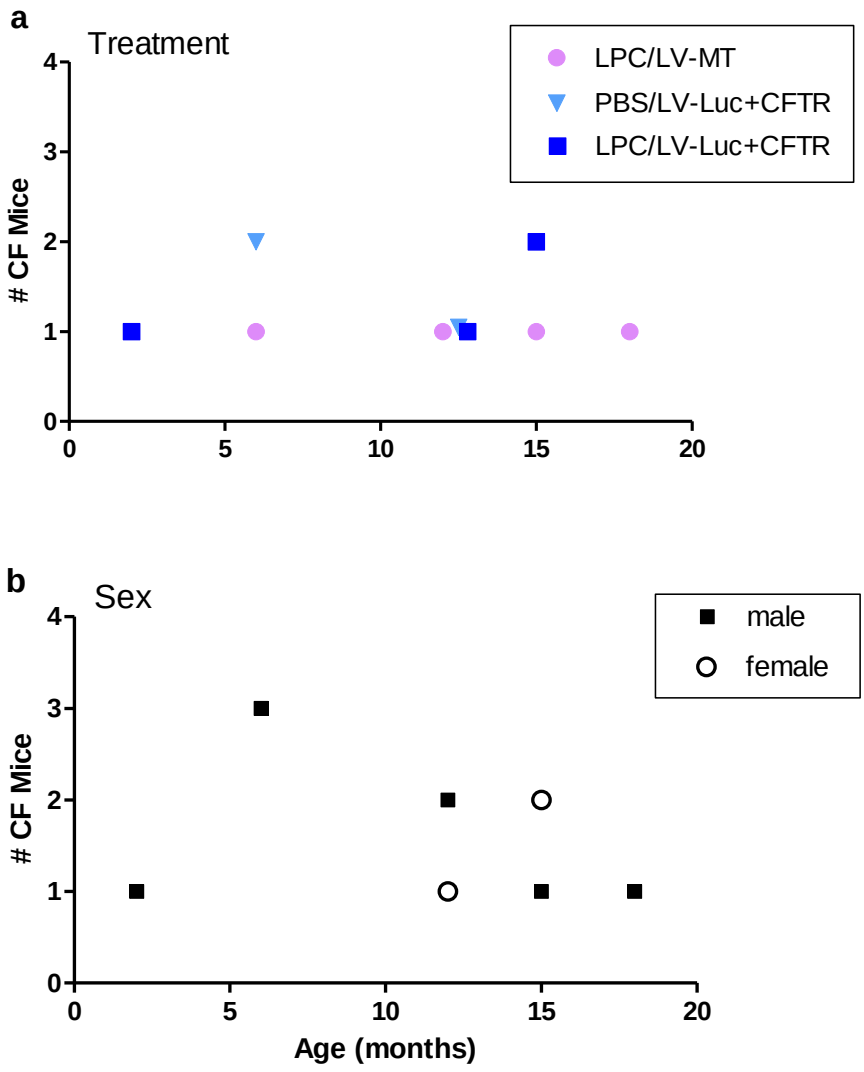


Figure 4-23 Unexplained Mice Deaths
The age at which CF mice died unexpectedly and had no known aetiology was not related to a) type of treatment or b) gender (r^2=0.38 and r^2=0.11, respectively, Pearson's Correlation).

4.2.5 Discussion

This is the first reported study of a LV gene therapy employing a repeated-measures design for long term airway gene expression in individual CF mice over their lifetime. Non-invasive imaging of *in vivo* gene expression by the Luc transgenes' production of luminescence has allowed for the monitoring of individual animals over long periods of time. Extended (9 months – 2 years) luciferase reporter airway gene expression in normal mice has previously been described using viral vectors such as HD-AdV[369], SIV[270, 361, 370]and FIV[275, 293]. However, simultaneous monitoring of long term Luc (reporter) and CFTR (therapeutic) gene expression in the same CF mice has not been previously reported.

Although CF mouse studies began with larger than normal group sizes, the natural attrition of the less robust CF mice, smaller initial experimental numbers in the two control groups, and the repeated gene expression assessments led to inadequate sample size in these control cohorts for statistical analysis at the later time points.

As observed in normal mice, no Luc expression was detected in the control group of CF mice that received a LV vector without an included transgene (LV-MT). Although a low level of Luc gene expression was detected in the nasal airways of the control group that received PBS prior to a LV-Luc gene vector at the early time points, the level of gene expression was no different to the LPC/LV-MT control group. For those CF mice treated with LPC prior to the Luc gene, high level and extended luciferase reporter gene expression was present in nasal airways, at levels significantly greater than both control groups for up to 1 year after a single LPC/LV vector dose. This further supports the need for LPC for effective transduction of nasal airways due to possible increased vector residence time from reduction in MCC[371] and access to the receptors on the basolateral surface.

For mice treated with LPC and Luc the resultant nasal luminescence expression in CF mice persisted for half of the period it was present in normal mice, however statistical significance could not be analysed after the 15 month time point in CF mice due to low numbers in the control groups. When CF mice in the LPC/LV-Luc treated group were considered alone, nasal luminescence persisted for 15 months, ~ 75% of the longevity as observed in normal mice, given the same airway gene transfer treatment. The persistence of expression in the LPC/LV-Luc+CFTR mice may be due to transduction of terminally differentiated cells with slow turnover times[328] or replenished expression from airway progenitor cells[333].

Three months after LPC/LV-Luc+CFTR instillation, nasal luminescence slowly reduced over time. The reduction in nasal luminescence was due to the loss of nasal luminescence in particular mice possibly due the process of normal cell turnover of nasal epithelial cells. This reduction was similar to that observed in nasal reporter gene expression in normal mice, but occurred earlier at 3 months compared to 6 months in normal mice. The turnover of nasal cells and the types of progenitor/stem cells is currently unknown and the majority of knowledge about respiratory epithelia half-life and proliferative capability comes from lung airways. The proposed half-life of lung respiratory cells range from 3-17 months[328, 329], but translating this directly to nasal respiratory epithelia may not be appropriate. The nasal airways are the first line of defence which are subject to inhaled pathogens and injury and would therefore likely have a shorter cell turnover time and possibly a variety of stem/progenitor populations primed for proliferation, which may also differ in proportions in CF mice compared to normal mice.

To confirm the hypothesis that stem/progenitor cells have been transduced by a gene transfer treatment, and if these cell populations vary from normal and transgenic mice, would be the next logical step. The ability to identify the possible transduced stem/progenitor cells using immunohistochemical

staining techniques may be possible for lung airways[328], but have not been successfully translated to nasal airways due to the inference of the necessary decalcification processing of the murine skull.

Lung luminescence was also detected in CF mice that received the Luc gene, regardless of airway pre-treatment. Of particular interest in the context of potential transduction of adult airway stem cells was the finding of a reduction in lung luminescence at the 6-9 month assessments in mice that received Luc, completely disappearing in all mice in the PBS pre-treated group, but re-emerging 12 months after the initial nasal instillation. Re-emergence of gene transfer in the lung airways in both strains of mice further supports the hypothesis that airway stem/progenitor cells[372] may have been transduced. The loss of transduced terminally differentiated epithelial cells by normal cell turnover processes[328] can explain the loss and reduction of lung gene expression. However the re-emergence of lung gene expression 3-6 months later suggests that some progenitor cells[329], such as basal cells[332, 333] that reside in protected specialised niches[331] were transduced and therefore the re-emergence of expression may come from the proportion of resultant daughter cells that arise from these transduced stem cells. Since re-emergence of lung expression also occurred in the PBS treated group, LPC may not be necessary for lung transduction, nor for access to basal cells in the lung airway. A proportion of Clara cells may also perform as stem/progenitor cells for the lung[373], or transient amplifying cells[374] arising from 'niche protected' stem cells, may be transduced and therefore LPC may not be essential for longevity, especially in lung airways[375].

Future examination of the possible gene transduction of stem/progenitor cells in both nasal and lung airways by the utilization of separative clonogenic assays[366] may help elucidate the mechanisms of longevity of gene expression and would be of major significance for future gene therapy studies.

Reporter gene expression in CF mice can provide valuable evidence for any differences in the level or distribution compared to normal mice. In this case, there was a difference in the duration of reporter

gene expression between the two strains, and a complete loss of lung luminescence was observed in CF mice. The profile of antibodies to the Luc protein in both strains of mice differed in terms of both the level detected in the sera, as well as the persistence of the immune response. The differences in reporter gene expression observed in the CF null strain highlights the differences in nasal anatomy[376] pathophysiology[302, 306, 377, 378] including inflammatory and immune responses[379] compared to normal mice. This reporter gene evidence provides the next step towards translation of gene therapy protocols utilising a therapeutic/functional gene treatment for a transgenic mouse model.

As previously stated, the ultimate goal for a CF gene therapy is to successfully insert a functional copy of the defective gene to enable correction of the CFTR defect[380]. There is still debate on how much CFTR expression is required (from 5% - 25%[324-326]) to achieve correction for a therapeutic effect in humans.

The results showed that the control treatments PBS/LV-Luc+CFTR and LPC/LV-MT had no effect on the change in TPD and were similar to that measured in non-treated CF mice. In contrast, there was a sustained partial correction in the bioelectrical defect in the nasal airways of CF mice that received the therapeutic gene CFTR, when given in conjunction with LPC pre-treatment, for up to 1 year. This correction of the chloride response was significant compared to the response in age-matched UnRx CF mice, and the improved function ranged from 12-54% towards normal. That is, if CFTR correction translates to a similar level of lung disease correction, this effect would likely be sufficient as a therapeutic treatment. The significance of these results is that this is the first instance of long term airway CFTR therapeutic correction of the electrophysiological defect monitored in the same CF mice over their lifetime, further supporting similar findings in timed-group studies[297].

The long term partial correction of the CFTR defect as functionally measured by nasal TPD was strongly correlated to the presence of the Luc reporter gene expression in the same cohort of LPC/LV-

Luc+CFTR treated CF mice (Spearman correlation r = -0.92). The higher the level of luminescence detected in the nasal airways of the same mice was proportional to the more negative (i.e. closer to normal values) ΔPD_{Cl^-} of the chloride response. This provides further support that the use of reporter gene transfer studies can be useful as a therapeutic gene expression surrogate.

Introduction of a foreign protein (Luc or CFTR) into the airways of mice to achieve successful gene therapy has the potential to elicit an unwarranted host immune response. The immune response can be to the viral vector and to the transgene product (the expressed protein). The majority of early viral vectors used for CF gene therapy such as AdV have been ineffective. In large part this has been due to the host's immune response[381] resulting in transient expression and the inability to effectively re-dose. Lentiviral vectors are less immunogenic[382] than other viral vectors such as AdV, but an immune response is nevertheless raised against the transferred gene product[364, 383] and still is to be satisfactorily addressed.

The sustained increase in antibodies to the LV vector envelope protein from 1-9 months after a single nasal delivery was not substantial enough to completely block nasal gene expression, indicating that repeated gene vector dosing may be possible to maintain gene expression levels if needed. An increase in circulating antibodies to the VSV-G protein, a constituent of the gene vector structure, was detected in the blood of mice that received a single dose of the Luc/CFTR gene, but not in those mice that received the empty vector. The presence of a "live" antigen (such as Luc or CFTR) that integrates into the genome after a single administration could provoke an immune response to a viral capsid protein[384] whereas an empty vector or inactive virus particle may provoke an immune response but not sufficient to induce an Immunoglobulin G neutralization humoral response[385, 386].

Only the mice that were treated with LPC/LV-Luc had a significant increase in Luc antibodies, this was present 3 - 9 months after treatment. The peak of antibody presence was 3 months after treatment in a

small proportion of CF mice in which nasal and lung luminescence (i.e. gene expression) disappeared completely. Circulating antibodies were at undetectable levels by 12 months which coincided with some re-emergence of lung expression in this cohort. However the re-emergence of lung luminescence also occurred in the PBS/LV-Luc treated group, which had no detectable levels of circulating antibodies to the Luc protein product at any time point, suggesting that the loss of gene expression may have been due to normal cell turnover and loss of terminally differentiated transduced cells rather than a host immune response to the introduced protein.

Following airway administration of the therapeutic CFTR gene, no increases in antibodies to the CF peptide PDKZ1 detected in blood from mice that received LPC as a pre-treatment. The only CFTR protein molecule available at the time was the PDKZ1 peptide used to coat the ELISA plates, which may have been ineffective in forming an antigen complex with the primary CFTR antibodies from the mouse sera, due to the intracellular location, tissue specificity and synthetic composition. The recombinant fragment of the human CFTR of known amino acid sequences with the N-terminal tag is now available and worth pursuing in future studies for its potential to detect circulating antibodies to the CFTR protein product via ELISA.

As this was the first study to employ a repeated-measures design with CF knockout mice and required multiple gene transfer assessments, the impact on survival was closely monitored. The survival statistics of untreated CF (-/-) null mice from our *cftr*^{tm1unc} colony in Adelaide is anecdotal as mice are bred for experimental purposes only and death as an endpoint is not an ethically acceptable outcome in this instance. Separate cohorts of untreated CF mice were used as age-matched controls for TPD assessments (including those that survived up to 18 and 21 months of age) however the proportion of CF null mice that are long lived in our colony is unknown. All CF mice receive a mild diarrhoeal agent and high caloric diet to overcome the fatal gut obstruction in newly weaned mice, but it is still a major

cause of morbidity and mortality in this particular strain. All treated CF mice were adults (7 - 15 weeks of age) at time of LV instillation and unfortunately could not be randomised into equal ratios of gender in each treatment group.

The LPC and CFTR treated group had a significantly longer median survival (20 months) than the control groups that received either PBS prior to CFTR (15 months) or an empty vector (9 months). The two control group's shorter survival could be attributed to smaller starting numbers (n=6) than the LPC/LV-Luc+CFTR treated group (n=12), however statistical analysis should have accounted for these discrepancies. Alternatively, the successfully integration of the introduced CFTR into the genome had a protective effect. However, as Luc expression (via luminescence) was not detected in any other organs, nor detected by qPCR, a beneficial 'off target' transduction (e.g. intestinal epithelia) effect is unlikely. This study was not intended to correct the CF phenotype only attempt to correct the nasal CFTR defect, unlike other gene transfer studies that specifically targeted the intestinal defect via *in utero* delivery[387]. However as little as 5% CFTR can improve overall health in humans with CF[323] this may be a similar scenario with CF mice, that improvements in overall health and nutritional status improves survival. In previous nasal LV-CFTR treated CF mice, employing the strain with the FABp correction of the intestinal defect, the anecdotal evidence of yellowing of the usual white enamel of the incisors has been noted in 1-2 animals many months following LV delivery (personal communication, Dr K Kremer). Further suggesting an overall beneficial effect from the introduction of functioning CFTR may have far reaching effects not just limited to the nasal epithelia.

Despite an increased number of procedures and anaesthetics, there was no apparent significant negative impact on animal survival. Animal mortality that did occur was due to typical morbidity issues such as lymphomas in older aged animals that occurred at a similar incidence to normal mice[367].

Intestinal obstruction is common in CF transgenic mice[301] and most sudden CF deaths had no known aetiology, and were most likely the result of natural aging processes.

4.3 CHAPTER SUMMARY

These studies provide convincing evidence for robust "lifetime" reporter gene expression in the nasal airways of normal C57Bl/6 mice after only a single nasal gene transfer event. Employing a two-step nasal delivery protocol of LPC followed 1 hour later by a LV vector containing the reporter gene luciferase, sustained nasal airway luminescence was detected consistently for the entire 2 years of the study. Pre-treatment with LPC enabled this longevity of nasal airway LV gene expression. Although not directly tested here, this finding is consistent with published data indicating that LPC improves vector access to the appropriate airway cell surface receptors as well as transient disturbance of epithelial surface barrier function[300]. The latter permits vector particle access to the basolateral receptors and to deeper-lying basal cells that may have progenitor-like qualities[333]. The persistence of airway gene expression has been reported in normal mice after single and multiple doses of a SIV-based LV vector[361]. However the volume delivered in that study was five times that reported here. The large 100 µl volume would flood the nasal and lung airways with fluid, increasing the permeability of cells and effectively disrupting the tight junctions between the cells, explaining the longevity of airway expression seen in that study, similar to using the pre-conditioning agent reported here. In CF mice LPC pre-treatment provided extended nasal Luc gene expression for at least 1 year.

After nasal airway delivery with Luc, gene expression was also detected in the *lungs* of both strains of mice, regardless of pre-treatment. The 20 µl volume of LV vector used in these nasal delivery experiments has been shown to reach the lung in synchrotron phase-contrast X-ray imaging studies[365], whereas the 4µl pre-treatment volume remains exclusively in the nasal region. Lung luminescence reduced after 1-6 months, in some instances completely disappearing, but re-emerged 12 months after the initial gene transfer delivery. The re-emergence of lung luminescence provides evidence that airway stem/progenitor cells[372] may have been transduced. As re-emergence of lung expression occurred

when PBS was used, or with LPC which at this volume does not penetrate to the distal airways, the stem/progenitor cells may not only be basal cells in protected niches, but transient amplifying cells arising from these cells or sub populations of Clara cells[373-375] and do not need an agent that disrupts the tight junction between cells.

