INVESTIGATING A COMBINED CELL AND GENE THERAPY FOR CYSTIC FIBROSIS: WORKING TOWARDS A PROOF OF PRINCIPLE

AFRODITI AVGERINOU

Great Ormond Street Institute of Child Health
University College London

A thesis submitted for the degree of Doctor of Philosophy

June 2020
DECLARATION

I, Afroditi Avgerinou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
ACKNOWLEDGEMENTS

I would initially like to thank Dr. Adam Giangreco, who was the person who developed the idea of this project and selected me to work on it, albeit without him.

My deepest gratitude goes to Dr. Paola Bonfanti, my primary supervisor, who accepted me in her lab and guided me through to the end of my PhD. Her input in the epithelial biology and tissue engineering parts of my project were invaluable. Her feedback on my work and her expectations challenged me, but have definitely contributed to my development as a researcher and as a person.

I would like to thank Professor Stephen Hart, my secondary supervisor, for the continuous support and encouragement. He contributed greatly to the gene editing and molecular aspect of this work, by generating fascinating ideas and suggestions.

Special thanks goes to Professor Robin McAnulty for helping me with the in vivo experiments of my PhD. He was especially supportive and understanding while he was teaching me new techniques and it was a shame there was not enough time to investigate in vivo cell engraftment further.

I would also like to thank my tutor, Dr. Claire Thorne for being there when I needed her, for listening to my concerns and for guaranteeing that my PhD progresses evenly.

I am very grateful to Professor Chris O’Callaghan, for providing me with CF patient cells from his biobank and to Asllan Gjinovci, for providing me with the rat tracheas for decellularisation and for his valuable contribution to our weekly lab meetings.

Further, I would like to thank Dale Moulding, Ayad Eddaoudi, Dani Lee, John Counsell, Sahira Khalaf, the histopathology STP in the Francis Crick Institute and the team at the Animal House in the Institute of Child Health, for their help and contribution to various aspects of my project. I would like to express my gratitude to Ruhina who assisted me in the animal house numerous times and Dave who did not give up trying to make the human cells stain work, only to discover no engrafted cells in my lung sections. Nevertheless, his devotion to science is truly admirable.

I am sincerely grateful to Ellie, my lab-buddy, partner in crime and great friend for all the times we spent together, not discussing science. Also to Sara, who has been a core member of the lab,
sharing advice, support and making things happen. Lastly, to Constance, who made the lab a fun place with her joy and playful nature.

I would like to acknowledge the rest of the members of the Bonfanti lab during the period of my project, Luca, Marco, Roberta and Mattias. Working alongside you has been a pleasure!

I am really grateful to Amy, for the most valuable troubleshooting discussions during the full course of our parallel PhDs. Also to Max, Martin, Ileana and Ahmad for all the bits of help and advice.

Thank you to the rest of the members of our SRC, Karen, Patrick and Debbie for contributing to insightful scientific discussions in our meetings and for all the fun during the conferences.

I would like to thank all past and present members of the Stephen Hart and Paolo de Coppi groups, for their contributions to various lab meetings and for some great times during parties and dinners.

Many thanks to Marisa and Emma, my MSc students, who helped with a number of experiments of this work and with the development of my supervising skills.

I am grateful to my parents, who through the years have contributed to the development of my confidence and never doubted that I was going to succeed in anything I tried. Equally grateful I am to my two grandmothers, who are 94 and 98 as I am writing these words. Together with my parents, they helped raise me, and I miss them as much as they miss me. Surprisingly, even though they know I am not a medical doctor they trust me more than they trust their children.

My deepest thank you goes to Toby, who is the reason I chose to stay in London after my MSc internship and has been the most valuable support during my PhD. Together, we have solved many molecular issues and have trumped over demons, dragons and domestic disorderliness.

Last but not least, I would like to thank the CF Trust for funding my PhD generously and giving me the opportunity to work on a project that I was very passionate about. My hope is that I get the chance to work in the CF field again, as a researcher or otherwise, and to be able to contribute further in the efforts to find a cure for cystic fibrosis.
ABSTRACT

Cystic fibrosis (CF) is the most common heritable disease among people with Northern European ancestry. CF is a life-limiting condition affecting multiple systems, however its morbidity and mortality are associated with progressive lung disease followed by respiratory failure. Currently, there is no available cure for CF, as most drugs work only for patients with specific mutations and show limited efficacy. We believe that a combined cell and gene therapy approach (ex vivo gene therapy) where the patient’s own airway cells are corrected by CFTR gene editing in vitro and subsequently transplanted to the lungs, could provide a cure for all patients with CF.

Here, we show that we can successfully expand and differentiate primary human airway epithelial cells while maintaining physiological marker expression, electrical responses and CFTR expression.

We optimised nucleofections and CRISPR/Cas9 RNP mediated double strand breaks in order to correct the ΔF508 CFTR mutation in patient cells. Correction was achieved through the homology directed repair (HDR) pathway with the help of a donor repair plasmid with a puromycin selection cassette. A second correction approach which included scarless removal of the selection cassette was not successful.

We also created an isogenic CFTR knockout in Normal Human Bronchial Epithelial (NHBE) cells, which, after single cell cloning, maintains the ability to form a monolayer and differentiate towards different cell types such as ciliated and mucus cells.

Furthermore, we have optimised a protocol for full decellularisation of rat tracheas via a system, which includes perfusion of solutions through the trachea lumen with a pump. CF primary airway epithelial cells engraft in these acellular scaffolds and form a cell layer positive for airway epithelial cell markers.

Finally, we have successfully created injury in mouse lungs by intratracheal instillation of polidocanol, which will promote engraftment of delivered airway epithelial cells among the native epithelium.
IMPACT STATEMENT

Cystic fibrosis (CF) is the most common heritable disease among people with Northern European ancestry and affects an estimated 70,000 people worldwide. CF is a life-limiting condition involving multiple systems; however, its morbidity and mortality are associated with progressive lung disease followed by respiratory failure. The life expectancy of people with CF is under 40 years and the financial burden for families and the healthcare systems is very high.

With this study, we worked towards a combined cell and gene therapy (ex vivo gene therapy) as a possible cure for CF. In the future, this type of therapy has the potential to offer a one-off, universal cure for every CF patient, irrespective of the genetic mutation they are carrying. This would be beneficial for both patients themselves and the healthcare payers.