Of particular significance was that this is the first reported instance of long term functional correction of the CFTR electrophysiological defect in the airways of the same CF mice for much of their lifetime. There was a sustained partial correction of the bioelectrical defect in the nasal airways of CF mice that received the therapeutic CFTR gene, in conjunction with LPC pre-treatment, for up to 1 year. This persistent correction of the CFTR defect ranged from 12-54% towards normal chloride ion transport, a level likely to be an effective therapeutic[325].

An increase in circulating antibodies to the LV vector protein and the transgene proteins was detected in the blood of mice; however the immune response did not completely block gene expression.

Survival of C57Bl/6 mice was unaffected by gene transfer treatment and by the repeated assessments of gene expression. Mice received over 20 anaesthetic events with non-invasive and intrusive airway gene assessment procedures. More caution maybe needed when applying the repeated-measure design for gene assessment in CF mice due to their pathophysiology and less robust nature[302], with extra numbers of CF mice required in each treatment group to allow for natural attrition and morbidity due to their genetic modification.

Surprisingly, CF mice that received the LPC/LV-Luc+CFTR treatment had a statistically significant improvement in survival. No off target transduction of genomic DNA in other non-airway tissues nor luminescence was detected in any other organs in this study. The successful integration of CFTR into airway epithelia in a null CF mouse model may in itself provide a protective effect, even though there is

no CF lung disease associated with this CF strain. The expression of CFTR in nasal epithelial cells may increase survival due to normalised ion transport that would provide protection from foreign particles by improvement in MCC, mucus viscosity and increased volume of the ASL layer.

As little as 5% CFTR function improves survival outcomes in humans with CF due to nutritional and overall health status and this may be a similar phenomenon observed in CF mice treated with functional CFTR.

CHAPTER 5

REPEAT LENTIVIRAL AIRWAY GENE TRANSFER *IN VIVO*

CHAPTER 5 REPEAT LENTIVIRAL GENE TRANSFER

5.1 MULTIPLE LENTIVIRAL GENE TRANSFER

5.1.1 Introduction

For a life-long disease, such as for CF, longevity of an effective gene therapy treatment is a necessity. The level needed for an effective gene transfer that results in clinical improvement is still under debate. Regardless, to achieve a clinical treatment the need for effective re-administration of a gene therapy over an individual's lifetime will be likely. Repeated dosing of a gene transfer could enable levels of gene expression necessary for therapeutic treatment as well as the potential for renewal of gene expression if or when initial gene expression wanes. In the previous chapter nasal gene expression in both normal C57Bl/6 and CF mice reduced after 3 months and in some cases completely disappeared. Delivery of additional airway gene transfer doses at these time points would be appropriate to replenish gene expression to therapeutic levels.

Repeat dosing has been performed by other groups using different systems. Short term dosing at 2-3 week intervals with an AAV/serotype 2 vector into the lungs of rhesus macaques produced increased gene expression and elicited a host immune response, but the immune response did not inhibit gene transfer[388]. Acute repeat dosing to achieve initial effective levels of gene expression using a GP64 / FIV LV vector in the airways of mice has already been reported without generation of significant host immune blocking responses[293]. Early repeat dosing with a GP64 glycoprotein and VSV-G glycoprotein pseudotyped HIV LV vector in foetal and neonatal lungs of mice was successful but led to an increase in transduced macrophages[348]. Weekly or monthly repeat dosing of a transmission incompetent (ΔF)/SeV vector in murine or ovine lung airways significant reduced gene expression compared to a single administration[184, 269].

These diverse findings show that comparisons of repeat viral gene transfer studies are difficult to interpret due to the wide variation in animal species, vector species, envelope pseudotype and titre; but especially due to the differences in number and timing of repeat airway administrations.

This chapter will describe the effect of repeated nasal administration of a LV vector on airway gene expression compared to that produced by a standard single dose.

5.1.2 Aims

The aim of the following studies was to determine if LV vector gene transfer dosing could be successfully repeated to achieve improved gene expression levels compared to our standard single gene treatment regime, and also to determine if repeat dosing of a LV vector would extend or boost long term gene expression in the nasal airways of mice.

5.1.3 Methods

5.1.3.1 Short Term Multiple LV Dosing

Multiple doses of a LV vector were instilled into the right nostril of female C57Bl/6 mice using our standard two-step protocol: a 4 μl bolus of 0.3% w/v LPC was delivered 1 hour prior to a 20 μl aliquot of a LV vector containing the LacZ gene. Multiple doses were compared to our standard single dose outcomes and LacZ gene expression was analysed 1 month after the initial dose (n=8/group). Short term multiple doses were 2 doses 12 hours apart (2 doses); 2 doses per day over 2 days for a total of 4 doses; three doses 8 hours apart over 1 day (3 doses); or 3 doses/day over 2 days for a total of 6 doses. All nasal doses were performed on anesthetised mice as described in Methods section 2.3.2.3.

Final titre of LV-LacZ_{Co} was 20 μl at 1.6 x10^{10} tu/ml. LacZ gene expression was analysed 1 month after the initial LPC/LV-LacZ dose, as described in sections 2.3.3.1.1- 2.3.3.1.2.

5.1.3.2 Long Term Repeat LV Dosing

In this study, the control group received 1 dose of LPC/LV-LacZ$_{Co}$ and LacZ gene expression was measured 1 year later. The re-dose treatment groups mice received a second LPC/LV-LacZ$_{Co}$ at 1, 3, 6 or 9 months following the initial LPC/LV-LacZ$_{Co}$ (n=8/group). All doses of LV-LacZ$_{Co}$ used the standard 20 µl volume at the maximum titre of 1.6 x 10^{10} tu/ml and LacZ gene expression was analysed 1 year after initial LV-LacZ instillation (see Methods sections 2.3.2, 2.3.3.1.1- 2.3.3.1.2).

5.1.3.2.1 Circulating antibodies

Blood was removed by cardiac puncture at study termination and the sera were stored at -70°C until analysis for the presence of circulating antibodies to the VSV-G envelope and to the reporter gene LacZ (see section 2.2.7). The positive control for the VSV-G envelope was obtained from previous experiments that used multiple doses of an LV-MT vector[357] and the positive control used for the transgene LacZ was from 3 samples with the highest level of antibody detection from this multiple long-term LacZ dosing experiment (5.1.3.2) and the short-term multiple dosing in the above study 5.1.3.1.

5.1.3.3 Repeat Transgene Dosing Studies

In the previous experiments, repeat dosing of the LV vector was performed using the same reporter transgene, LacZ. In the next series of experiments the Luc gene was administered i.n., and one month later mice were instilled with either PBS (as a vector sham control), the *same* Luc transgene vector, or a LV vector containing a *different* reporter transgene LacZ (n=8). Utilising the reporter gene Luc allowed for immediate observation of successful LV gene expression by bioluminescence and assessment of the effects on nasal luminescence after a second LV dose.

The right nostril of female C57Bl/6 mice was instilled with LPC and LV-$Luc_{EF1-\alpha}$ at the maximum titre of 0.9 x 10^{10} tu/ml as described in section 2.3.2. For the second dose delivery one month later the control group received a sham dose of 4 µl of PBS pre-treatment and 20 µl dose of PBS. The same-transgene cohort received the second dose with the same LPC/LV-$Luc_{EF1-\alpha}$ treatment and the different-transgene cohort received LPC/$LacZ_{Co}$ administered i.n. at 1.6 x 10^{10} tu/ml.

Luciferase gene expression was measured non-invasively one week after the first nasal administration and 1 month later, as described in section 2.3.3.1.3. Nasal luminescence was also measured 1 week after the second dose (i.e. at 5 weeks) and then at 2 and 3 months following the initial nasal instillation. LacZ gene expression was measured at the 3 month study termination point after humane killing and Xgal processing as described in 2.3.3.1.1 to determine the number of blue stained LacZ cells in the nasal respiratory epithelia at 3 designated levels (2.3.3.1.2).

5.1.3.3.1 Circulating antibodies after repeat transgene dosing

Blood was removed via cardiac puncture at the termination of the study, 3 months after the initial LV-Luc dose, and sera was analysed for circulating antibodies to the VSV-G protein, recombinant Luc protein and to the β-galactosidase protein by ELISA (2.2.7).

5.1.4 Results

5.1.4.1 Short Term Multiple Dosing

Early repeat doses of LPC/LV-LacZ resulted in no significant increase or decrease in nasal gene expression with increasing doses of a LV vector over 1-2 days (Figure 5-1).

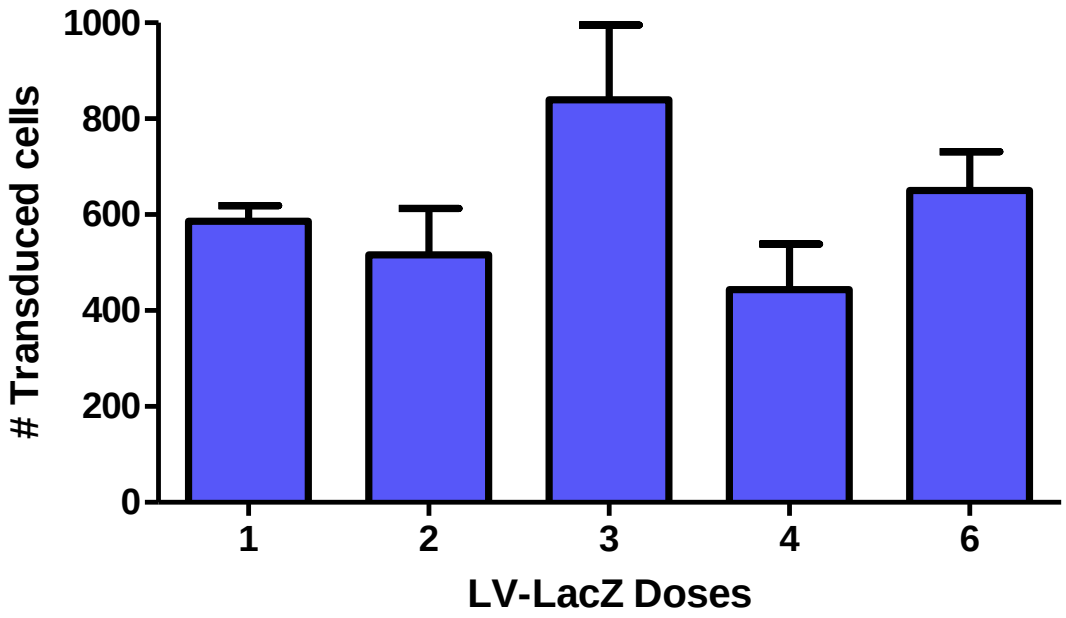


Figure 5-1 LacZ Gene Expression from Multiple Doses of LPC/LV-LacZ
No difference in nasal gene expression was observed at 1 month with increasing number of LPC/LV-LacZ doses (n.s, ANOVA compared to 1 dose, $\bar{X} \pm SEM$, n=8).

5.1.4.2 Long Term Repeat LV Dosing

In the longer interval repeat dosing study, the level of gene expression after 1 year was very low in all groups and there was no difference in the number of transduced nasal epithelial cells detected between a single and two doses of LV-LacZ (Figure 5-2). Only 1 out of 8 mice (12.5%) displayed LacZ expression at 1 year in the single dose group. The same proportion (12.5%) of mice displayed LacZ expression in each of the groups that received a second dose at 1, 3 and 6 months. No LacZ transduction of the nasal epithelia was detected in the 9 month re-dose cohort.

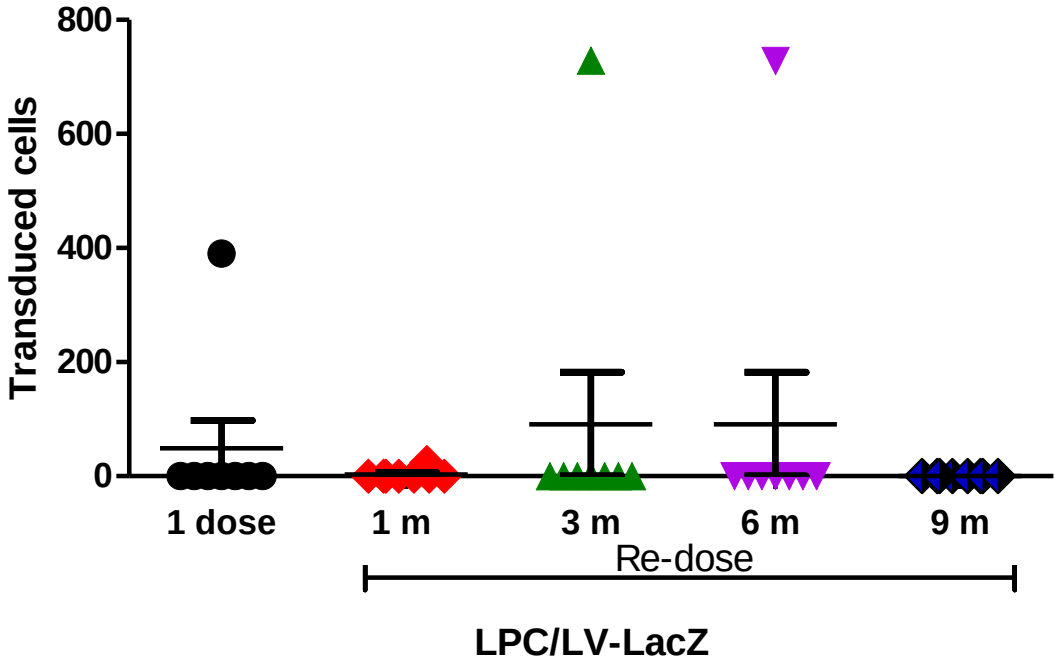


Figure 5-2 LacZ Gene Expression from Long Term LPC/LV-LacZ Re-dose
One year LacZ gene expression after 1 or 2 doses of LPC/LV-LacZ (n.s., ANOVA vs single dose, bar represents data as $\bar{X} \pm SEM$, n=8). 1 dose = single dose, second dose at 1, 3, 6, or 9 months.

5.1.4.2.1 Circulating antibodies

5.1.4.2.1.1 Anti-VSV-G protein

At the 12 month final assessment there was a statistically significant increase in circulating antibodies to the LV vector VSV-G protein in the mice that received a second dose of LPC/LV-LacZ at 6 months compared to a single LPC/LV-LacZ dose (Figure 5-3a, $p<0.01$ ANOVA, n=8). The absence of antibodies to the LV envelope protein did not correlate to those mice that displayed LacZ expression at 12 months (r^2 =0.49, Pearson's Correlation, n=4 pairs, see Appendix 8.5-1).

5.1.4.2.1.2 Anti-β-galactosidase

There was also an increase in circulating antibodies to the β-galactosidase protein in the sera of mice that received the second dose at 6 months compared to a single LPC/LV-LacZ dose ($p<0.05$, ANOVA, n=8). There was no difference in circulating antibodies to the β-galactosidase protein at 1 year for any of the mice that received the second dose of the LacZ transgene delivered at 1, 3 or 9 months compared to a single dose (Figure 5-3b).

There was no significant correlation between the mice that displayed LacZ gene expression and the absence of circulating antibodies to the β-galactosidase protein (n.s., $r^2 = 0.03$, Pearson's Correlation, n=4 pairs). The individual data points are presented in Appendix 8.6-1.

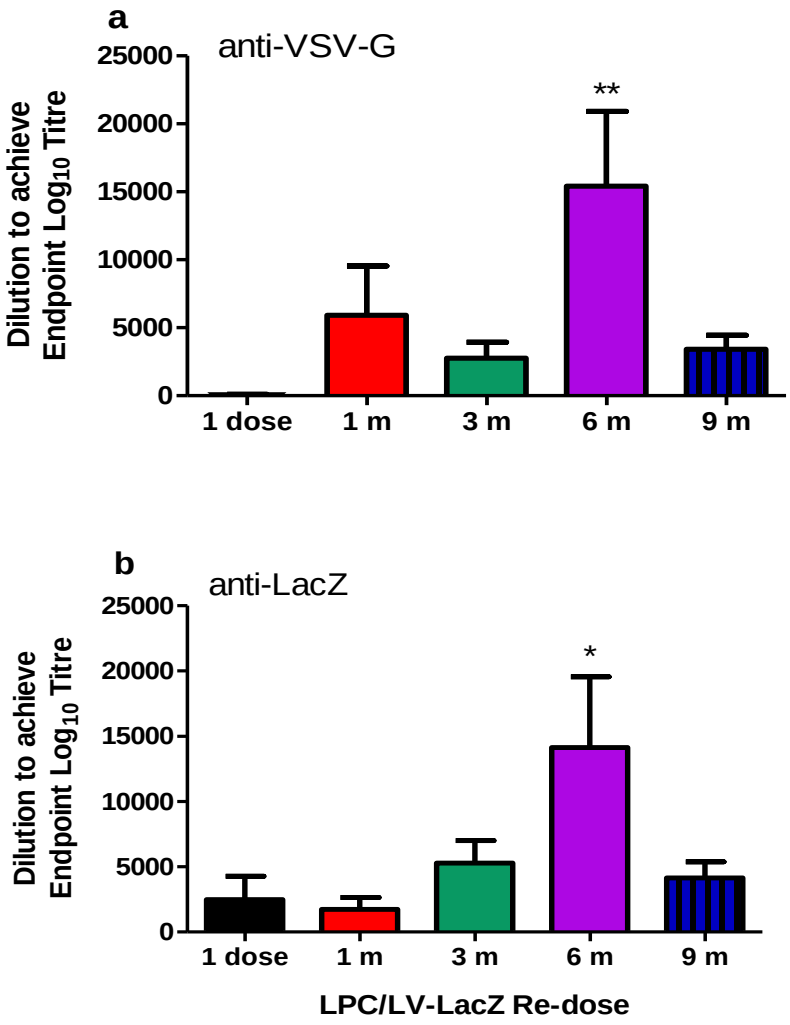


Figure 5-3 Circulating Antibodies to Repeat LV Dosing
*Presence of circulating antibodies detected in sera after 12 months to the a) LV envelope protein VSV-G and b) recombinant β-galactosidase (LacZ) protein over time. (**p<0.01, *p<0.05., Dunnett's ANOVA compared to single dose, $\bar{X} \pm SEM$, n=8).*

5.1.4.3 Repeat Transgene Dosing Studies

5.1.4.3.1 Nasal luminescence gene expression

For the experiments utilising the same or a different transgene as a second dose, one week after the administration of the second dose (i.e 5 week assessment) no difference in nasal luminescence was

detected between all 3 groups. However, 2 and 3 months after re-administration of the same transgene (i.e. Luc) a significant decrease in the flux of nasal luminescence was present (Figure 5-4, p<0.001 Dunn's RM ANOVA, n=8) compared to that produced by a single dose of Luc. The administration of a second LV vector dose, but containing a different reporter gene (LacZ), produced no significant difference in nasal luminescence compared to a single dose of LV-Luc (n.s., Dunn's RM ANOVA, n=8).