More specifically, we have achieved progress in the expansion and maintenance of primary airway epithelial cells, a cell type suitable for ex vivo gene therapy. We have optimised transfection and successfully corrected the CF mutation in primary airway epithelial cells from CF patients with the CRISPR/Cas9 tool. We have also created an isogenic CFTR knockout, which can be used to answer questions relevant to cell therapy as well as regarding the fundamental differences between CF and healthy cells. We demonstrated that single cell cloning is possible for primary airway epithelial cells and that, after expansion, single cells maintain the ability to form a monolayer and differentiate towards different cell types. This technique will be useful for the potential identification of a long-lived stem cell progenitor population of the airway epithelium. Lastly, we have created a fully decellularised rat trachea scaffold on which primary airway epithelial cells can engraft, as a precursor to in vivo cell transplantation. This scaffold can additionally be used for tissue engineering and regenerative medicine studies.

This work has been presented in many national and international conferences, generating interest from both academia and industry. It has also been presented in nonprofessional language for the engagement of the CF patient community. We believe that this work will have impact in the wider CF, gene editing and gene therapy research fields.
TABLE OF CONTENTS

DECLARATION .................................................................................................................. 2
ACKNOWLEDGEMENTS ...................................................................................................... 3
ABSTRACT .......................................................................................................................... 5
IMPACT STATEMENT .......................................................................................................... 6
TABLE OF CONTENTS .......................................................................................................... 7
LIST OF FIGURES ................................................................................................................... 12
LIST OF TABLES ................................................................................................................... 16
ABBREVIATIONS .................................................................................................................. 17

CHAPTER 1. INTRODUCTION ................................................................................................. 21

1.1. Cystic fibrosis .............................................................................................................. 22
1.2. History of cystic fibrosis ............................................................................................. 22
1.3. CFTR function and dysfunction ................................................................................. 23
1.4. CFTR mutations ......................................................................................................... 24
1.5. Epidemiology and diagnosis of CF ............................................................................. 25
1.6. Lung disease in CF ...................................................................................................... 27
1.7. Disease management ................................................................................................. 28
1.8. Models for CF research .............................................................................................. 29
  1.8.1. Cell lines .............................................................................................................. 29
  1.8.2. Primary cells ........................................................................................................ 29
  1.8.3. Mice ........................................................................................................................ 30
  1.8.4. Other in vivo models ............................................................................................ 30
1.9. Techniques to assess CFTR ......................................................................................... 31
  1.9.1. ALI cultures ......................................................................................................... 31
  1.9.2. Ussing chambers ............................................................................................... 31
  1.9.3. Western blots ........................................................................................................ 32
1.10. Current therapies: Ion channel modulators (potentiators and correctors) .............. 32
1.11. New therapeutic approaches .................................................................................... 34
1.12. Gene therapy ............................................................................................................ 35
  1.12.1. In vivo gene therapy for CF ................................................................................ 35
2.20. Assessment of puromycin/TK selection cassette excision.................................................. 77
2.21. Lentivirus production ........................................................................................................... 78
2.22. Lentivirus titration and optimisation .................................................................................... 80
2.23. Lentivirus transduction ......................................................................................................... 80
2.24. RNA extraction .................................................................................................................... 80
2.25. qRT-PCR ................................................................................................................................ 80
2.26. Protein extraction .................................................................................................................. 81
2.27. Western Blots ....................................................................................................................... 81
2.28. Decellularisation of rat tracheas .......................................................................................... 82
2.29. Airway epithelial cell seeding on decellularised rat trachea scaffolds ................................. 83
2.30. In vivo experiments in small animal models ........................................................................ 84

CHAPTER 3. CULTURE AND CHARACTERISATION OF PRIMARY HUMAN AIRWAY EPITHELIAL
CELLS ................................................................................................................................................. 86

3.1. Introduction ............................................................................................................................ 87
3.2. Aims .......................................................................................................................................... 89
3.3. Isolation, culture and characterisation of Human Fetal Tracheal Cells ................................. 90
3.4. Liposome transfection of Human Fetal Tracheal Cells .......................................................... 95
3.5. Culture and characterisation of Adult Airway Epithelial Cells ............................................ 98
3.6. Detection of CFTR protein in NHBEs and ΔF508 NECs by Western Blot ......................... 110
3.7. Nucleofection of Adult Airway Epithelial Cells .................................................................. 111
3.8. Discussion ............................................................................................................................... 116

CHAPTER 4. CRISPR/CAS9 CORRECTION OF CFTR IN ΔF508 NASAL EPITHELIAL CELLS .......... 120

4.1. Introduction ............................................................................................................................ 121
4.2. Aims .......................................................................................................................................... 122
4.3. Construction of two repair templates for CRISPR/Cas9 correction of the ΔF508 mutation
via the HDR pathway .................................................................................................................. 123
4.4. Optimisation of CRISPR/Cas9 mediated Double Strand Breaks in ΔF508 NECs with guide
7 ...................................................................................................................................................... 128
4.5. Puromycin selection optimisation in ΔF508 NECs ................................................................. 138
4.6. Creation of puromycin-resistant 3T3-J2 cells ...................................................................... 141
4.7. CRISPR/Cas9 correction of ΔF508 NECs via the HDR pathway with sgRNA, Cas9 and
repair plasmid ................................................................................................................................. 144
4.8. Discussion ............................................................................................................................... 159
CHAPTER 5. CRISPR/CAS9 KNOCKOUT OF CFTR IN NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS

5.1. Introduction .................................................................................................................. 166
5.2. Aims ............................................................................................................................... 167
5.3. Optimisation of CRISPR/Cas9 mediated Double Strand Breaks in NHBE cells with guide 1 .......................................................................................................................... 168
5.4. Characterisation of NHBE single cell clone 2.2 and single cell clone 2.20.............. 177
5.5. Discussion ..................................................................................................................... 183

CHAPTER 6. SEEDING OF ΔF508 NASAL EPITHELIAL CELLS ON DECELLULARISED RAT TRACHEA SCAFFOLDS ........................................................................................................................................... 186

6.1. Introduction .................................................................................................................. 187
6.2. Aims ............................................................................................................................... 188
6.3. Optimisation of a protocol for full decellularisation of Rat Tracheas ....................... 189
6.4. Seeding of ΔF508 Nasal Epithelial Cells (NECs) on decellularised rat trachea scaffolds. 195
6.5. Discussion ..................................................................................................................... 200