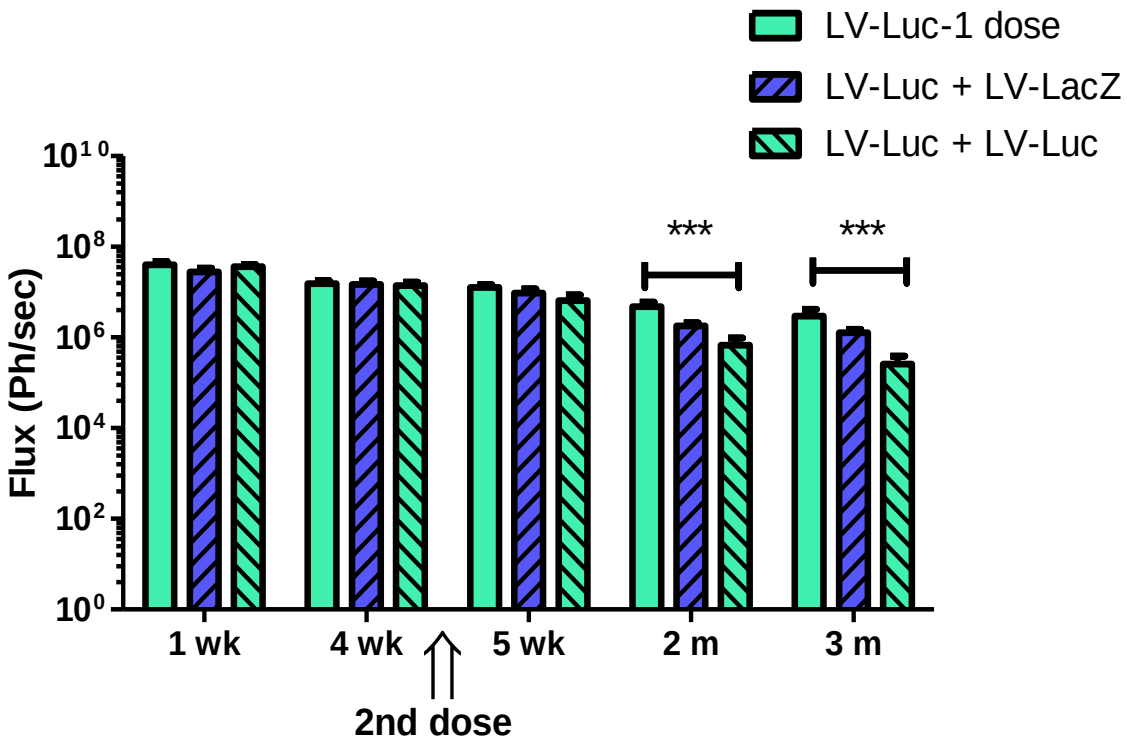


Figure 5-4 Nasal Luminescence from Repeat LV dosing
*Nasal luciferase gene expression, after one or two doses of nasal LV gene transfer deliveries, imaged over 3 months (***p<0.001, Dunn's RM ANOVA, two doses of LV-Luc compared to a single LV-Luc dose, $\bar{X} \pm SEM$, n=8). Time of second dose administration is arrowed.*

5.1.4.3.2 Nasal LacZ gene expression

As expected, only those mice that received the LacZ gene as the second dose, displayed evidence of LacZ transduction of the respiratory epithelia (Figure 5-5, p<0.01, Dunn's ANOVA, n=8). However the

level of LacZ gene expression was very low compared to a single LacZ delivery at the same 3 month time assessment, albeit at a lower concentration than in previous published data[297] ($\bar{X} \pm SEM$, 13.8 ± 8.4 compared to 120 ± 50, respectively).

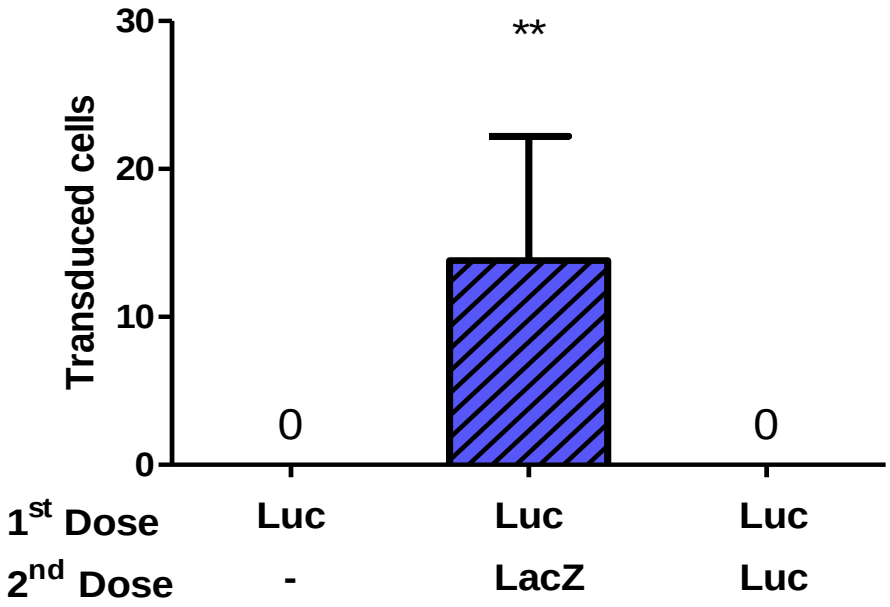


Figure 5-5 LacZ Reporter Gene Expression after LV Re-dosing
*Nasal LacZ gene expression 2 months after a second nasal gene transfer delivery (**p<0.01, Dunn's ANOVA, $\bar{X} \pm SEM$, n=8).*

5.1.4.3.3 Circulating antibodies after repeat transgene dosing

5.1.4.3.3.1 Anti-VSV-G protein

Circulating antibodies to the LV vector envelope VSV-G protein were present in the sera of mice who received a single dose of LPC/LV-Luc and either of the two doses of the LV vector, irrespective of the

transgene delivered (Figure 5-6a). A trend of increasing levels of antibodies to the VSV-G protein with repeated doses was observed, but due to the wide variability did not reach statistical significance (n.s., Dunnett's ANOVA, n=8). This increase in antibodies to the LV vector was not greater than that from the positive controls obtained from mice that received four multiple LV vector doses over a 2 month period[357].

5.1.4.3.3.2 Anti-Luciferase

One dose of the Luc gene vector delivered to the nasal airways produced an increase in antibodies to the Luc protein (Figure 5-6b). This Luc antibody response was also detected in those cohorts of mice that received another LV vector dose with either a different transgene or the same Luc transgene. Mice that received two doses of the same Luc reporter gene had double the levels of circulating antibodies to the Luc protein as mice that received only once dose of the Luc gene, but this did not reach statistical significance (n.s., Dunnett's ANOVA, n=8).

5.1.4.3.3.3 Anti-β-galactosidase

Unsurprisingly, no circulating antibodies to the β-galactosidase protein were detected in the sera of mice that did not receive the reporter gene LacZ (Figure 5-6c) at the 3 month assessment time point. For the mice treated with LacZ as the reporter gene in the second LV vector dose the amount of anti-β-galactosidase in the sera was just above the limit of detection of the ELISA assay. Antibodies to the β-galactosidase protein were not significantly different in mice that received LacZ compared to those mice that did not receive LacZ as a nasal instillation (n.s, Dunnett's ANOVA, n=8).

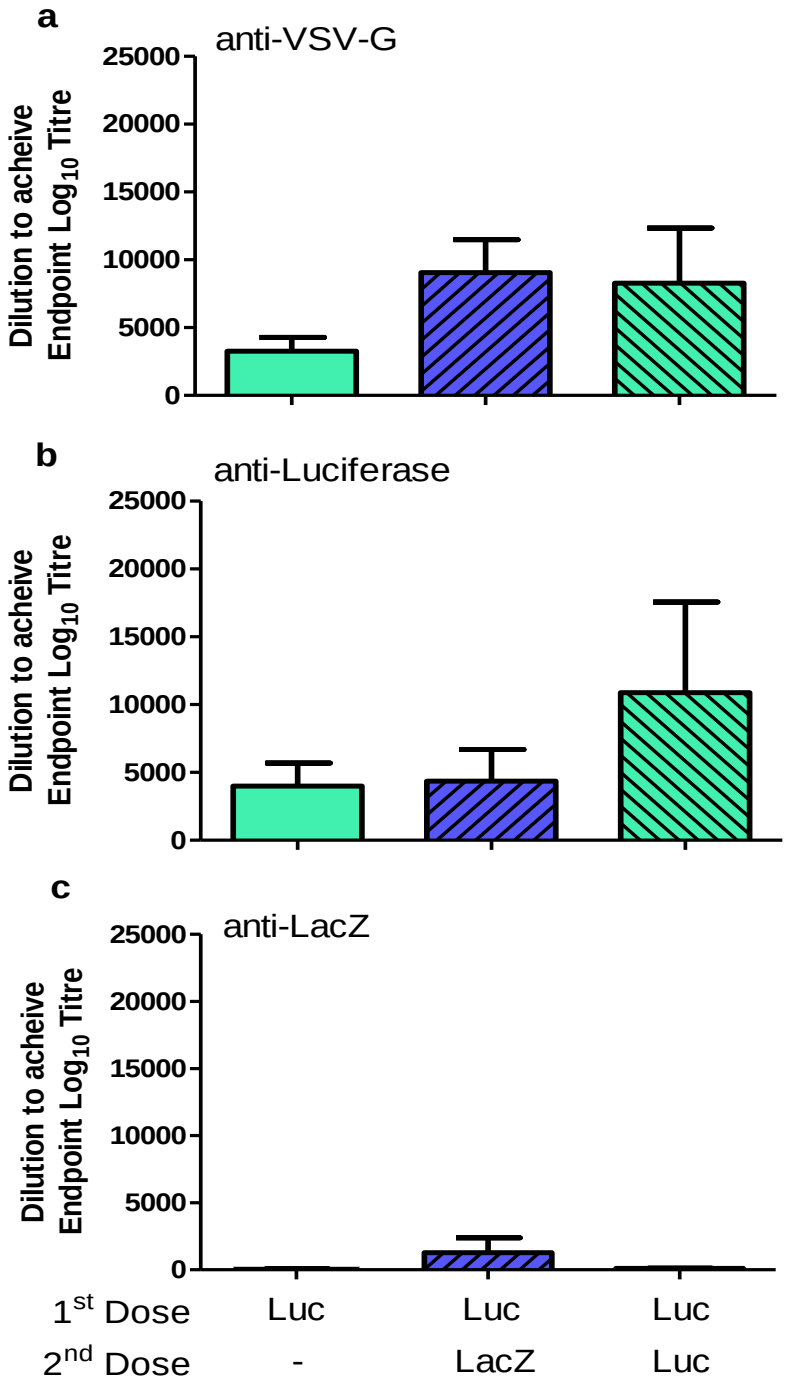


Figure 5-6 Circulating Antibodies to Repeat Transgene Dosing

Circulating antibodies after 3 months for the a) LV envelope protein VSV-G and b) recombinant luciferase protein c) recombinant β-galactosidase (LacZ) protein over time. (n.s, Dunnett's ANOVA compared to single dose, $\bar{X} \pm SEM$, n=8).

5.1.5 Discussion

Airway gene therapy has great potential to halt or even prevent lung disease in CF patients. To achieve an effective genetic therapeutic treatment for CF the correct cells must be targeted at sufficient levels and remain effective over a lifetime. Re-administration of an airway gene therapy may be required to achieve the necessary level of transduction for correction of the genetic defect, as well as to achieve the necessary longevity of expression. However, the host immune response against the LV vector constituents and the transferred gene protein are major barriers to successful re-administration of a gene therapy[382].

The immunological defence mechanisms evolved to protect the host are the innate and adaptive immune responses. The innate (non-specific) immune response can be pre-existing or an immediately generated response to a delivered viral vector, and is the first line of immunological defence. The innate immune cells are phagocytic (such as dendritic cells, macrophages, neutrophils and natural killer T-cells) and these recognise and respond within minutes of pathogen presentation, but do not provide long-lasting immunity[196]. The adaptive (specific) immune response is activated by the innate response and is the next line of defence that can take days to weeks to develop. The adaptive defence involves both antibodies (the humoral response) and T-cell lymphocytes (cell-mediated response) that both recognise and most importantly remember the specific pathogen, to provide long lasting immunity to a particular antigen. This specific immune response may be responsible for the subsequent blocking of gene expression from repeat doses of an airway gene therapy[200].

The ability to re-administer LPC/LV-LacZ over short periods of time daily, twice and thrice daily over 1-2 days was successful, however the level of LacZ transduction achieved after a single dose of LPC/LV-LacZ was the same as the maximum level detected after re-administration. The use of a LPC pre-

treatment and a single dose of a LV vector are sufficient to provide enhanced and effective levels of gene expression necessary for a therapeutic effect. The ability to re-administer a LV gene therapy to provide initial gene expression likely to have a therapeutic effect has been demonstrated in murine nasal epithelia[293]. However in that case, mice received 7 daily or 7 weekly doses of a feline LV (FIV) vector to achieve gene expression, whereas a single FIV dose did not produce significant gene expression. Recently, the daily re-administration of a SIV LV vector over 5 or 10 doses produced increased lung luminescence compared to a single dose in a murine model[361].

To maintain effective gene expression by boosting expression that has waned over time, re-administration of a LV vector dose in a longer and more therapeutically relevant time frame was examined. A second nasal dose of a LPC/LV-LacZ treatment was administered at 1, 3, 6, and 9 months after the initial airway gene transfer dose. When analysed at 1 year, gene expression was only present in 12.5% of animals in each treatment cohort, including the group of mice that received only one dose of LV gene transfer. The high proportion of mice that had no LacZ transduction in the single dose group was unexpected and perplexing, as the LV-LacZ virus batch employed was the same as that used in the short term repeat dosing study that produced highly effective gene transfer. Previous published single-dose LPC/LV-LacZ studies revealed only 20%[297] or as shown in chapter 4 a maximum 50% of mice that had zero nasal Luc expression 12 months after nasal administration. The very low percentage of animals that displayed gene expression here may be due various factors, such as a low initial LacZ transduction, a defective LV vector production batch, loss of titre due to thawing issues, normal cell turnover or an immune response to either the vector envelope proteins or the transgene itself.

Increased antibodies to the LV vector VSV-G protein were detected in mice that received two doses of the LV vector compared to the cohort of mice that received only one dose. However the level of VSV-G antibody presence in the sera was only significantly greater at the 6 month re-dose compared to a single

dose. As discussed in Chapter 4 low levels of antibodies to the VSV-G envelope persisted for 1-9 months following a single dose that did not block gene expression but may produce an immune memory response to a second LV dose. The level of antibodies to the VSV-G protein increased 10 fold at the 6 months re-dose suggesting a humoral response to the repeat dose possibly did occur at this re-dose timing. This data shows that the LV vector envelope constituents result in a low level immune response; and an adaptive immune response to the viral capsid may still present 6 months after an initial LV delivery. However, as the level of antibodies to the VSV-G protein from the single LV vector dose (which also resulted in no gene expression) was below detection, an immune response may not be solely responsible for the lack of gene expression observed in this study.

Also, there was no correlation between the level of antibodies to the VSV-G protein and the presence of LacZ expression, further suggesting that LV vectors have a low immunological response[382]. LV vectors pseudotyped with the VSV-G envelope have been shown to not elicit major pro-inflammatory signals in human airway epithelial cells[280].