CHAPTER 7. IN VIVO DELIVERY OF ΔF508 NASAL EPITHELIAL CELLS IN MOUSE AIRWAYS ....202

7.1. Introduction .................................................................................................................. 203
7.2. Aims ............................................................................................................................... 204
7.3. Optimisation of upper airway injury in mice ............................................................... 205
7.4. Labelling of ΔF508 NECs with mCherry by lentiviral vector transduction .............. 206
7.5. Characterisation of mCherry+ ΔF508 NECs ............................................................... 211
7.6. In vivo transplantation of mCherry+ ΔF508 NECs in mouse upper airways .......... 213
7.7. Discussion ..................................................................................................................... 217

CHAPTER 8. DISCUSSION .................................................................................................... 219

8.1. Main findings ............................................................................................................... 220
8.2. Achievements, limitations, challenges and future work ........................................ 220
  8.2.1. Cell type, expansion and differentiation ............................................................. 221
  8.2.2. Correction .............................................................................................................. 222
  8.2.3. Knockouts and single cell cloning ...................................................................... 224
  8.2.4. Engraftment ......................................................................................................... 225
  8.2.5. General limitations ............................................................................................. 226
8.3. From bench to bedside ............................................................................................... 226
8.4. Conclusion ................................................................................................................................. 226
REFERENCES ..................................................................................................................................... 228
APPENDIX ........................................................................................................................................ 251
LIST OF FIGURES