Mice that received the re-dose at 6 months also had significantly higher levels of antibodies to the transgene compared to a single dose. From the Chapter 4 long term studies, antibodies to the transgene after a single dose peaked at 3 months and were sustained for 9 months after a single reporter gene delivery. An increase in antibodies to the reporter gene protein was observed at all the re-dose time points, with 5 times the level at the 6 month re-dose event compared to a single dose. However, even one dose of LV-LacZ produced an immune response that may in part explain the lack of LacZ expression observed at the 1 year assessment period (see Appendix 8.6-1) although this has never been observed before. Of the 4 out of 8 mice that had very low levels of antibodies to the LacZ transgene in this single-dose cohort, only one of these mice displayed LacZ gene expression, implying

that cell turnover may also be a contributing factor in the lack of gene expression observed at the 1 year assessment.

Since the number of mice successfully transduced with the LacZ reporter gene was too low to provide an unequivocal answer that the repeat dosing was blocked by a specific immune response, another experiment was performed using the Luc reporter gene in which nasal gene expression could be assessed immediately. One to two months after a second dose of the same reporter gene, nasal gene expression was significantly reduced compared to a single LV dose. Nasal luminescence after a second dose of a different reporter gene (LacZ) was no different to a single instillation. Gene expression of the different reporter gene LacZ was not blocked by the previous LV vector dose. A similar observation that has been reported after 2 monthly repeat dosing events of a LV vector with a different transgene (GFP) followed by the Luc gene showed no difference in lung expression compared to a single Luc dose[361]. An effective repeated gene treatment is more likely to be hampered by the hosts' destructive T cell response specific to the foreign transgene protein.

As in the long term repeat study using the same reporter gene, circulating antibodies to the VSV-G envelope were detected in the sera of mice that received either one or two doses of a LV vector. The level of anti-VSV-G was lower than that detected in positive controls and did not completely block the expression from subsequent LV doses regardless of the transgene, suggesting that the LV vector is less immunogenic.

The increase in levels of anti-luciferase in the sera of mice that received two doses of the same transgene were double that observed from the single dose, but did not reach statistical significance. The antibody response to the Luc transgene did not completely block gene expression, but did reduce the nasal luminescence detected in the group of mice that was re-dosed with the same Luc gene.

Nasal luminescence in the group of mice that received a different reporter gene (LV-LacZ) as the second dose, displayed no difference in expression to that observed from the single LV-Luc cohort. The subsequent LacZ gene expression in the mice that received a LV-LacZ dose was evident in the nasal epithelia and the level of antibodies to the LacZ protein was no different to negative controls or those mice that did not receive a LV-LacZ dose.

These results confirm the feasibility of repeat dosing of a LV vector, but highlight the possibility of an immune response being raised against the foreign transgene. In the case of a genetic therapy for CF an immune response to the CFTR protein may need to be overcome before effective repeat dosing can be achieved. As majority of mutations responsible for CF have the CFTR protein present but non-functioning, the host immune system may not recognise the introduced human CFTR protein as foreign and therefore the LV vector remains the most promising tool for introducing a fully functioning copy of the CFTR protein into defective airway cells.

5.2 CHAPTER SUMMARY

The studies reported in this chapter provide first-time evidence of the effects of repeat dosing of a HIV-1 based LV vector for producing effective airway gene transfer. The initial level of gene expression from a single dose was sufficient to achieve levels of transduction required for correction of a genetic defect, irrespective of the number of early short-term repeat dosing events. The innate immune response was not activated, possibly due to the absence of pre-existing immune responses to the particular (HIV-1 based) LV vector employed, or because LV vectors are less immunogenic than other viral antigens. The enhanced efficacy of the LV airway gene transfer was most likely due to the 2 step regime employing the surfactant LPC prior to LV vector administration for either single or multiple dosing events.

To maintain effective gene expression and eventually boost expression that has possibly waned over time, re-administration of a LV vector dose over a longer time frame was examined. The ability to boost gene expression levels was not demonstrated; however gene expression was not completely blocked during subsequent LV doses. The immune response detected in the circulating sera was raised mainly against the foreign protein expressed from the transgene and even though an antibody response to the LV vector protein product constituents was detected, it was not robust enough to completely block subsequent gene expression. As the majority of mutations responsible for CF have the CFTR protein present but non-functioning, the host immune system may not recognise the introduced human CFTR protein as foreign. These results confirm the feasibility of repeat dosing of a LV vector to provide further evidence towards a potential clinical gene therapy for CF airway disease.

CHAPTER 6

IMMUNOSUPPRESSION FOR LENTIVIRAL AIRWAY GENE TRANSFER *IN VIVO*

CHAPTER 6 IMMUNOSUPPRESSION FOR LV GENE EXPRESSION

6.1 IMMUNOSUPPRESSION

6.1.1 Introduction

In the previous chapter the ability to repeatedly dose a LV vector to produce an effective airway gene therapy was examined, and demonstrated mixed results. Multiple doses of a LV vector delivered over a short time frame (days-weeks) were without any detrimental effect on gene expression, but did not increase gene expression. For repeated doses separated by months, the results were inconclusive.

The need for maintaining effective therapeutic levels of a gene therapy or the potential to boost waning gene expression remains a pre-requisite for an effective treatment for CF lung diseases. An immune response to the transgene itself was raised by gene transfer. This specific transgene immune response reduced gene expression after the second dose and that dose did not boost gene expression to a higher level. An immune response to a gene transfer procedure has also been noted by others[382, 389].

Immunosuppressive drugs have been developed for preventing organ rejection in transplantation medicine. The use of immunosuppression in conjunction with a gene transfer to allow effective repeat dosing has been documented for AdV vectors[241, 390, 391] and AAV vectors[392, 393]. The immunosuppressive drug sirolimus (rapamycin)[394] has been used successfully in models of lung transplants[395] and lung cancer[396] in mice. In this chapter the effectiveness of administering sirolimus at and around the time of airway gene therapy delivery was examined.

6.1.2 Aims

The aim of the studies described here was to determine if oral administration of an immune suppression therapy in conjunction with delivery of a LV vector gene transfer dose could achieve higher levels of gene expression in the nose or lungs of mice, compared to our standard single airway gene dose. A secondary aim was to reveal whether the administration of an immune suppression treatment could improve the longevity of gene expression.

6.1.3 Methods

6.1.3.1 Immunosuppression Studies

The immune suppression drug sirolimus (Rapamune®) was administered orally to female C57Bl/6 mice aged 8 weeks of age (n=8). Sirolimus was swallowed spontaneously by the animal as the viscous liquid was slowly pipetted into the corner of the mouth. An 80 µl volume of sirolimus (4 mg/kg/day) was administered as a priming dose daily for 4 days prior to nasal instillation of the LV vector containing the $Luc_{EF1\alpha}$ gene. Our standard nasal gene transfer delivery method was used: a 4 µl aliquot of 0.3% LPC 1 hour prior to 20 µl of LV-Luc at the maximum dose of 0.9×10^{10} tu/ml (as described in section 2.3.2).

Sirolimus was not administered on the day of LV vector instillation (day 0), but a double-strength dose was given on the following day (day1). Sirolimus was then administered as an oral dose at 2 mg/kg/day (40 µl volume) for the next 6 days (Table 6-1).

A control group of mice (n=8) received a sham oral dose of water (H_2O) at the same volume and frequency as that delivered to mice that received sirolimus. Control mice received the same standard LPC/LV-Luc nasal gene therapy dose on day 0.

Mice were imaged for nasal and lung gene expression at 1, 2, 3, and 4 weeks and then monthly for up to 6 months after LV instillation (2.3.3.1.3). Luminescence was calculated as flux (Ph/sec) using contour parameters.

DAY	-4	-3	-2	-1	0	1	2	3	4	5	6	7
SIROLIMUS (mg/kg/day)	4	4	4	4	-	4	2	2	2	2	2	2
H_2O (µl)	80	80	80	80	-	80	40	40	40	40	40	40

Table 6-1 Sirolimus Dosing Schedule
Mice received orally either sirolimus or water (H_2O, as a control) at the same volume, on the same days. All mice received the standard nasal LPC/LV-Luc dose on Day 0.

6.1.3.2 Circulating Antibodies

Blood was removed via submandibular bleeding (2.3.4) prior to and 3, 4, 5 months following LV vector instillation and via cardiac puncture at study termination (6 months), and analysed by ELISA (see section 2.2.7) for the presence of circulating antibodies to the VSV-G envelope and the recombinant Luc protein.

6.1.4 Results

6.1.4.1 Nasal Luminescence

Nasal Luc gene expression was no different between mice that received water orally (sham control) or the immunosuppressive drug sirolimus orally (Figure 6-1, n.s., Bonferroni's RM ANOVA, n=8) on the

days prior to, and for 1 week after the nasal instillation of the LPC/LV-Luc gene treatment. No significant difference in nasal luminescence was seen at any of the time points tested.

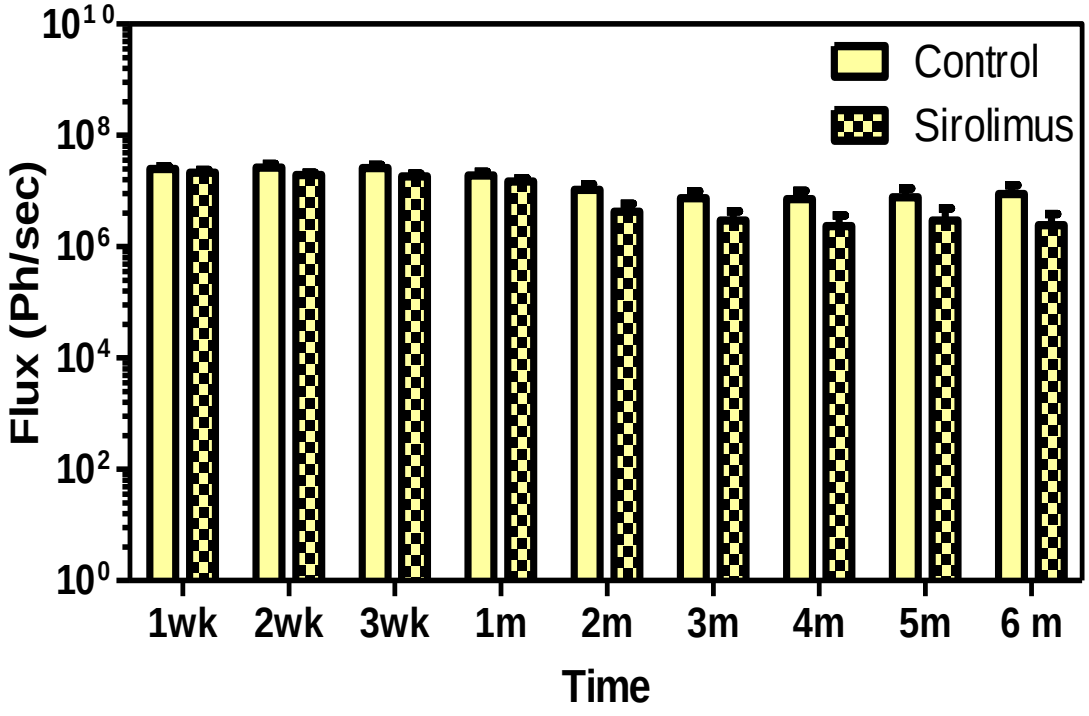


Figure 6-1 Nasal Luminescence – Immunosuppression Treatment
LPC/LV-Luc nasal luminescence with or without oral administration of the immunosuppressive drug sirolimus (n.s., Bonferroni's RM ANOVA, $\bar{X} \pm SEM$, n=8).

When treatment groups were examined individually, mice that received the standard pre-treatment gene regimen of LPC prior to LV-Luc displayed a significant decrease in nasal luminescence from 2-6 months compared to the level of luminescence detected at 1 week (Figure 6-2a, $p<0.01$, Dunnett's RM ANOVA, n=8). This corresponded to a reduction in nasal luminescence as a whole cohort and not due to a complete loss of nasal expression in some animals.

A decrease in nasal luminescence at the later time points was also observed in the group of mice that received the same nasal LPC/LV-Luc gene treatment but with the addition of the immunosuppressive drug sirolimus (Figure 6-2b). Sirolimus treated mice displayed a decrease in nasal gene expression from 1 month on, compared to nasal flux measured at 1 week ($p<0.05$, Dunnett's RM ANOVA, n=8). This overall significant reduction in nasal luminescence was partially due to the complete loss of nasal gene expression in 25% of mice as assessed 4 months after immunosuppressant and LV vector administration.

In summary, the addition of a short term oral immunosuppressive treatment with sirolimus had no overall effect on initial or long term nasal gene expression, with both groups displaying a significant reduction of nasal gene transfer, over the 6 month study.

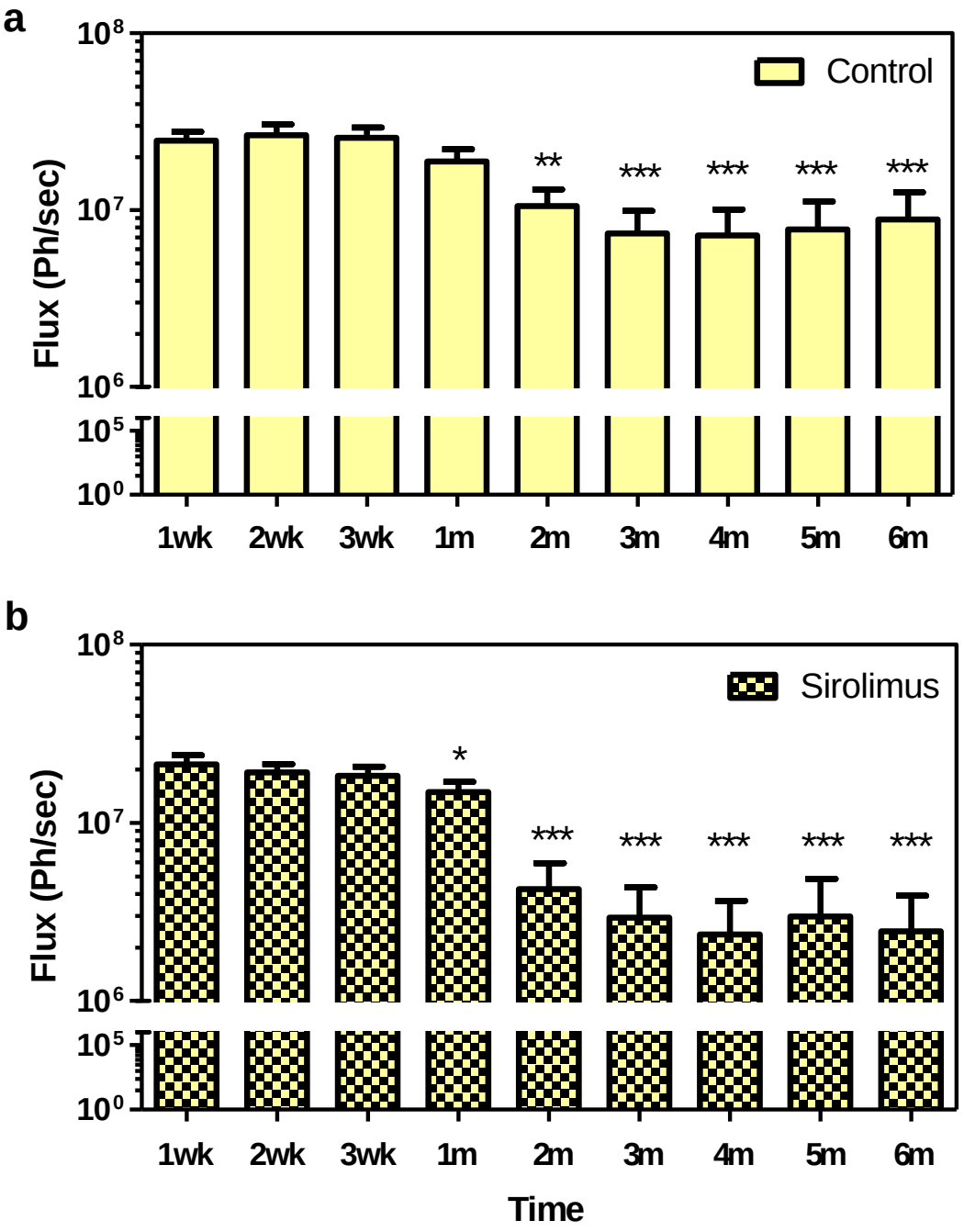


Figure 6-2 Nasal Luminescence – With and Without Immunosuppression

*Nasal luminescence over time a) without and b) with sirolimus immunosuppression (*p<0.05, **p<0.01, ***p<0.001, Dunnett's RM ANOVA compared to 1 week, $\bar{X} \pm SEM$, n=8). Same data as presented in Figure 6-1, but separated into individual treatments using expanded y axis for clarity.*

6.1.4.2 Lung Luminescence

As reported in previous chapters, dosing for nasal airway gene transfer also produced gene expression in mouse lung airways. In comparison to the nasal gene expression results, there was a significant difference in the initial luminescence detected in the lung airways of mice that were treated with the immunosuppressive drug sirolimus. A significant increase in the level of lung luminescence at 1 week – at the conclusion of the short-term oral administration of sirolimus – was present in the cohort of mice that received immunosuppression compared to mice that received the standard LPC/LV-Luc nasal gene transfer dose (i.e. water sham control) (Figure 6-3, $p<0.05$, Bonferroni's RM ANOVA, n=8).