Figure 1.1. Effects of Cystic Fibrosis Transmembrane conductance Regulator (CFTR) dysfunction ................................................................. 24
Figure 1.2. CFTR mutations can be divided into six classes. ................................................................. 25
Figure 1.3. Schematic of Cas9 nuclease DNA targeting ........................................................................ 38
Figure 1.4. Gene editing is promoted by DSB repair ............................................................................. 38
Figure 1.5. Classifications of epithelia ................................................................................................. 43
Figure 1.6. The respiratory system .................................................................................................. 43
Figure 1.7. Layers of the respiratory system ..................................................................................... 44
Figure 1.8. Lineage of airway epithelial stem cells and their progeny. ................................................... 46
Figure 1.9. Cell therapy for cystic fibrosis .......................................................................................... 50
Figure 2.1. Air Liquid Interface (ALI) Culture Airway Epithelial Cells .................................................. 56
Figure 2.2. pX330-U6-Chimeric_BB-CBh-hSpCas9 vector ................................................................ 62
Figure 2.3. SGK-007 Multivector from Transposagen .......................................................................... 67
Figure 2.4. Schematic of plasmid assembly design for the correction of the ΔF508 mutation in ΔF508 Nasal Epithelial Cells ........................................................................................................ 69
Figure 2.5. pGEM-T easy vector ........................................................................................................... 72
Figure 2.6. pUC19 vector .................................................................................................................... 73
Figure 2.7. Position of screening primers for successful HDR with the puromycin/TK selection cassette ................................................................................................................................. 76
Figure 2.8. pSicoR-EF1a-mCh-Puro vector .......................................................................................... 79
Figure 2.9. Preparation of decellularised rat trachea scaffolds for cell seeding. .................................... 84
Figure 3.1. Human trachea isolated from a 20 weeks post conception (wpc) fetus. ............................. 91
Figure 3.2. Human trachea isolated from a 13 weeks post conception (wpc) fetus. ............................. 91
Figure 3.3. Human Fetal Tracheal Epithelial cells expanded in co-culture with irradiated feeder layer (3T3) ................................................................................................................................. 92
Figure 3.4. Growth curve of HuFeTRC01 ............................................................................................ 93
Figure 3.5. Immunofluorescence of HuFeTRC01 expanded in vitro. .................................................. 94
Figure 3.6. Human Fetal Tracheal Epithelial cells are expanded in co-culture with irradiated feeder layer (3T3) ................................................................................................................................. 95
Figure 3.7. Transfection of HuFeTRC with pEGFP-N1 using Lipofectamine 3000 .............................. 98
Figure 3.8. Expansion of Normal Human Bronchial Epithelial Cells (NHBEs) .................................................. 99
Figure 3.9. Growth curve of NHBE .................................................................................................................. 100
Figure 3.10. ΔF508 Nasal Epithelial Cells (ΔF508 NECs) expanded in co-culture with irradiated feeder layer (3T3) ................................................................................................................................. 101
Figure 3.11. Comparison of two independent ΔF508 NEC growth curves. ...................................................... 102
Figure 3.12. Immunofluorescence of NHBEs passage 4, expanded in vitro on irradiated feeder layer without Y-27632 ................................................................................................................................................ 104
Figure 3.13. Immunofluorescence of ΔF508 NEC passage 10, expanded in vitro on irradiated feeder layer with Y-27632 ........................................................................................................................................... 106
Figure 3.14. 3D reconstruction of confocal images of NHBEs and ΔF508 NECs differentiated in vitro in air liquid interface (ALI) cultures with BEBM/DMEM + Retinoic Acid+ BulletKit. .......... 107
Figure 3.15. 3D reconstruction of confocal images of NHBEs and ΔF508 NECs differentiated in vitro in air liquid interface (ALI) cultures with Pneumacult media. ......................................................... 108
Figure 3.16. Effects of epithelial sodium channel (ENaC) inhibitor (amiloride), CFTR activator (forskolin + IBMX) and CFTR inhibitor 172 in stimulated short circuit current (Isc) of NHBE and ΔF508 NEC cultures ................................................................................................................................... 109
Figure 3.17. Western blots of NHBEs and ΔF508 NECs.................................................................................. 110
Figure 3.18. Phase contrast and fluorescence microscopy images of GFP+ NHBE nucleofected with 5μg pmaxGFP .............................................................................................................................................. 111
Figure 3.19. Nucleofection of NHBEs with pmaxGFP. .................................................................................. 113
Figure 3.20. Nucleofection of ΔF508 NECs with GFP-N1. ........................................................................... 115
Figure 4.1. ΔF508 NECs Sanger sequencing result of CFTR exon 10. ......................................................... 125
Figure 4.2. Schematic of CRISPR/Cas9 approach for the correction of the ΔF508 mutation in ΔF508 NECs ........................................................................................................................................... 125
Figure 4.3. Agarose electrophoresis of fragments used for Gibson Assembly. ................................. 126
Figure 4.4. Agarose electrophoresis of repair plasmids digested with ECORI. ................................. 127
Figure 4.5. Agarose electrophoresis of repair plasmid digested with ECORI. ................................. 127
Figure 4.6. Nucleofection of ΔF508 NECs with px330 and GFP-N1. ......................................................... 130
Figure 4.7. Indels created by nucleofection of ΔF508 NECs with px330 and GFP-N1. .............................. 131
Figure 4.8. Growth curve of GFP+ ΔF508 NECs nucleofected with px330 and GFP-N1 versus standard non-nucleofected culture ......................................................................................................................... 132
Figure 4.9. Indels created by nucleofection of ΔF508 NECs with sgRNA (Sigma) and Cas9 protein (NEB). ............................................................... 135
Figure 4.10. Growth curve of ΔF508 NECs nucleofected with sgRNA (Sigma) and Cas9 protein (NEB), versus standard non-nucleofected culture. ............................................................... 135
Figure 4.11. Indels created by nucleofection of ΔF508 NECs with sgRNA (Synthe~ go) and Cas9 protein (Thermo). ........................................................................................................... 138
Figure 4.12. Growth curve of ΔF508 NECs nucleofected with sgRNA (Synthego) and Cas9 protein (Thermo), versus standard non-nucleofected culture............................................................... 138
Figure 4.13. Puromycin selection of ΔF508 NECs. ............................................................................................................................... 141
Figure 4.14. mCherry/puromycin lentivirus titration in HEK293. ................................................................. 143
Figure 4.15. mCherry/puromycin lentivirus optimisation in 3T3-J2 cells. ................................................................. 144
Figure 4.16. Puromycin selection of ΔF508 NECs nucleofected with sgRNA (Synthego), Cas9 protein (Thermo) and repair plasmid without sgRNA recognition sites. ΔF508 NECs are treated
with 1 μg/mL puromycin. ................................................................................................................................. 147
Figure 4.17. Genotyping of puromycin-resistant ΔF508 NECs nucleofected with sgRNA (Synthego), Cas9 protein (Thermo) and repair plasmid without sgRNA recognition sites. ...... 149
Figure 4.18. ICE analysis of ΔF508 NECs after piggybac excision................................................................. 150
Figure 4.19. ICE analysis of ΔF508 NECs after improved HDR and piggybac excision. ...................... 153
Figure 4.20. Puromycin selection of ΔF508 NECs nucleofected with sgRNA (Synthego), Cas9
protein (Thermo) and Donor-G repair plasmid.................................................................................................... 155
Figure 4.21. Genotyping of puromycin resistant ΔF508 NECs nucleofected with sgRNA
(Synthego), Cas9 protein (Thermo) and Donor-G repair plasmid................................................................. 157
Figure 4.22. Western blot of ΔF508 NECs corrected with Donor-G. ............................................................. 158
Figure 5.1. Indels created by nucleofection of NHBE cells with sgRNA (Synthego) and Cas9 protein
(Thermo). ......................................................................................................................................................... 168
Figure 5.2. Percentage of sequences presenting with indels in NHBE cells nucleofected with 9:1
sgRNA/Cas9 ratio, after the first, second and third passage. ................................................................. 169
Figure 5.3. Indels created by 3 repeated nucleofections of NHBE cells with a 9:1 ratio of sgRNA
(Synthego) and Cas9 protein (Thermo). ............................................................................................................ 171
Figure 5.4. Phase contrast images of selected NHBE single cell clones during passage 1........ 172
Figure 5.5. TIDE plots of selected NHBE single cell clones of cloning experiment 1. ............ 175
Figure 5.6. NHBE single cell clones expanded in co-culture with irradiated feeder layer (3T3) and Y-27632. .......................................................... 177
Figure 5.7. Immunofluorescence of NHBE single cell clone 2.2, expanded in vitro on irradiated feeder layer with Y-27632. .......................................................... 178
Figure 5.8. Immunofluorescence of NHBE single cell clone 2.20, expanded in vitro on irradiated feeder layer with Y-27632. .......................................................... 178
Figure 5.9. 3D reconstruction of confocal images of NHBE single cell clones 2.2 and 2.20 differentiated in vitro in air liquid interface (ALI) cultures. ........................................ 181
Figure 5.10. Rt-qpcr of NHBE single cell clones 2.20 and 2.2. ........................................ 181
Figure 5.11. Western blot of NHBE single cell clone 2.2 ........................................ 182
Figure 6.1. Hematoxylin and Eosin images of decellularised rat tracheas. .................. 191
Figure 6.2. Protocols 7 and 8 for the decellularisation of rat tracheas. ....................... 193
Figure 6.3. Histological comparison of decellularised rat trachea scaffolds (protocol 8) with fresh rat tracheas. .......................................................... 195
Figure 6.4. Hematoxylin and Eosin stain of decellularised rat trachea scaffolds seeded with ΔF508 NECs in submerged conditions. .................................................. 196
Figure 6.5. Immunofluorescence of decellularised trachea scaffold seeded with ΔF508 NECs for 3 days in submerged conditions. .................................................. 197
Figure 6.6. Immunofluorescence of decellularised trachea scaffold seeded with ΔF508 NECs for 6 days in submerged conditions. .................................................. 199
Figure 6.7. Hematoxylin and Eosin stain of decellularised rat trachea scaffolds seeded with ΔF508 NECs in exposure conditions .................................................. 199
Figure 7.1. Airway injury with 2% w/v polidocanol in mice. ........................................ 206
Figure 7.2. mCherry/puromycin lentivirus optimisation in ΔF508 NECs. ....................... 209
Figure 7.3. mCherry/puromycin lentivirus transduction of ΔF508 NECs. .................... 211
Figure 7.4. Immunofluorescence of mCherry+ ΔF508 NECs, expanded in vitro on irradiated feeder layer with Y-27632. .......................................................... 212
Figure 7.5. 3D reconstruction of confocal images of mCherry+ ΔF508 NECs, differentiated in vitro in air liquid interface (ALI) cultures. .................................................. 213
Figure 7.6. Optimisation of antibodies and staining protocols for the detection of mCherry+ ΔF508 NECs in mouse lungs. .......................................................... 215
Figure 7.7. In vivo transplantation of mCherry+ ΔF508 NECs in mouse airways. .......... 216
LIST OF TABLES