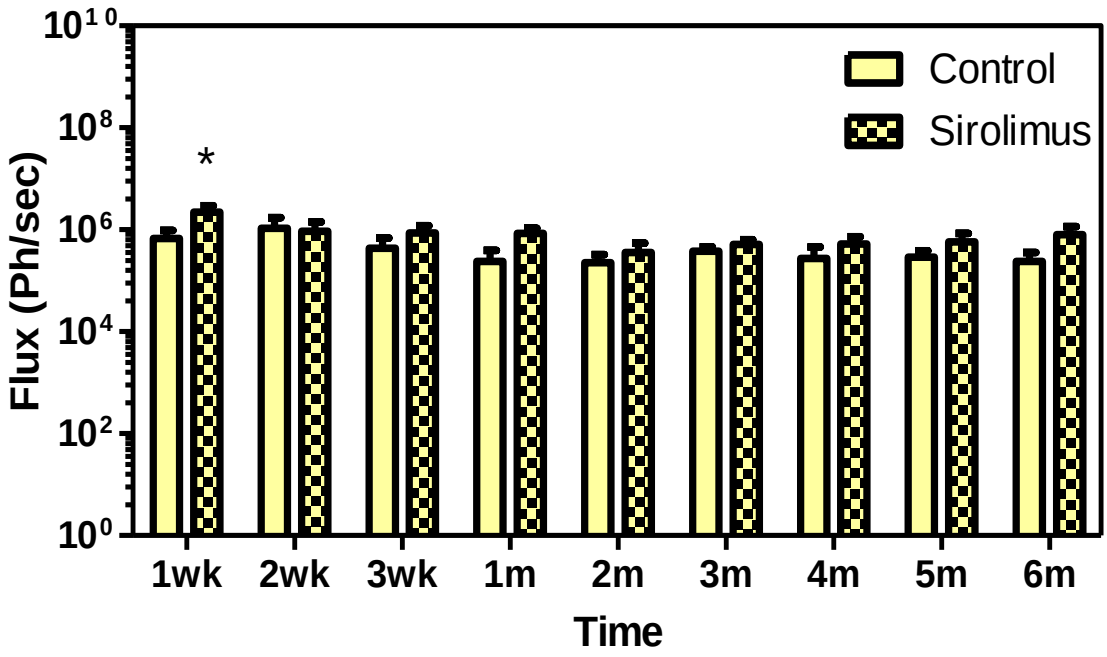


Figure 6-3 Lung Luminescence – Immunosuppression Treatment
*LPC/LV-Luc lung luminescence with or without oral administration of the immunosuppressive drug sirolimus (*p<0.05, Bonferroni's RM ANOVA, $\bar{X} \pm SEM$, n=8).*

When treatments were examined individually, there was no difference in lung luminescence in mice that received only the standard LPC/LV-Luc nasal administration (control) at all of the time points assessed (Figure 6-4a). The sustained longevity of lung gene expression was a combination of sustained expression, loss and re-emergence of luminescence in this cohort.

However, since sirolimus immunosuppressive treatment increased lung luminescence detected at 1 week after nasal instillation of LPC/LV-Luc, there was a significant decrease in lung gene expression detected at 2 weeks to 6 months after nasal gene transfer compared to lung luminescence displayed at 1 week (Figure 6-4b, $p<0.05$, Dunnett's RM ANOVA, $n=8$). The pattern of early reduction and re-emergence of lung expression was noted in the sirolimus treated mice as the same trend noted in normal C57Bl/6 mice in Chapter 4 (Figure 4-6b). Again the overall lung expression was a combination of sustained expression, no expression, reduced expression, loss and re-emergence of lung expression.

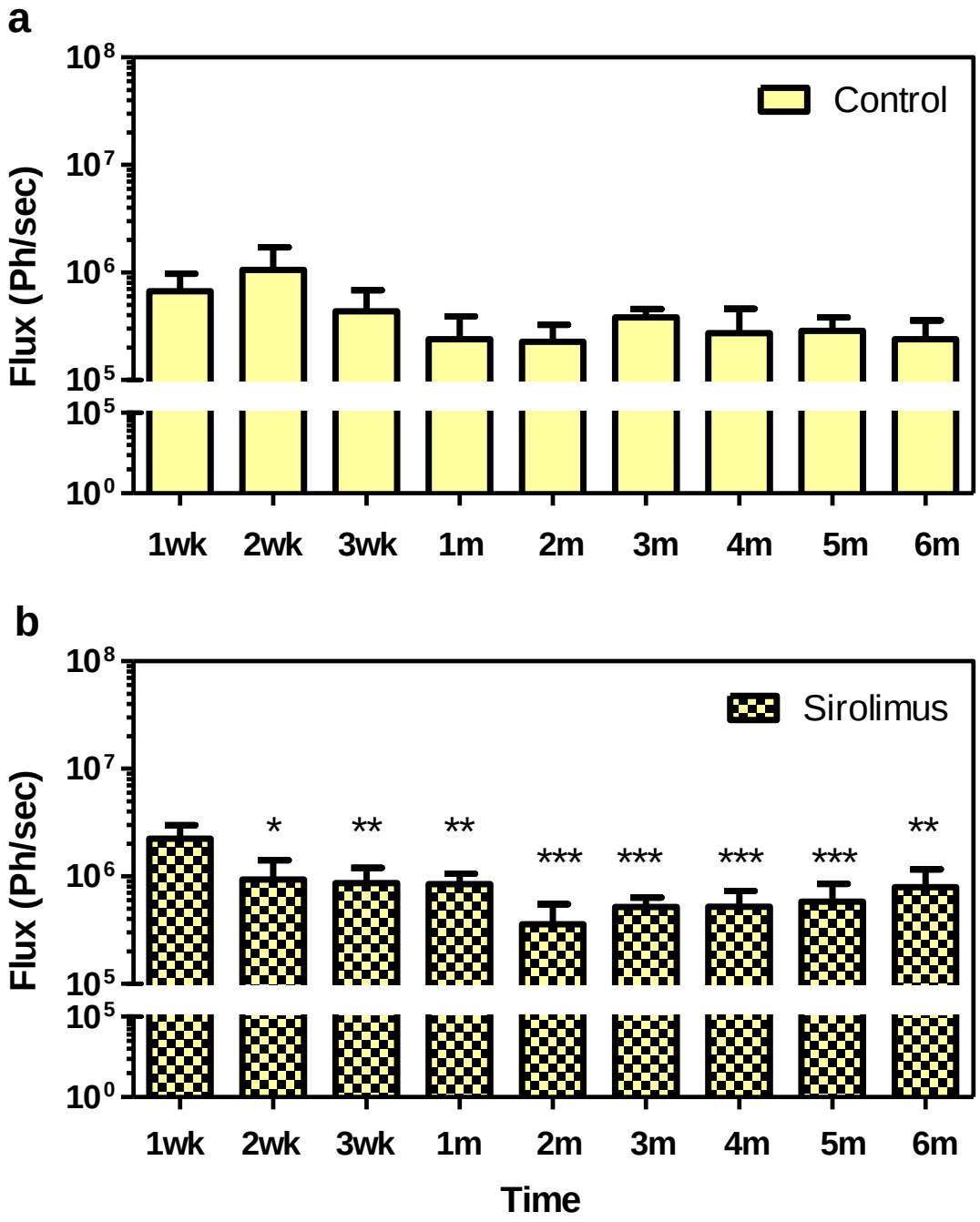


Figure 6-4 Lung Luminescence – With and Without Immunosuppression

*Lung luminescence over time a) without and b) with sirolimus immunosuppression (*p<0.05, **p<0.01, ***p<0.001, Dunnett's RM ANOVA compared to 1 week, $\bar{X} \pm SEM$, n=8). Same data as presented in Figure 6-3, but separated into individual treatments using expanded y axis for clarity.*

6.1.4.3 Circulating Antibodies

6.1.4.3.1 Anti-VSV-G protein

There was an increase in circulating antibodies to the LV vector VSV-G protein five months after delivery in the mice that received a single administration of LPC/LV-Luc, compared to pre-administration levels (Figure 6-5a, $p<0.05$, Dunnett's RM ANOVA compared to time zero (baseline), n=8). There was no increase in circulating antibodies to the VSV-G protein in the mice that received one dose of LPC/LV-Luc in conjunction with oral administration of sirolimus, compared to pre-administration of LV vector delivery (baseline) at all of the time points tested.

6.1.4.3.2 Anti-Luciferase

Four months after mice were treated with water as a control (no-immunosuppression) prior to and following nasal delivery of LPC/LV-Luc, a significant increase in circulating luciferase antibodies was observed compared to pre-treatment levels (Figure 6-5b, $p<0.05$, Dunnett's RM ANOVA, n=8). In contrast, there was no significant increase in antibodies to the luciferase protein in mice that received a single dose of LPC/LV-Luc with immunosuppressive treatment at any of the assessment time points.

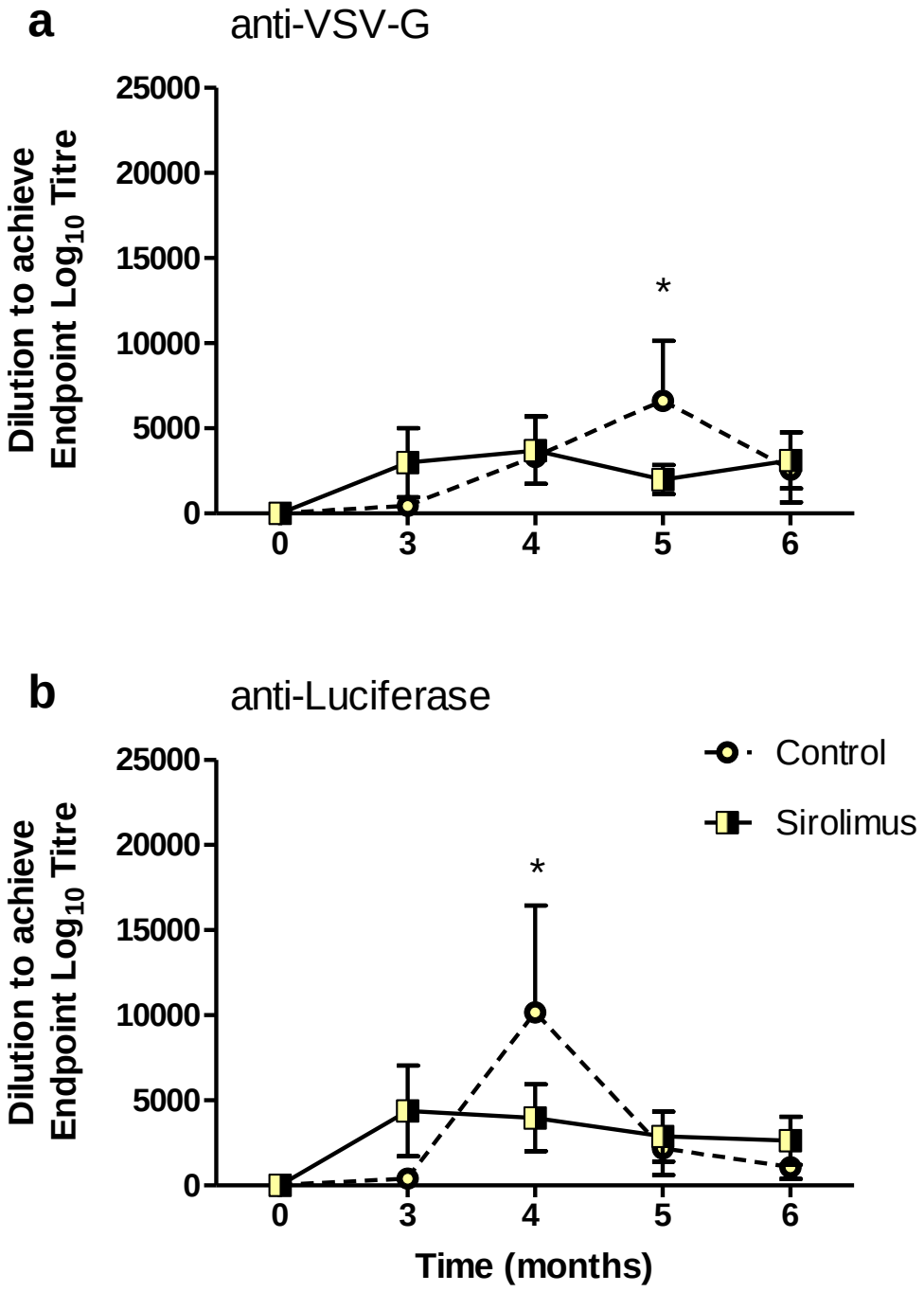


Figure 6-5 Circulating Antibodies – Immunosuppression

*Circulating antibodies to the a) LV vector VSV-G envelope protein and b) recombinant luciferase (Luc) detected in mice that received a single dose of LPC/LV-Luc with or without transient immunosuppression (sirolimus) administration (*p<0.05, Dunnett's RM ANOVA compared to zero (pre-treatment baseline) time point, $\bar{X} \pm SEM$, n=8).*

6.1.5 Discussion

Sirolimus (rapamycin) is an immunosuppressive agent that was isolated from a soil sample of a strain of *Streptomyces hygroscopicus*, collected from Easter Island (Rapa Nui). It was first used as an antifungal agent[397] and then for its immunosuppressive activity[398]. Oral sirolimus is currently used clinically as a prophylactic treatment for post-transplantation organ rejection, primarily in renal[399] and cardiac[400, 401] patients. Sirolimus acts by inhibiting T-cell proliferation while selectively increasing the number of Treg cells that actively suppresses the activation of the immune system [402]. In this experiment the addition of a transient immune suppression treatment at the time of LV airway gene transfer was examined, in an effort to modulate the host's immune response and enhance airway gene expression.

Daily oral administration of sirolimus prior to and for 1 week following LV vector delivery had no significant effect on the level of Luc gene expression measured in the nasal airways, compared to mice receiving no immune suppression. The lack of effect reported here has been observed by others using sirolimus alone[403]. Success in reducing organ transplantation rejection is achieved by employing intraperitoneal administration of sirolimus, but only in conjunction with co-stimulatory blockade agents[403, 404] including anti-CD4 and anti-CD8. The potent synergistic benefit of sirolimus and cyclosporine has also been documented[405, 406]. The absence of co-stimulatory and/or other immunosuppressive agents may explain the ineffectiveness of oral sirolimus in this setting; however differences in the route and the timing of administration may also have contributed.

The decrease in nasal luminescence at 2-6 months from the level of Luc gene expression measured one week after nasal gene transfer was consistent with the normal loss of a proportion of terminally-differentiated transduced epithelial cells, as part of cell turnover process.[328, 407, 408] This effect was also observed in the long term single dose studies in both normal and CF mice reported in Chapter 4.

Sirolimus treated mice also displayed a decrease in nasal gene expression from 1-6 months, compared to nasal flux measured at 1 week. The addition of a short term immunosuppressive treatment had no overall effect on initial or long term nasal gene expression.

No difference in luminescence was detected in the lung airways of mice that received the standard LPC/LV-Luc treatment only, over all time points assessed. Immunosuppression induced by sirolimus did briefly increase initial lung luminescence, but could not be sustained over the remainder of the study.

The later and more significant reduction in lung gene expression observed from 2-5 months following LV instillation could be due to an antibody response to the Luc protein or the loss of transduced cells from apoptosis. The increase in lung luminescence observed at the 6 month time point in sirolimus treated mice was consistent with the re-emergence of lung gene expression noted in earlier studies described in Chapter 4.

The presence of circulating antibodies to the VSV-G protein constituents of the LV vector envelope and to the Luc protein was significantly increased only in the sera of mice that received the LV vector without an immune-suppression treatment. The result confirms that the transient immune suppression treatment employed in this study could reduce antibody production but it was not a sufficient treatment to enhance nasal gene expression over the standard protocol. It is tempting to speculate that this immune-suppressive treatment may have allowed enhanced gene expression from a second dose administered at the later time points had that been tested.

The results from this experiment highlight the continuing need to develop an effective transient immune-suppressive treatment that enhances initial gene expression, but more importantly permits the boosting of gene expression levels when repeat dosing is required to obtain the long term therapeutic benefits

promised by an effective genetic therapy. Other approaches such as the use of sirolimus via the intraperitoneal route, and in conjunction with co-stimulatory agents[409] warrants further examination.

6.2 CHAPTER SUMMARY

The addition of a transient immunosuppressive regime using oral sirolimus did not improve nasal Luc gene expression over the standard gene transfer protocol (of LPC pre-treatment and LV-Luc) administration. The ineffectiveness of oral sirolimus in this setting maybe due to several factors, including the route and timing of immunosuppression treatment, and also the absence of co-stimulatory and/or other immunosuppressive agents known to have a powerful synergistic effect.

However, sirolimus was effective in increasing the initial level of gene expression detected in the *lung* airways compared to our standard gene transfer protocol. An early reduction in lung luminescence was consistent with the high level of lung luminescence unable to be maintained without the continued use of the immunosuppression treatment.