Table 1.1. Clinical manifestations of cystic fibrosis. ................................................................. 27
Table 2.1. Primary and secondary antibody information. ....................................................... 58
Table 2.2. sgRNA information. .................................................................................................. 63
Table 2.3. Primers and PCR conditions used for the assessment of Double Strand Breaks. ...... 64
Table 2.4. PAM sites and respective sgRNA sequences 130 bp upstream and 130 bp downstream of the ΔF508 mutation. ................................................................................. 65
Table 2.5. Primers used for PCR amplification and Sanger sequencing of a region 1000 bp upstream and 1000 bp downstream of the ΔF508 mutation....................................................... 66
Table 2.6. Sequence of the Homology Arms used for the construction of the repair template. 68
Table 2.7. Primers and PCR conditions used for the addition of overlapping fragments required by Gibson Assembly. ............................................................................................................ 70
Table 2.8. Primers used for confirmation of construct sequence by Sanger sequencing. ........ 74
Table 2.9. Primers and PCR conditions used for the creation of the fragment required for the construct with the sgRNA sites. ............................................................................................................. 75
Table 2.10. Primers used for in-out PCR’s for the confirmation of the incorporation of the puromycin/TK cassette and the correction of the ΔF508 mutation......................................................... 76
Table 2.11. Primers used for in-out PCR’s for the confirmation of the incorporation of the CFTR-Donor G cassette and the correction of the ΔF508 mutation. ....................................................... 77
Table 2.12. Rat trachea decellularisation protocols 1-5. .......................................................... 82
Table 2.13. Rat trachea decellularisation protocols 6-8. .......................................................... 83
Table 3.1. Details of human fetal tracheas processed............................................................... 90
Table 3.2. Transepithelial resistance of cell monolayers on Ussing chambers, calculated using Ohm’s law from stimulated short circuit (Isc) measurements, before treatment. ......................... 109
Table 5.1. NHBE single cell clones isolated by two cloning experiments. ............................... 175
ABBREVIATIONS

3T3 – mouse embryonic fibroblasts
AAV – Adeno-Associated Virus
AC-TUB – Acetylated Tubulin
ADA-SCID – Adenosine Deaminase Deficiency
ALI – Air Liquid Interface
ASL – Airway Surface Liquid
ATI – Type I Pneumonocytes
ATII – Type II Pneumonocytes
BEGM – Bronchial Epithelial Cell Growth Medium
BMI – Body Mass Index
BMI-1 – B Lymphoma MO-MLV Insertion Region 1 Homolog (oncogene)
cAMP – cyclic Adenosine Monophosphate
(sp)Cas9 – (Streptococcus pyogenes) CRISPR associated protein 9
cDNA – complementary DNA
CF – Cystic Fibrosis
cFAD – Keratinocyte Culture Medium
CFTR – Cystic Fibrosis Transmembrane conductance Regulator
CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CK – Cytokeratin
COPD – Chronic Obstructive Pulmonary Disease
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
crRNA – CRISPR RNA
DAPI – 4′,6-diamidino-2-phenylindole
DET – Detergent Enzymatic Treatment
DDR – DNA Damage Response
DMEM – Dulbecco's Modified Eagle Medium
DNA – Deoxyribonucleic Acid
DNase-I – Deoxyribonuclease I
DSB – Double Strand Break
E-CAD – E-Cadherin
ECM – Extracellular Matrix
EDTA – Ethylenediaminetetraacetic Acid
EF1a – Elongation factor 1-alpha
EGF – Epidermal Growth Factor
EMT – Epithelial-Mesenchymal Transition
ENac – Epithelial Sodium Channel
EpCAM – Epithelial Cell Adhesion Molecule
ESCs – Embryonic Stem Cells
FACS – Fluorescence Activated Cell Sorting
FBS – Fetal Bovine Serum
FEV₁ – Forced Expiratory Volume in 1 second
FOXI1 – Forkhead Box I1
GFP – Green Fluorescent Protein
GMP – Good Manufacturing Practice
H&E – Haematoxylin and Eosin
HBECs – Human Bronchial Epithelial Cells
HBSS – Hanks Balanced Salt Solution
HCO₃⁻ – Bicarbonate
HDBR – Human Developmental Biology Resource
HDR – Homology Directed Repair
hEGF – human Epidermal Growth Factor
HEK293 – Human Embryonic Kidney 293 (cells)
HIV – Human Immunodeficiency Virus
HITI – Homology-Independent Targeted Integration
hPIV – Human Parainfluenza Virus
HuFeTRC – Human Fetal Tracheal Epithelial Cells
IBMX – 3-isobutyl-1-methylxanthine
ICE – Inference of CRISPR Edits
indel – insertion- deletion
iPSCs – Induced Pluripotent Stem Cells
IPTG – Isopropyl β-D-1-thiogalactopyranoside
IRT – Immunoreactive Trypsinogen
$I_{sc}$ – short circuit current
ITR – Inverted Terminal Repeat
LCA – Leber Congenital Amaurosis
LHA – Left Homology Arm
LPLD – Lipoprotein Lipase Deficiency
mRNA – messenger RNA
MOI – Multiplicity Of Infection
MUC5AC – Mucin SAC
NEB – New England Biolabs
NHBE – Normal Human Bronchial Epithelial (cells)
NHEJ – Non-homologous End Joining
NMD – Nonsense Mediated Decay
OTC – Ornithine Transcarbamoylase
p53 – Tumor protein 53
p63 – Tumor protein 63
PACB – McGill University Primary Airway Cell Biobank
PAM – Protospacer Adjacent Motif
PBS – Phosphate Buffer Saline
PCR – Polymerase Chain Reaction
PD – Population Doubling
PEI – Polyethyleneimine
PET – Polyethylene Terephthalate
PFA – Paraformaldehyde
Puro – Puromycin
RHA – Right Homology Arm
RNA – Ribonucleic Acid
RNP – Ribonucleoprotein
RPMI – Roswell Park Memorial Institute (RPMI) 1640 Medium
rt-qPCR – Quantitative reverse transcription PCR
SCID – X linked Combined Immune Deficiency Disorder
SDC – Sodium Deoxycholate
SDS – Sodium Dodecyl Sulfate
SeV- Sendai Virus
sgRNA – single guide RNA
SIV – Simian Immunodeficiency Virus
ssODN – single-stranded oligo DNA nucleotide
T7E1 – T7 Endonuclease I
TALENs – Transcription activator-like effector nucleases
TBS – Tris-buffered saline
TEER – Transepithelial Electrical Resistance
Thermo – Thermo Fisher Scientific
TIDE – Tracking Indels by Decomposition
TK – Thymidine Kinase
tracrRNA – trans-activating crRNA
TU – Transducing Units
V-ATPase – Vacuolar H+-ATPase
wpc – weeks post conception
WT – Wild Type
X-Gal – 5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside
Y-27632 – Rho-associated protein kinase (ROCK) inhibitor
ZNFs – Zinc finger proteins
ZO-1 – Zonula Occludens-1
ΔF508 NECs – Nasal Epithelial Cells with the ΔF508 CFTR mutation
CHAPTER 1. INTRODUCTION
1.1. Cystic fibrosis