Immune suppression reduced the presence of circulating antibodies to both the LV vector VSV-G protein product and to the recombinant Luc protein, but had no significant effect on enhancing Luc expression compared to the standard LPC/LV-Luc administration. In summary, the use of sirolimus on its own was an ineffective immunosuppressive agent in this gene transfer setting. The potential for enhancing LV gene transfer may be realised when administration of sirolimus is delivered during re-dosing events of a gene transfer protocol.

CHAPTER 7

CONCLUDING REMARKS

CHAPTER 7 CONCLUDING REMARKS

7.1 FINAL DISCUSSION

The recent success in the field of gene therapy for inherited genetic disorders such as ADA-SCID, chronic granulomatous disease, X-linked adrenoleukodystrophy, haemophilia, inherited blindness and other diseases has renewed hope for a treatment for the genetic disease CF. The difficulties experienced in providing an effective and a sustained gene therapy for the correction of the CF gene defect has highlighted the complexity involved in airway gene therapy protocols. However, the huge potential for a gene therapy strategy to treat CF airway disease, regardless of the class of mutation, makes it a worthwhile candidate for further experimentation towards clinical usage.

The level, proportion and distribution of CFTR correction in epithelial cells needed to provide a therapeutic benefit are still under debate. Heterozygote carriers for CF with one functioning copy of the CFTR gene are considered normal, but much less that 50% of cells need to be transduced. Even if only 5-20% of cells are required to be corrected to produce a therapeutic benefit, the question remains what proportion of respiratory epithelial cells is required, how many genome copies per cell are adequate, and what percentage of particular epithelial cell types must be targeted.

The ability to assess the effectiveness of a gene therapy for CF is also crucial for clinical outcomes. The detection of DNA or mRNA in tissue may confirm successful gene and protein delivery, but the ability to assess functional benefit is paramount. Clinical success must be able to be shown via lung function tests, potential difference measurements, chest X-rays, alterations in microbial flora, antibiotic usage, reduction in exacerbations, and improvements in quality of life. However, to detect a significant improvement in these parameters from only a small change in genetic correction may prove challenging, especially if lung disease is already established. Further confounded by the treatments CF patients are

already taking, increasing the difficulty in determining which treatment is providing an effective treatment. The optimal treatment age for a successful gene therapy would be in infants before CF disease has established. Gene therapy in infants is another major ethical issue, compounded by the lack of acceptable outcome measures by which to determine success in infants, and the challenges of consent for a disease that is no longer inevitably fatal, which may hinder gene therapy for CF.

LV vectors can be targeted to respiratory epithelia, can effectively transduced non-dividing cells, and have relatively low immunogenicity, making them promising candidates for a CF airway gene therapy. Despite good progress with a non-viral vector, the UK Gene Therapy Consortium has committed to a gene therapy with another LV vector, confirming the emergence of LV vectors as lead candidates for CF airway gene correction therapies. Furthermore, the current efficiency and safety of LV vectors that have now reached Phase I and II clinical trials for other diseases provides a strong rationale for the use of a LV vector system for clinical CF airway disease.

The work presented in this thesis has attempted to address some of the major issues that have hampered gene therapy for CF disease. The ability to produce large volumes of high-quality and high-purity LV vectors with the capacity to contain the large CFTR gene in its complete form, and at titres sufficient to transduce airway epithelia in a small animal model of CF also makes this a practical approach. Although our group has reported partial functional correction of the electrophysiological defect in nasal airways of CF mice after a single dose of a LV-CFTR vector using separate timed cohorts, this is the first examination of persistence of LV reporter and functional CFTR gene expression in individual CF mice over their lifetimes.

The advantage of utilizing the Luc reporter gene is that it allows non-invasive assessments to be performed at multiple time points, not only reducing the total experimental animal numbers, but also

reducing within and between animal variability. It is also more sensitive than the LacZ gene, and allows nasal and lung gene expression to be assessed in the same animal. The differences in persistence of gene expression between nasal and lung luminescence emphasized the caution required in interpreting nasal expression as a surrogate for lung gene therapy.

Nevertheless, the only CF animal model currently available in Australia is the CF mouse; in this model the CF pathophysiology is present only in the nasal airway epithelial cells and not in the lung. To detect a functional correction of the bioelectrical defect caused by mutations in the CFTR gene the nasal airway of CF mice must be studied using nasal TPD measurements for quantification.

For effective nasal airway gene transfer of a VSV-G pseudotyped LV vector, a pre-treatment (in this case LPC) is required to reduce mucociliary transport and transiently open the tight junctions between cells to reach the appropriate basolateral receptors for cell transduction. When PBS was used as a sham pre-treatment very poor or absent LV vector transduction was present. The use of LPC may enable the longevity seen for both reporter and functional CFTR nasal gene expression due to the transduction of stem/progenitor cells, a proportion of which are thought to be basal cells that reside in the protective niches of the basolateral membrane. Alternatively or concomitantly, long term expression could also be due to transduction of terminally differentiated airway cells with a slow turnover rate.

The reduction in nasal expression that was observed after 3 months may be due to normal cell turnover in the respiratory epithelia or the result of host immune responses to the introduced transgene(s). Although the detection of circulating antibodies to the transgene corresponded with a reduction in nasal gene expression, it was not sufficient to completely block gene expression. However, whether an immune response would be raised against the introduction of human CFTR in a vector in to CF patients

is still unknown. The detection of antibodies to the VSV-G envelope protein occurred rapidly but had no significant effect on gene expression confirming the low immunogenicity of the LV vector.

The sustained partial correction of the electrophysiological defect in CF mice for up to 1 year after a single LPC/LV-CFTR dose provides strong support for the potential of a LV gene therapy for CF. Our findings are in contrast to those of other groups working in this area of functional CFTR gene transfer. None have detected significant correction of the defect via the nasal TPD technique for more than a few weeks. This lack of robust correction may be due to ineffectiveness of particular viral and non-viral vectors, inefficient nuclear uptake after apical transduction, transduction of olfactory epithelia (never seen with our LV vector) that confounds bioelectrical measurements, or the protein produced by the introduced CFTR reaching the apical surface but not functioning effectively.

The complete disappearance and re-emergence of lung gene expression was unexpected because the lung airways were not intentionally targeted with the small volume of vector used for nasal airway transduction. The re-emergence of lung luminescence suggests stem/progenitor transduction might have occurred and that LPC may not be necessary as transduction was just as effective in the PBS pre-treated cohort. Lung progenitor cells may not be just a proportion of basal cells likely to be accessed by LPC, but some Clara cells or (unidentified) transient amplifying cells derived from protected niches and are ready for differentiation. Further experimentation using a LV gene delivery to lung epithelia in the newer animal models with relevant CF lung disease - the CF pig or CF ferret - is the next logical step towards testing and fulfilling the potential for an effective life-long treatment for CF airway disease.

CF mice treated with LPC/LV-CFTR also had statistically significantly longer survival than their control CF counterparts, suggesting that the presence of functional CFTR gene correction in nasal cells alone may provide overall beneficial effects. The normalised ion transport could provide protection from

inhaled pathogenic particles by improving MCC, altering mucus viscosity and increasing the depth of the ASL layer. How nasal airway CFTR correction could have an impressive effect on survival is not yet known and given the limitations in this study from the small animal group numbers caution in interpretation of results is required. The majority of mortality in this CF mouse strain is due to intestinal obstruction but as no luminescence was detected in off target tissue such as the gut, this seems to be an unlikely explanation for the increase in survival. However as little as 5 % CFTR can improve overall health in humans and this may be a similar scenario with CF mice, so only small amounts of CFTR may be required to produce improvements in overall health and nutritional status, leading to increased survival.

Although early integrating retroviral vectors resulted in cases of insertional mutagenesis (e.g Leukaemia after use of γ-retroviral vectors in SCID-X1 patients), the increased safety of this LV vector system was supported by the normal rates of spontaneous lymphomas in LV vector treated mice. Most unexpected deaths had no known aetiology and could be ascribed to death within the normal aging process.

Even though long-term gene expression after a single LV dose was demonstrated in many mice, a proportion of mice did lose gene expression. This loss could be due to the normal cell processes of apoptosis or by an active removal via a host immune response to the introduced protein(s). The ability to boost expression by repeat doses was also examined in the short term (days) and over a longer (months) time frame. A FIV LV vectors given repeatedly (7 doses over 7 weeks) were needed to achieve effective nasal expression. Though multiple doses of this HIV-1 based LV vector over many days were feasible, gene expression was not boosted and was not significantly greater than that expressed from a single dose.

The result of the longer interval re-dose study was not so illuminating as gene transfer measured at 1 year was only detected in one animal after either one or two doses. However, the detection of circulating antibodies to both the envelope protein and the reporter gene protein in the 6 month re-dose cohort suggests timing of repeat dosing may be crucial for successful boosting of waning gene expression, and this may have implications for the longevity of transduction.

The effect of repeat dosing of the VSV-G pseudotyped LV vector containing either the same, or a different transgene, showed that the use of the repeat dose of the same transgene did not boost expression nor did it block subsequent gene expression when a different reporter gene vector was delivered. However, antibodies to the VSV-G protein product increased with increasing doses, as did antibodies to the same foreign transgene. Since the main host immunological response is directed against the foreign transgene protein this may not be an issue for a CF gene therapy as the majority of human CFTR mutations produce CFTR despite it being non-functioning. The introduced protein is derived from human CFTR and therefore may not be recognised by the immune cells as foreign.

Since there was no boost in respiratory cell gene expression from repeat LV dosing, despite being accompanied by increased levels of circulating antibodies to both the envelope and the transgene protein, the use of an immunosuppressive treatment was examined in an attempt to overcome this immunological barrier to gene transfer. Sirolimus delivery orally just prior and 1 week after LV nasal administration had no effect on nasal luminescence compared to our standard treatment. There was an increase in the initial (1 week) gene expression detected in the lung airways but this effect was not sustained for the remainder of the 6 month study period.

The significant reduction in circulating antibodies to both the envelope protein and to the transgene protein with the addition of the immunosuppression treatment suggests that the efficacy of this transient

immune suppression treatment may help sustain waning expression from immune responses, or may assist in boosting subsequent doses of a LV vector gene delivery for long term expression.

The ultimate goal for an effective gene therapy for CF airway disease is the ability to target the correct relevant cells before establishment of airway disease and maintain long term therapeutically relevant levels of CFTR function without serious adverse events occurring. The demonstration here of long term sustained partial functional correction of the CFTR dysfunction in CF mice, without major immune or safety issues, and with a substantial increase in survival, provides compelling evidence for the continued development of LV vectors towards their eventual use as an effective clinical treatment for CF airway disease, in all mutation classes.

7.2 CONCLUSION

The body of work summarized in thesis has addressed some of the major issues that have hampered the field of gene therapy for CF airway disease.

i) Overcoming the extracellular and intracellular barriers to airway gene therapy;

ii) The efficacy, safety and functional longevity of gene expression; and

iii) The ability to evade the host immune response using a single effective dose and the potential for effective re-dosing strategies.

This work confirmed that the use of the surfactant LPC greatly enhances LV gene expression in nasal airways of mice. The use of an endotoxin-free LV vector to safely integrate into the genome of the targeted respiratory ciliated cells provided effective and long term partial functional correction of the electrophysiological defect in CF mice nasal airways. The introduction of the CFTR gene increased the survival of CF mice and was not associated with long term insertional mutagenesis from LV vector delivery. Re-dosing was feasible and was not blocked by host immune responses. LV vector administration in conjunction with a trial of a transient immunosuppressive treatment reduced circulating antibodies to both the VSV-G pseudotyped envelope protein and to the foreign transgene protein.

In conclusion, the utilization of a LV vector as a gene transfer agent for CF airway disease has great potential, but must be carefully examined before introduction to the clinical setting. The use of more clinically-relevant animal models – for example non-human primate models such as the marmoset, and for CF lung disease models such as the CF pig and CF ferret – will move LV vectors a step closer towards clinical trials. The recent success of novel small molecule potentiators for a small proportion (~3%) of the CF population with the Class III mutation has renewed the development of potential novel therapies in the CF field. The reality of a gene therapy cure for the people with CF – with any type of

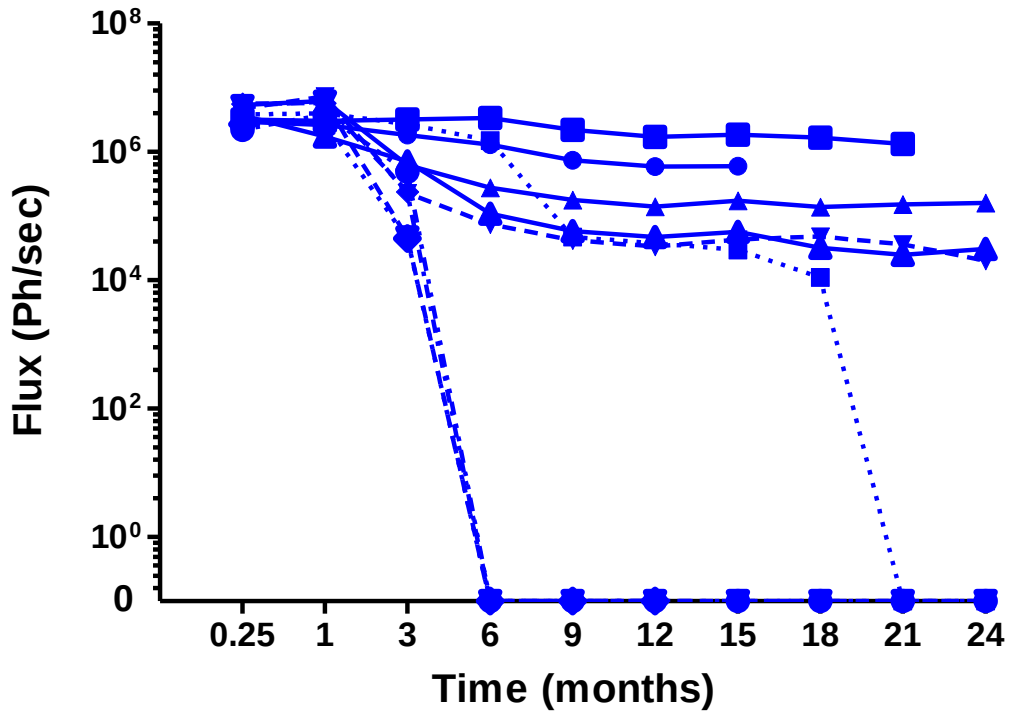
class mutation – has been advanced by the studies described here and remains an achievable goal for the future.

CHAPTER 8

APPENDICES

CHAPTER 8 APPENDICES

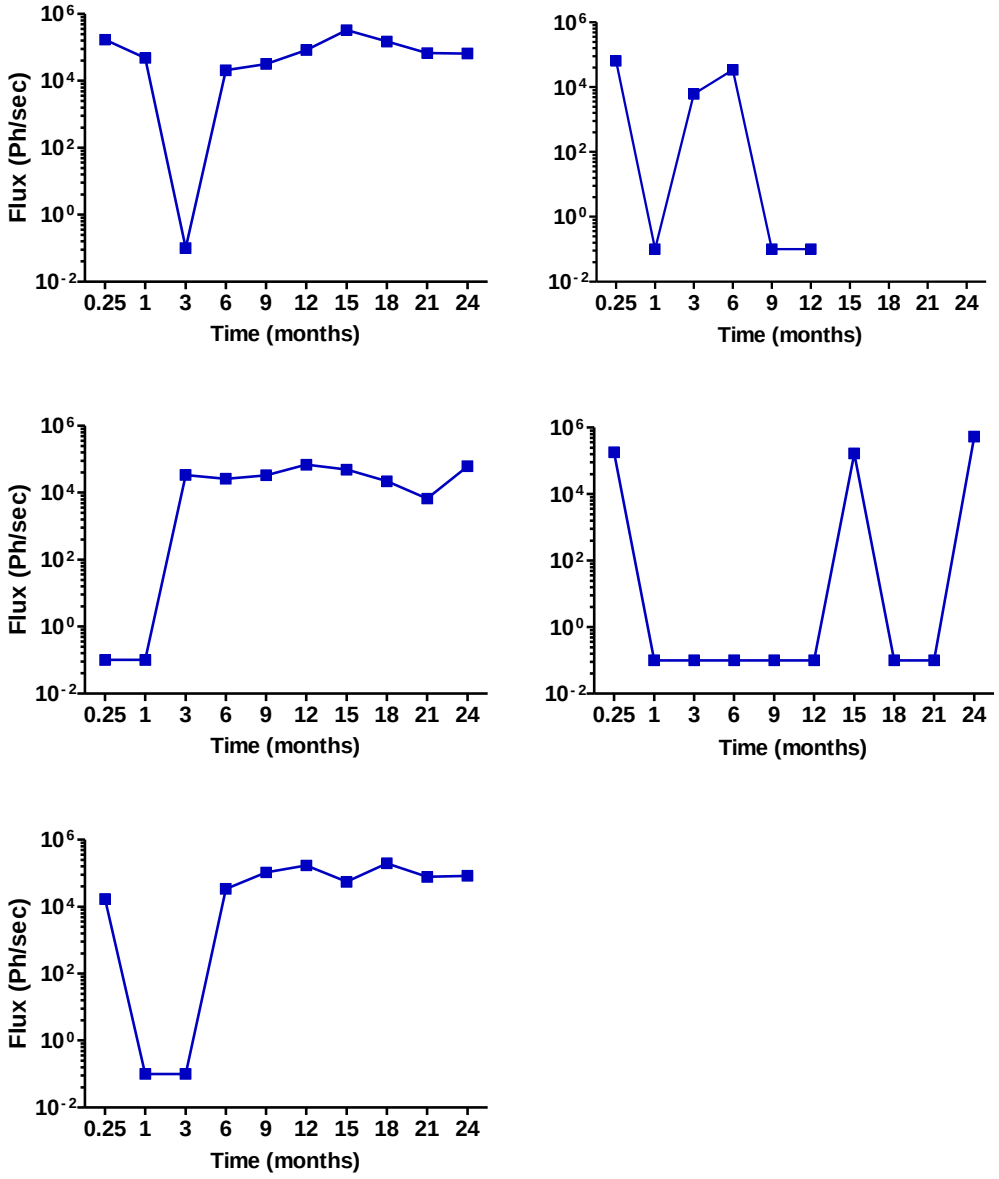
8.1 INDIVIDUAL NASAL LUMINESCENCE – NORMAL MICE



Appendix 8.1-1 Individual Nasal Luc Expression in LPC/LV-Luc+LacZ Treated Mice
Luciferase gene expression after nasal LV administration in the nasal airways of normal mice after LPC/LV-Luc+LacZ treatment (n=10). N.B. Proportion of mice displaying complete loss of nasal Luc gene expression at 6 and 21 months.