Cystic fibrosis (CF) is the most common Mendelian disorder with autosomal recessive inheritance in people with Northern European (Caucasian) ancestry. Its prevalence varies among countries from 1 in 2,000 to 1 in 100,000. The incidence in the UK is 1 in 2,415 live births and there are more than 10,000 people in the country with the disorder. CF is a life-limiting condition which affects multiple systems including the pancreas, the liver, the intestine, the sinuses, the bones and the male reproductive tract, however the morbidity and mortality of the disease are associated with progressive lung disease followed by respiratory failure (Antoniou and Elston, 2016). Median age of survival has been increasing during the last 60 years due to early detection and improvements in management and currently exceeds 40 years of age in developed countries (Elborn, 2016).

CF is caused by different mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene, which is located on chromosome 7 in the region 7q31.2. The CFTR protein codes for a regulated chloride channel which is localised on the surface of epithelial cells. The channel is mainly involved in the transport of chloride but also regulates sodium, bicarbonate (HCO$_3^-$) and water movement (Antoniou and Elston, 2016).

1.2. History of cystic fibrosis

Cystic fibrosis was first identified as a separate disease by Dorothy Andersen in 1938, when she described it as cystic fibrosis of the pancreas due to the mucus plugging of the glandular ducts. The disease characteristics included protein and fat malabsorption, steatorrhea, growth failure and lung infections. During a heatwave in New York in 1948, cystic fibrosis was associated with loss of salt and following, it was discovered that a fivefold excess of sodium and chloride was present in the sweat of CF patients. A sweat chloride test was subsequently used as a diagnostic test and in 1983 chloride transport was identified as the basic defect in CF. Finally, in 1989, the CF gene was discovered in cells derived from sweat ducts. Nowadays, it is known that CFTR is expressed in many epithelial cells, including these of the sweat ducts, the airways and the pancreas where it contributes to the clinical manifestations of the disease in these organs in CF patients (Davis, 2006).
1.3. CFTR function and dysfunction

In CF, the defect of the CFTR channel affects mainly the transport of chloride and bicarbonate. CFTR interaction with the epithelial sodium channel (ENac) and other ion channels, as well as interactions of CFTR with the inflammasome (cellular pathways related to inflammation) are considered important to the pathophysiology of CF (Stoltz et al., 2015). Recent studies show that in almost a third of children with CF, mucus obstruction, bronchiectasis, inflammation and recurrent infection episodes are present by the age of 3 (Sly et al., 2013; Ramsey et al., 2014; Rosenow et al., 2015). It is hypothesised that this clinical phenotype is a result of the decreased hydration of the airway surface liquid (ASL), the thin layer of fluid that lines the respiratory epithelium. Defective CFTR function reduces chloride secretion and decreases sodium absorption, leading to depletion of the ASL and in turn to impaired mucociliary action and mucus clearance reduction. Reduced mucus clearance is the cause for bacterial colonisation of the airways, which leads to recurrent infections, chronic inflammation and irreversible damage of the airways (Kumar et al., 2014). However, the decreased ASL hydration hypothesis has been recently challenged by findings in some animals, like pigs and ferrets with CF, where excessive sodium reabsorption was not observed (Stoltz et al., 2015). These findings suggest that simple hydration might not be important in the early impairment of mucociliary clearance.

Concerning the conduction of bicarbonate from CFTR, a defective bicarbonate transport results in a pH change of the ASL (Quinton, 2010). Experiments in the cystic fibrosis pig show that a change in the ASL pH can reduce the function of antimicrobial peptides and therefore result in impaired innate immunity (Hoegger et al., 2014; Keiser et al., 2015). The presence of bicarbonate is also important for the normal function of mucus, since reduced anion concentrations have been shown to dysregulate mucus tethering and detachment. CFTR defect, therefore, can have multiple consequences, including ASL hydration, mucociliary clearance, mucus function and tethering, impaired immunity as well as create predisposition for cellular inflammation (Hoegger et al., 2014) (Figure 1.1). These processes may contribute differently to the disease throughout its progression, may change with age and could possibly give insight to why drugs that target one of these effects, like mucolytics or antimicrobial agents, have modest effects (Graeber et al., 2013).
Figure 1.1. Effects of Cystic Fibrosis Transmembrane conductance Regulator (CFTR) dysfunction. The figure shows which functions are dependent on CFTR activity, how these are affected in cystic fibrosis and their contributions to the disease phenotype (Elborn, 2016).

### 1.4. CFTR mutations

More than 2,000 sequence alterations have been identified in the *CFTR* gene, however, as of October 2019, 346 are associated with the disease (https://cftr2.org/mutations_history). Most of the mutations are missense but nonsense mutations, splicing mutations, frameshift mutations and inframe insertions and deletions have also been reported (Bell et al., 2015). *CFTR* mutations are divided into six classes, which correspond to their effect on the function of the protein. Mutations of the classes I, II, and III result in no residual CFTR function and a severe disease phenotype in the patients, while class IV, V and VI mutations allow for some residual CFTR
function and in turn are associated with milder disease phenotypes (Figure 1.2). The most common mutation is ΔF508 (Phe508del) and is found in approximately 70% of the Caucasian population (Antoniou and Elston, 2016). The ΔF508 mutation is a missense mutation caused by the deletion of the phenylalanine amino acid at the position 508 of the protein. The amino acid deletion results in protein misfolding and subsequent destruction by the proteasome. Consequently, the protein does not get trafficked and the CFTR channel is absent from the membrane of the cells. ΔF508 is mainly a class II mutation, as only around 3% of the protein reaches the cell membrane. However, at the cell membrane the channel is not functional and therefore demonstrates properties of class III gating mutations and of class VI stability mutations.

<table>
<thead>
<tr>
<th>Normal</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR mRNA</td>
<td>CTR Protein</td>
<td>Defective CFTR channel</td>
<td>Defective CFTR mRNA</td>
<td>Decreased synthesis of CFTR</td>
<td>Decreased CFTR stability</td>
<td></td>
</tr>
<tr>
<td>No functional CTR protein</td>
<td>Absent functional CTR</td>
<td>Scarcely functional CTR</td>
<td>Decreased channel conductance</td>
<td>Scarcely functional CTR</td>
<td>Decreased CFTR stability</td>
<td></td>
</tr>
<tr>
<td>Type of mutations</td>
<td>Missense; frameshift; canonical splice</td>
<td>Missense; amino acid deletion</td>
<td>Missense; amino acid change</td>
<td>Missense; amino acid change</td>
<td>Missense; amino acid change</td>
<td></td>
</tr>
<tr>
<td>Specific mutation examples</td>
<td>G542X</td>
<td>Arg329X, Arg553X</td>
<td>G551D</td>
<td>Arg132P, Arg134V, 3845+1G→A</td>
<td>4275delTA, 4052delT</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1.2. CFTR mutations can be divided into six classes. Mutations of: class I result in no protein production, class II result in protein retention in the ER and degradation from the proteasome, class III impair channel opening, class IV cause reduced ion conduction, class V result in mRNA or protein reduction and class VI cause channel instability at the plasma membrane (Boyle and De Boeck, 2013).

1.5. Epidemiology and diagnosis of CF

During the 1950s, median survival of patients with cystic fibrosis was just a few months, with main causes of death the meconium ileus present at birth and malnutrition caused by pancreatic
malabsorption (Davis, 2015). Nowadays, treatment for meconium ileus where needed and nutritional repletion with pancreatic enzyme supplements coupled with the improvement of airway mucus clearance and controlling of the airway infections, has changed CF from being a childhood disease to now being also an adult condition, especially in developed countries (Elborn et al., 1991, Davis, 2015). The number of adults with cystic fibrosis is expected to continue to increase and in European countries to reach 70% by 2025 (Burgel et al., 2015). In the United States, cystic fibrosis survival improved at a rate of 1.8 per year from 2000 to 2010 and the projected median survival of a child born with the condition today is 56 years (MacKenzie et al., 2014).

Conversely, the diagnosis of cystic fibrosis is now prevalently done during childhood as a result of the universal newborn screening taking place in many countries (Castellani et al., 2009). Early detection and timely disease management has been proven to reduce the severity of the disease and the burden and cost of care. It is also important to prevent missed diagnoses (Sims et al., 2007). As a consequence, early diagnosis also contributes to increased survival as it can help children maintain good lung health into adulthood.

The different methods used for neonatal diagnosis usually involve measurement of immunoreactive trypsinogen (IRT), a marker of pancreatic injury consistent with CF, in blood taken from the baby (Castellani et al., 2009). In case of high concentrations of IRT, a sweat test and genetic testing will follow and will confirm the diagnosis in >95% of newborns with CF (Antoniou and Elston, 2016).

Despite newborn screening, individuals can present phenotypic characteristics associated with CF only later in life (Table 1.1). High sweat chloride and the identification of CFTR mutations in both gene alleles will confirm the diagnosis in the majority of these people. However, a normal or borderline sweat test could suggest need for further testing to evaluate CFTR dysfunction, such as nasal potential difference testing (Antoniou and Elston, 2016). Diagnosis of the disease later in life is usually associated with milder symptoms and a more positive prognosis, as many of these individuals carry mutations with residual CFTR function (Nick et al., 2010; McKone et al., 2015).
Table 1.1. Clinical manifestations of cystic fibrosis. The table shows Cystic Fibrosis characteristics in infants & children and adults (Antoniou and Elston, 2016).

<table>
<thead>
<tr>
<th>Infants &amp; children</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meconium ileus</td>
<td>Recurrent respiratory infections</td>
</tr>
<tr>
<td>Recurrent respiratory infections</td>
<td>Bronchiectasis</td>
</tr>
<tr>
<td>Failure to thrive or low body mass index</td>
<td>Atypical asthma</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>Sinus disease</td>
</tr>
<tr>
<td>Nasal polyps</td>
<td>Pancreatic insufficiency</td>
</tr>
<tr>
<td>Acute pancreatitis</td>
<td>Hepatobiliary disease</td>
</tr>
<tr>
<td></td>
<td>Pancreatitis</td>
</tr>
<tr>
<td></td>
<td>Malnutrition</td>
</tr>
<tr>
<td></td>
<td>Male infertility</td>
</tr>
</tbody>
</table>

1.6. Lung disease in CF

Inflammation is the main pathological symptom in the lung of CF patients. It is created by unsuccessful microorganism clearance, which leads to a pro-inflammatory microenvironment (Cohen and Prince, 2012). Bronchiectasis is present in a third of CF patients within the first few months of life and is further associated with raised neutrophil elastase. Raised neutrophil elastase in turn is disruptive for innate immunity, increases the production of mucus and by digesting the extracellular matrix of the lung, is the main driving force of injury (Sly et al., 2013; Collawn and Matalon, 2014; Mall and Schultz, 2014b).

Repeated respiratory tract infections with *Haemophilus influenzae* and *Staphylococcus aureus* lead to inflammatory damage as a response to the airway infection (Gilligan, 2014). With the progression of the disease and the development of bronchiectasis, CF patients become vulnerable to infections by Gram-negative bacteria which are only associated with human infections where either the host is immunocompromised or the integrity of the epithelium is compromised (Cohen and Prince, 2012; Parkins and Floto, 2015). The most prominent lung infection by Gram-negative bacteria in CF patients is caused by *Pseudomonas aeruginosa*.

The CF lung constitutes an inflammatory microenvironment (Cohen and Prince, 2012). Some studies suggest that CFTR mutations make epithelial cells more pro-inflammatory than healthy
cells, however the importance of this in the initiation of inflammation is not clear. Possibly, this characteristic of CF cells has a role in early life before inflammation becomes regular and driven mainly by microorganisms (Cantin et al., 2015).