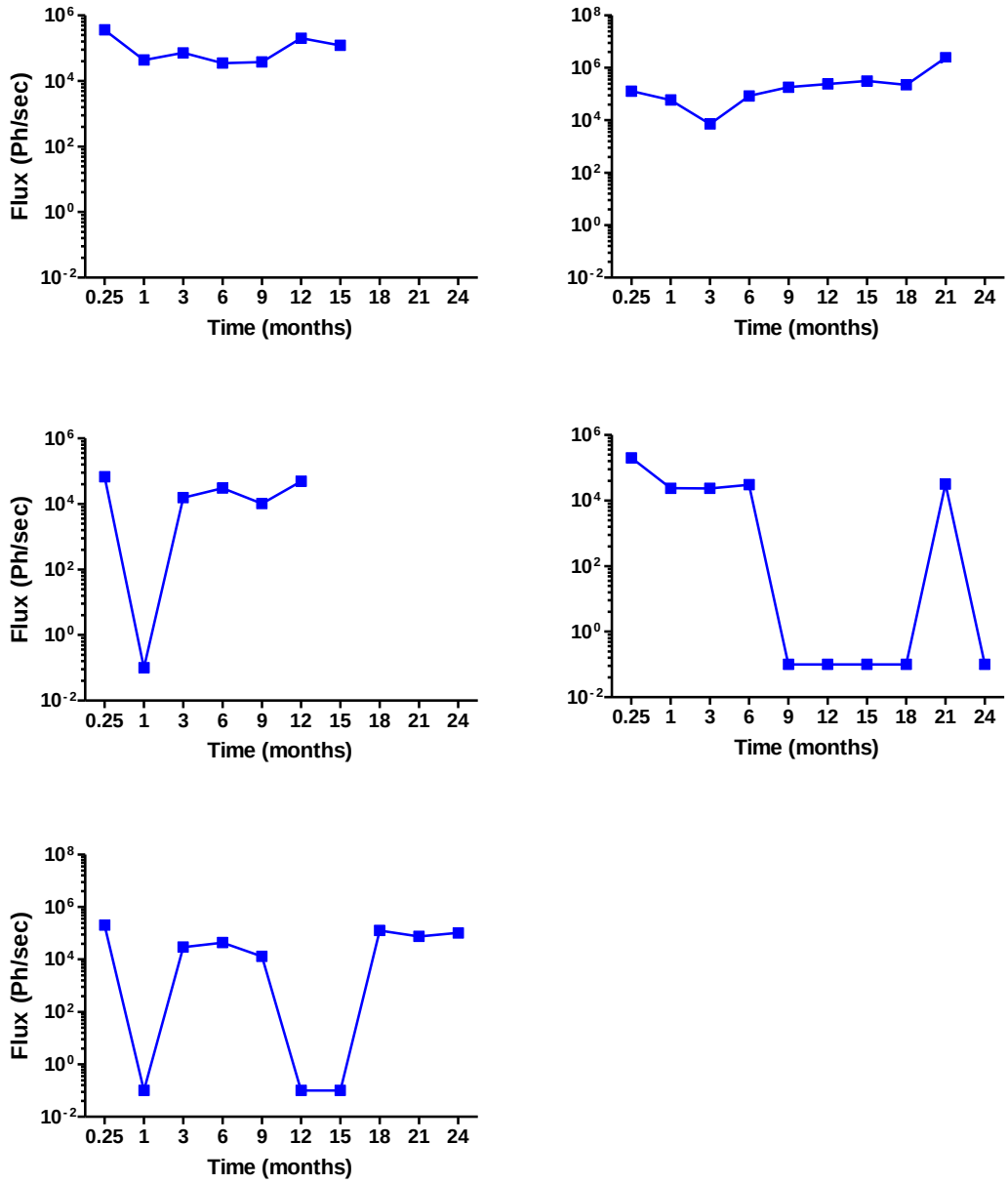
8.2 INDIVIDUAL LUNG LUMINESCENCE – NORMAL MICE

8.2.1 LPC/LV-Luc+LacZ



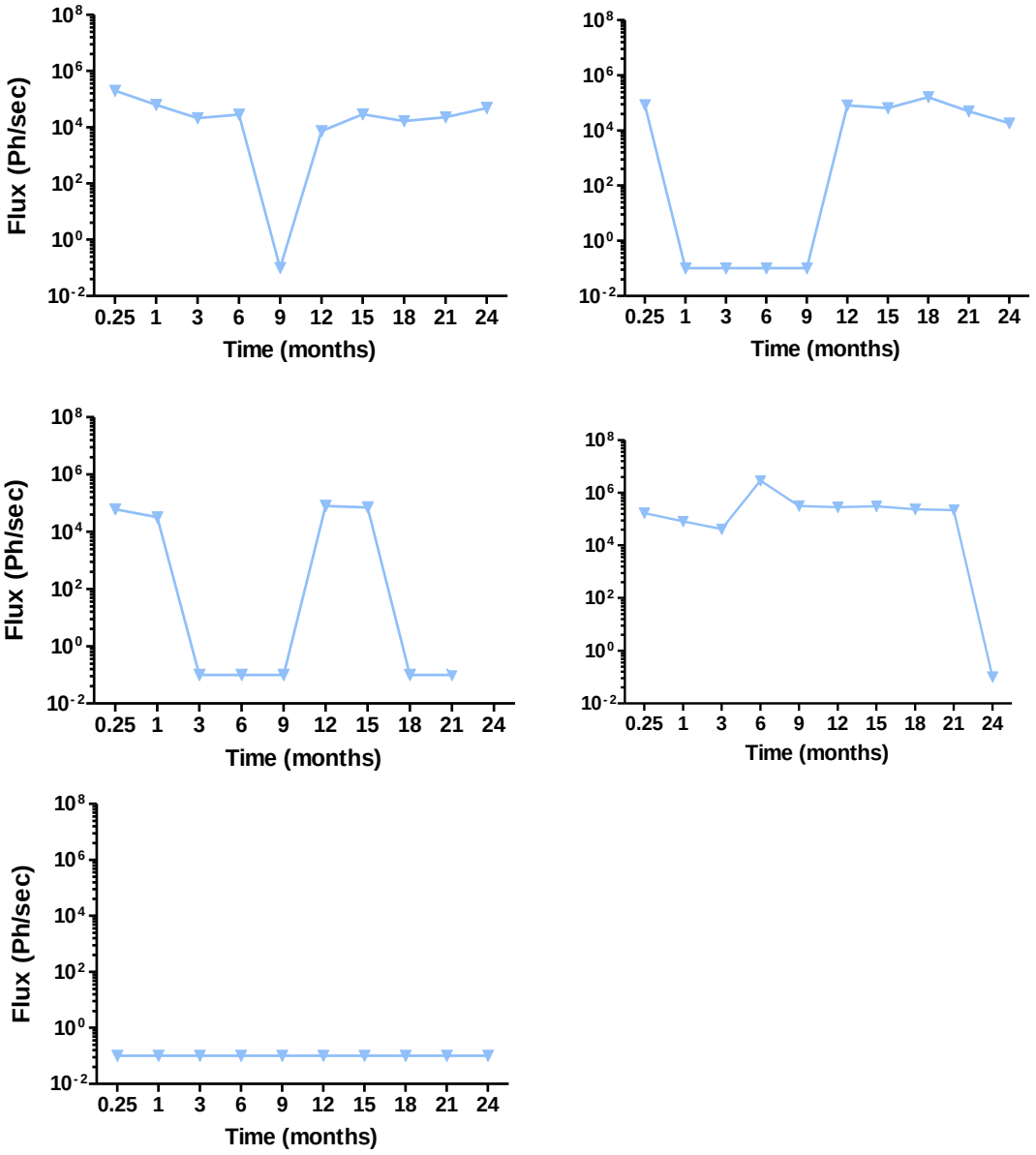
Appendix 8.2-1 Individual Lung Luminescence in LPC/LV-Luc+LacZ Treated Mice
Luciferase gene expression after nasal LV administration in the lung airways of normal mice after LPC/LV-Luc+LacZ treatment. N.B. Re-emergence of lung expression (n=5).

8.2.2 LPC/LV-Luc+LacZ continued



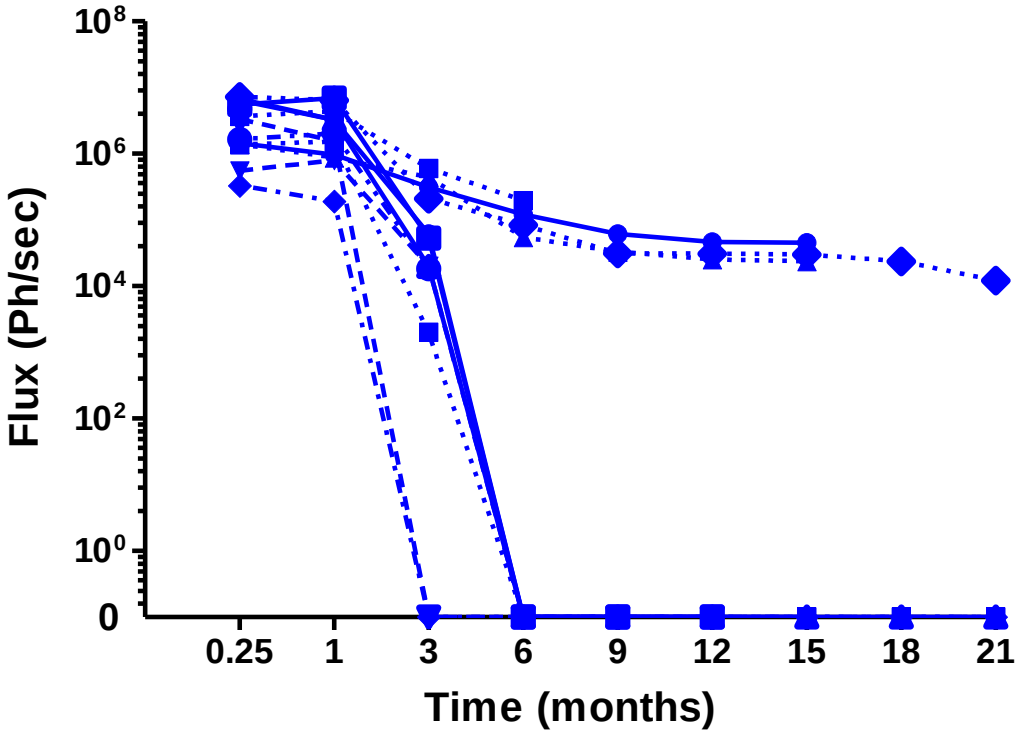
Appendix 8.2-2 Individual Lung Luminescence in LPC/LV-Luc+LacZ Treated Mice cont.
Luciferase gene expression after nasal LV administration in the lung airways of normal mice after LPC/LV-Luc+LacZ treatment. N.B. Stable (n=2) or re-emergence of lung expression (n=3).

8.2.3 PBS/LV-Luc+LacZ



Appendix 8.2-3 Individual Lung Luminescence in PBS/LV-Luc+LacZ Treated Mice
Luciferase gene expression after nasal LV administration in the lung airways of normal mice after PBS/LV-Luc+LacZ treatment. N.B. Re-emergence of (n=3), loss of (n=1) or no (n=1) lung expression.

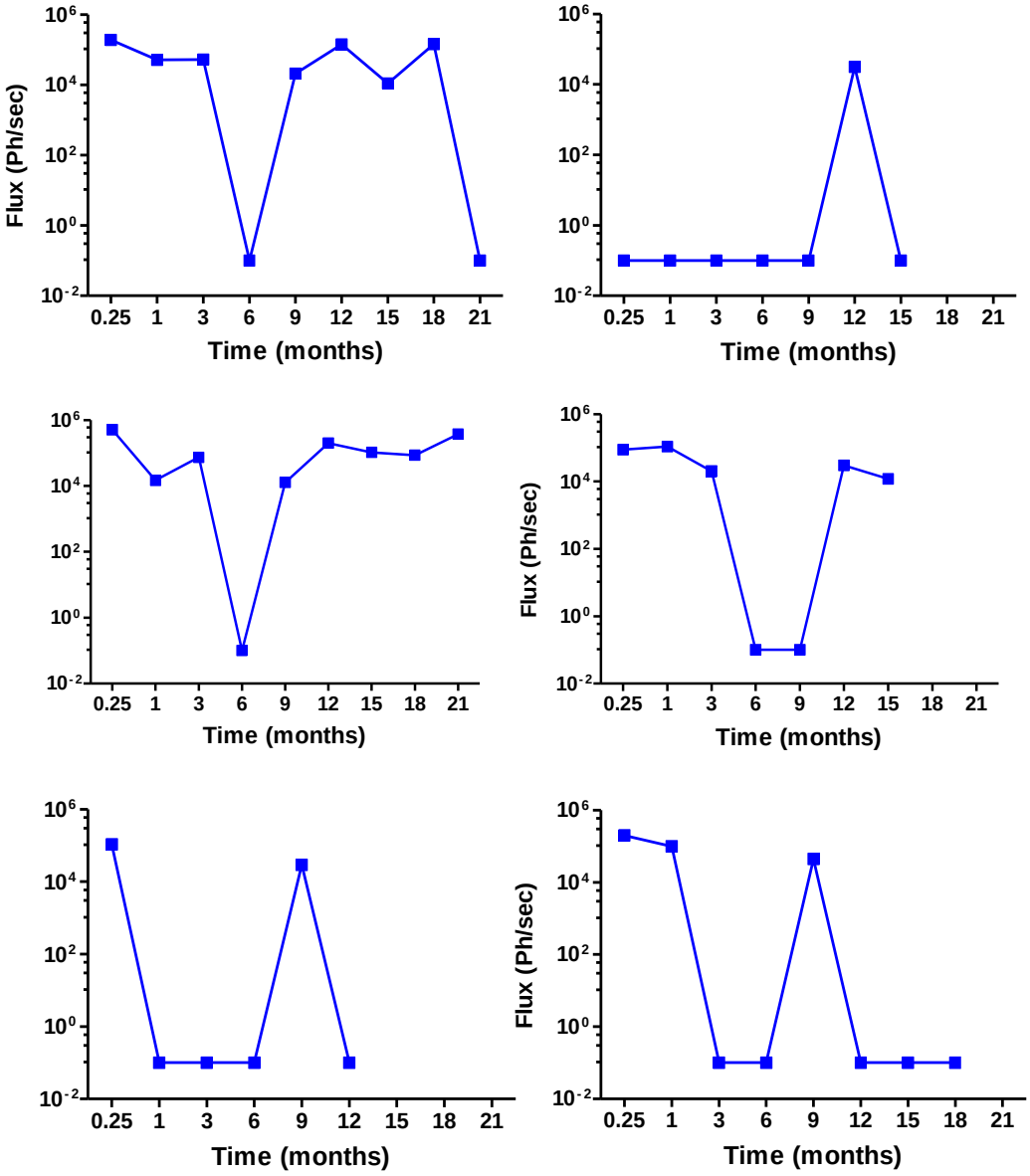
8.3 INDIVIDUAL NASAL LUMINESCENCE – CF MICE



Appendix 8.3-1 Individual Nasal Luc Expression in LPC/LV-Luc+CFTR Treated Mice
Luciferase gene expression after nasal LV administration in the nasal airways of CF mice after LPC/LV-Luc+CFTR treatment (n=12). N.B. Proportion of mice displaying complete loss of nasal Luc gene expression at 3 and 6 months.

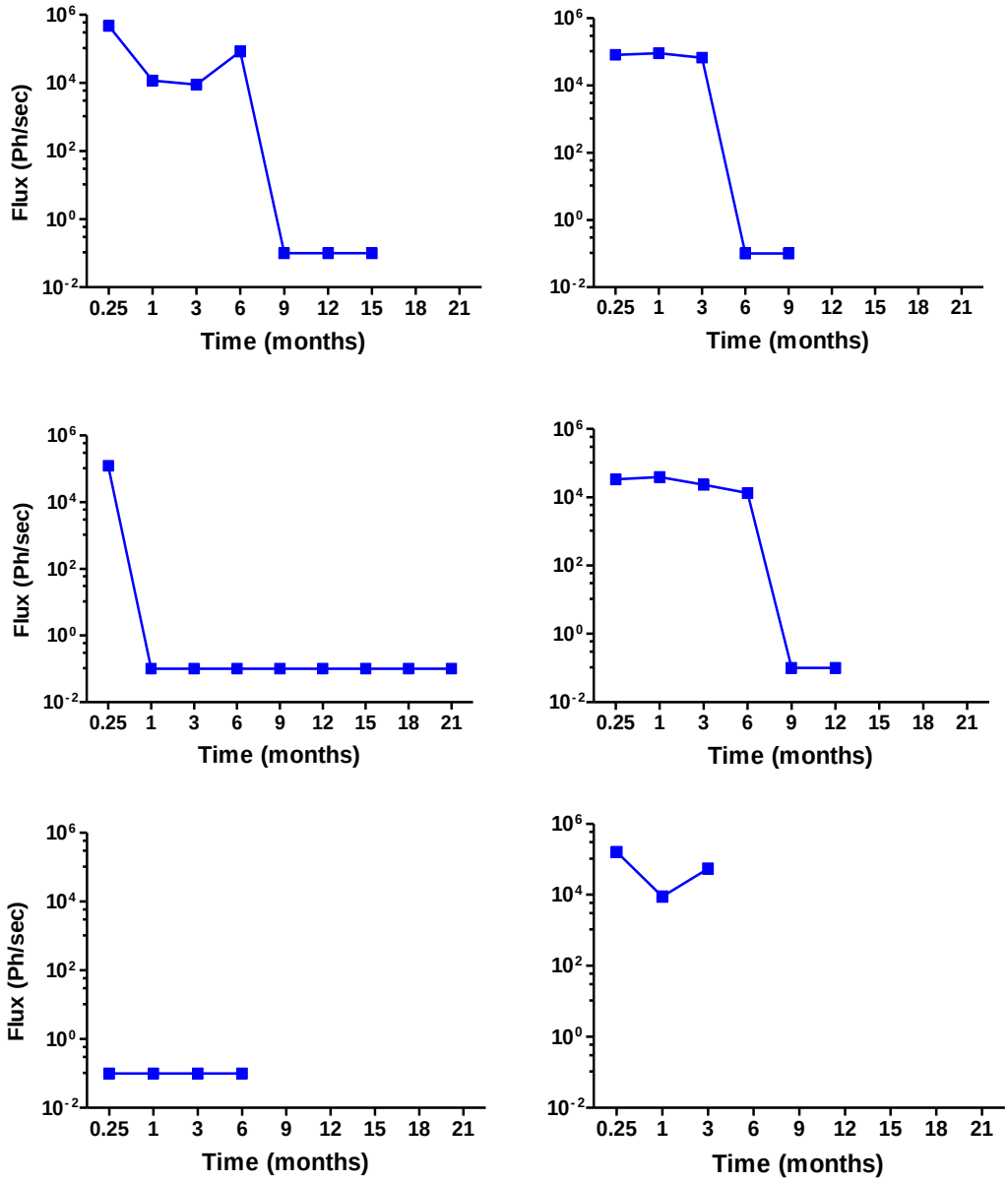
8.4 INDIVIDUAL LUNG LUMINESCENCE – CF MICE

8.4.1 LPC/LV-Luc+CFTR



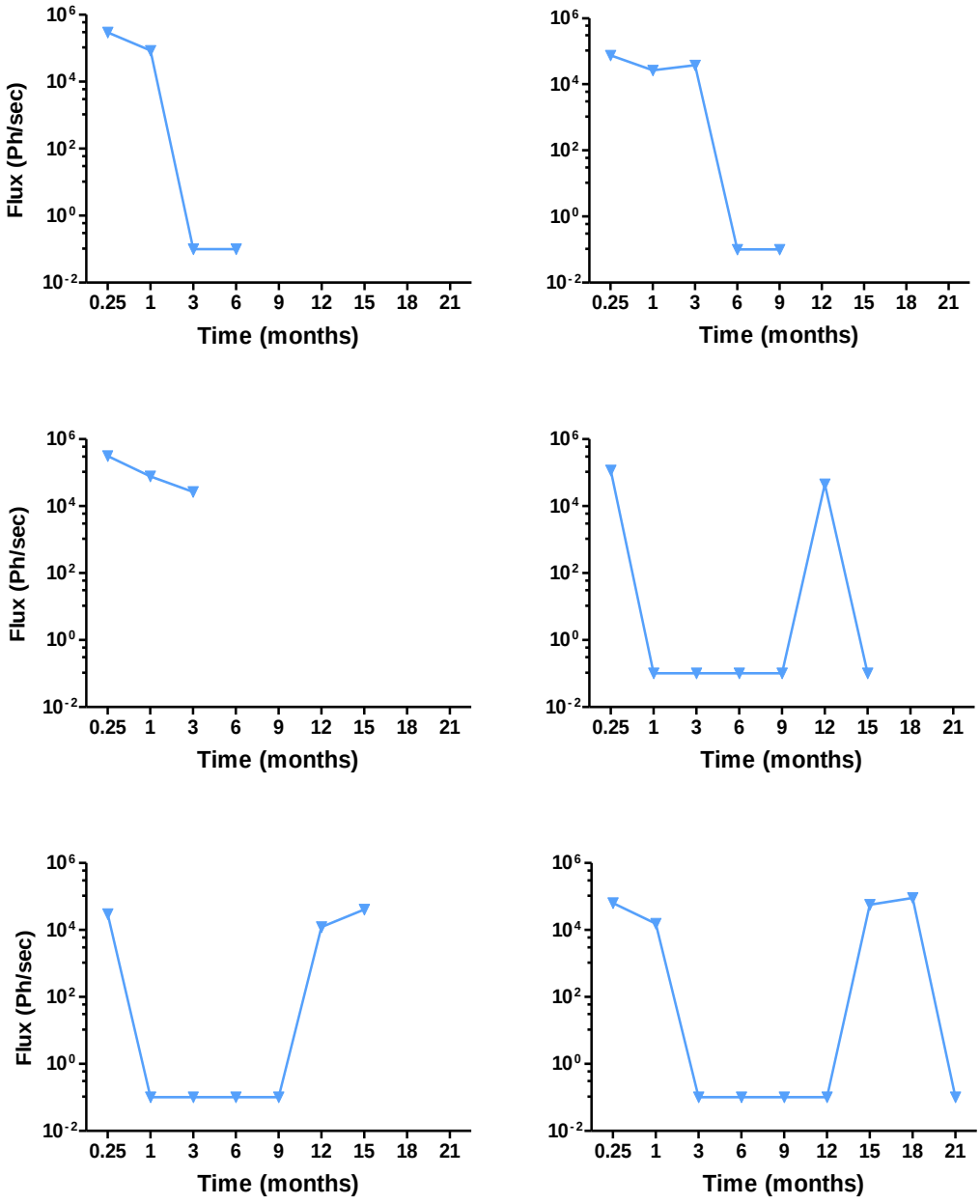
Appendix 8.4-1 Individual Lung Luminescence in LPC/LV-Luc+CFTR Treated CF Mice
Luciferase gene expression after nasal LV administration in the lung airways of CF mice after LPC/LV-Luc+CFTR treatment. N.B. Re-emergence of lung expression (n=6).

8.4.2 LPC/LV-Luc+CFTR Continued



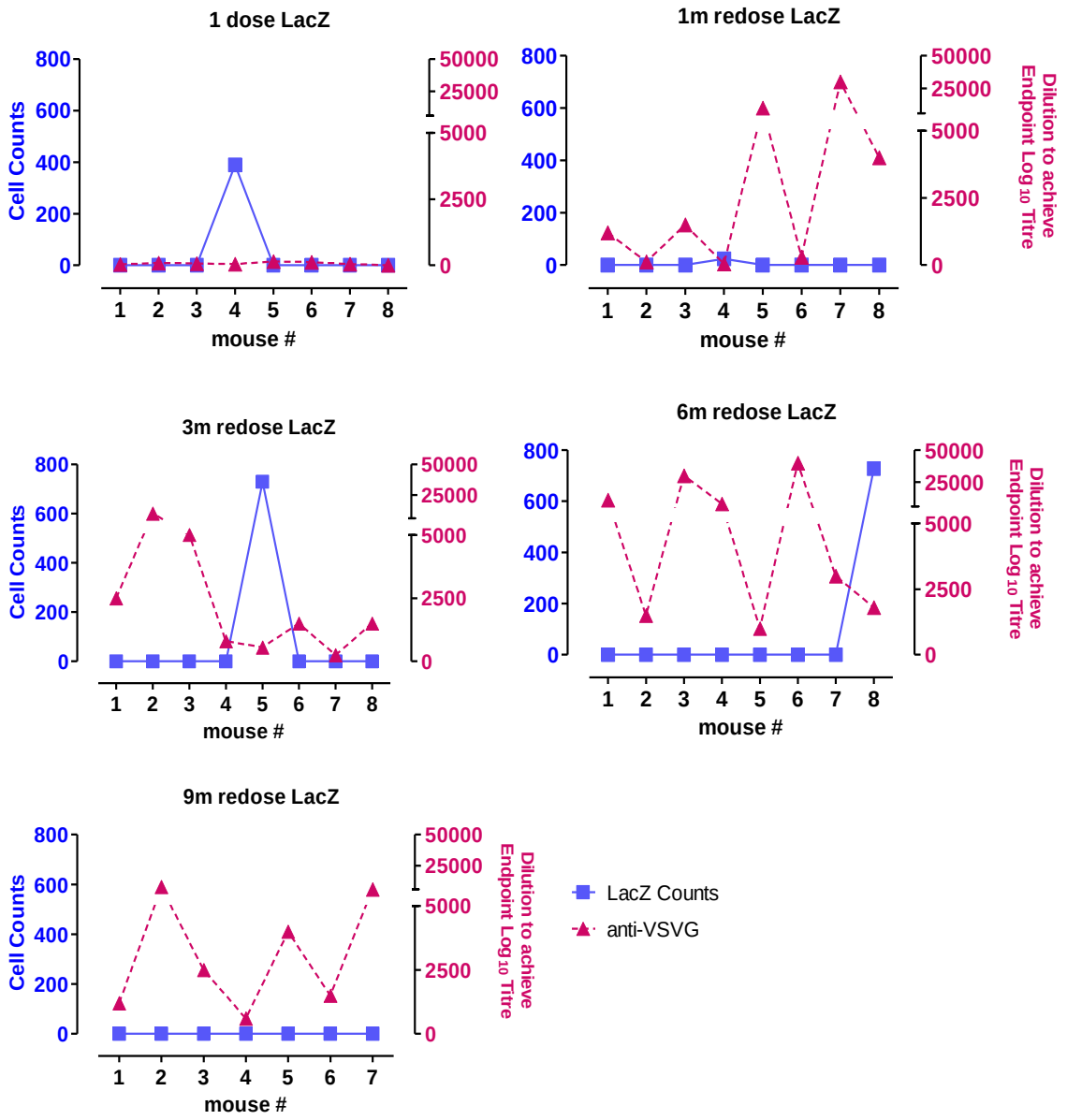
Appendix 8.4-2 Individual Lung Luminescence in LPC/LV-Luc+CFTR Treated CF Mice cont.
Luciferase gene expression after nasal LV administration in the lung airways of CF mice after LPC/LV-Luc+CFTR treatment. N.B. Loss of lung expression (n=4) or no lung expression (n=1), stable lung expression (only 3 time points, n=1).

8.4.3 PBS/LV-Luc+CFTR



Appendix 8.4-3 Individual Lung Luminescence in PBS/LV-Luc+CFTR Treated CF Mice
Luciferase gene expression after nasal LV administration in the lung airways of CF mice after PBS/LV-Luc+CFTR treatment. N. B. Re-emergence of lung expression after 9 months (n=3), reduced or loss of lung expression (n=3, only 3 - 9 months assessed).

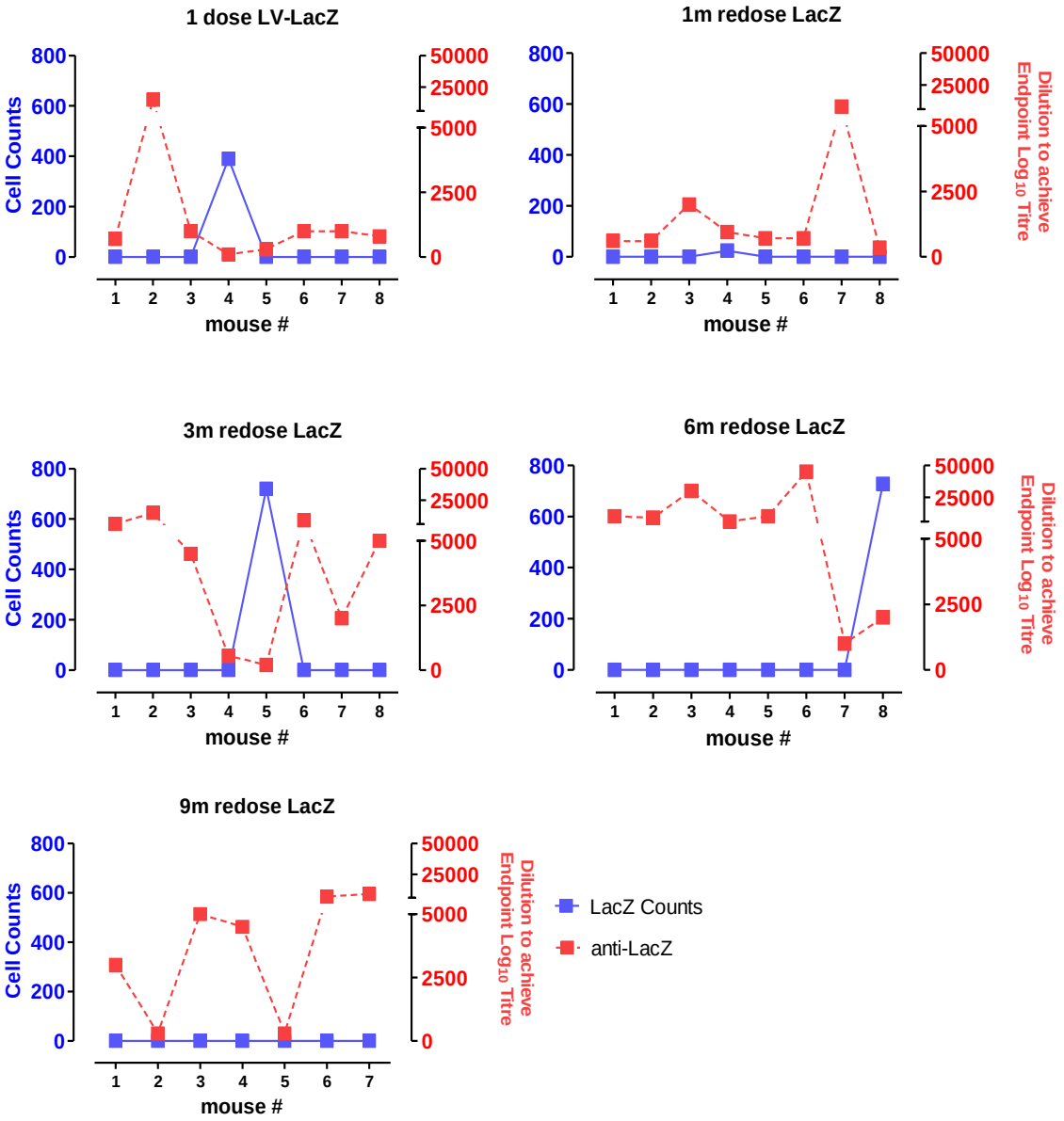
8.5 LACZ COUNTS AND VSV-G ANTIBODIES



Appendix 8.5-1 Individual LacZ Counts vs anti-VSV-G

Number of transduced LacZ cells detected at 1 year (left y axis, solid line) against sera antibodies to LV vector VSV-G envelope (right y axis, dashed line) following one or two doses of LacZ. Low level of circulating antibodies to the LV envelope detected in individual mice was irrespective of LacZ doses and LacZ cell counts.

8.6 LACZ COUNTS AND LACZ ANTIBODIES



Appendix 8.6-1 Individual LacZ Counts vs anti-LacZ

Number of transduced LacZ cells detected at 1 year (left y axis, solid line) against sera antibodies to β-galactosidase protein (anti-LacZ) (right y axis, dashed line) following one or two doses of LacZ. Low level of circulating antibodies to the LacZ protein detected in individual mice that displayed high numbers of LacZ transduced cells. Those mice that had no LacZ transduction had higher levels of antibodies to the LacZ protein.

CHAPTER 9

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