1.7. Disease management

The most favourable outcomes in CF result from centrally coordinated care in specialised centres and treatment from a team of experts including clinicians, nurses, physiotherapists, dieticians and psychologists. The patients should be reviewed approximately every 3 months and their respiratory function (assessed by forced expiratory volume in 1 second - FEV\textsubscript{1}), weight and sputum microbiology should be monitored. Screening for CF complications including diabetes mellitus, liver and bone disease and allergic bronchopulmonary aspergillosis, should be conducted annually. Since transmission of infection among CF patients has been observed, cohort segregation is now recommended as a standard of care (Antoniou and Elston, 2016).

More specifically, the classic management of CF has included addressing the respiratory disease to prevent or delay chronic infections, maintain the lung function and prevent pulmonary exacerbations. For this reason, drugs routinely used in CF are antipseudomonal antibiotics, mucolytics and osmotic agents administered through nebulization as well as orally administered azithromycin. Very important in the management of the lung disease is also chest physiotherapy. Ultimately, when the progression of the disease leads to respiratory failure, oxygen therapy and non-invasive ventilation can help the improvement of symptoms. At that stage the patients can be considered for lung transplantation, however many will die on the waiting list for a transplant (Antoniou and Elston, 2016).

About 85% of CF patients suffer from pancreatic insufficiency characterised by steatorrhea, low BMI and deficiency for vitamins A, D, E and K. These patients require pancreatic enzyme replacement and vitamin supplements (Antoniou and Elston, 2016).

Lastly, complications of CF, including haemoptysis, pneumothorax, distal obstruction syndrome and cystic fibrosis related diabetes, should be treated when they occur as this will improve quality and duration of life in patients (Antoniou and Elston, 2016).
1.8. Models for CF research

Even though the general pathophysiology of CF is established, there is a great variation in the severity and progression between patients and despite decades of research, there are still many unanswered questions. As already mentioned, it is not clearly understood how from dysfunction of CFTR the condition progresses to chronic lung disease and whether this is caused by bacterial colonisation or from inherent pro-inflammatory properties of the CF epithelium. It is also not understood whether the lung disease in CF is caused by hyper-absorption of fluid by sodium channels or by CFTR dependent secretion deficiency (Scholte et al., 2006). Answer to these questions is necessary for improvement of CF treatment, making model systems invaluable for research.

1.8.1. Cell lines

The study of CFTR in its natural environment, the membrane of a differentiated epithelial cell, is possible with immortalised cell lines (Gruenert et al., 2004). Such cell lines are derived either from airway epithelial cells of healthy donors and CF individuals after the introduction of a proto-oncogene or from other cell lines that do not express CFTR but have been stably transfected with a form of CFTR (mutant or normal). Such cell lines have been used successfully for many studies of CF function.

Epithelial cell lines can successfully demonstrate polarisation, tight junctions and ion transport. The downside of these immortalised cell lines is that they express oncogenes and as a result lack the complete phenotype of the cells they originated from. They also have unstable karyotypes and phenotypes when cultured long-term.

1.8.2. Primary cells

Primary human nasal and bronchial epithelial cells are used as a more realistic model of the airway epithelium. These cells can be acquired from nasal brushings and lung biopsies or autopsies. Primary cell models have been used extensively for CFTR study in relation to transport of fluid, secretion of mucin and production of interleukin. Data derived from primary cells are more relevant and robust than these coming from cell lines, however patient material is not always easy to access, primary cell culture is more challenging and time consuming and good differentiation can be hard to achieve. Primary cells from the mouse nasal epithelium could
provide a more accessible alternative but these cells reach early senescence in culture (Scholte et al., 2006).

1.8.3. Mice

Mouse models of CF have been generated to represent the different classes of mutations in the *Cftr*. Knockout mice have premature stop codons in the *Cftr* gene, produce no CFTR protein and represent Class I mutations. Mice with the ΔF508 mutation demonstrate trafficking defect of the protein and represent Class II mutations. Class III mutations are represented by G551D mice and have altered CFTR regulation by ATP, while Class IV mutations are represented by R117H mice and show altered channel opening. Other CF mouse cell lines are hypomorphic where the level of expression of the WT CFTR protein is reduced (Scholte et al., 2006).

The CF mouse models demonstrate the specific effects of the modeled mutation on the CFTR channel in epithelial tissues. However, the mouse and human Cftr genes, even though highly homologous, are not identical, and therefore interact differently with drugs. Mice with severe CF mutations have a gastrointestinal phenotype and die from intestinal blockage without dietary intervention. Conversely, CF mice do not develop pancreatic problems or CF lung disease. Chronic infection, inflammation, accumulation of mucus and tissue remodeling is not present in the lungs of CF mice, but the reasons behind this are not clear. Some theories include the scarcity of ciliated epithelium and submucosal glands in the mouse lung, compensation by other chloride channels in the mouse epithelium (Clarke et al., 1994), clean, pathogen-free animal facilities and more recently, the less acidic air surface liquid (ASL) of mouse epithelia compared to these of humans (Shah et al., 2016). Due to these differences, the relevance of CF mice in studying CF in humans is questionable.

A better mouse model for CF lung disease comes in the form of a transgenic mouse over-expressing ENaC, the amiloride-sensitive epithelial sodium channel. In the absence of bacterial infection, this mouse still demonstrates reduced ASL volume, metaplasia of goblet cells, inflammation and mucus obstruction (Frizzell and Pilewski, 2004).

1.8.4. Other in vivo models

Because of the inability of CF mice to develop lung disease, other CF animal were developed, and most notably, *Cftr*−/− piglets (Welsh et al., 2009). The lung anatomy of pigs is similar to these of humans, as are the bioelectric ion properties. Indeed, at birth, the CF piglet lungs are normal
without signs of inflammation, like those of CF infants (Rogers et al., 2008). Similarly to patients with CF, the CF piglet rapidly develops lung infections and at 2 months demonstrates signs of inflammation (Stoltz et al., 2010). However, it is difficult to study CF lung disease in pigs as 100% of them will die from meconium ileus, if left untreated (Stoltz et al., 2010). Surgical ileostomy is necessary for their survival and even though CF pigs demonstrate most of the human CF pathogenesis including pancreatic and intestinal features, studies have been limited due to the sheer cost and difficulty in keeping them alive (Semaniakou et al., 2019).

The lungs of ferrets also share similarities with the human lungs and the Cftr<sup>-/-</sup> ferrets develop CF lung disease soon after birth (Sun et al., 2010). Other similarities include airway obstruction, accumulation of mucus and presence inflammation (Sun et al., 2014). CF ferrets suffer from intestinal disease that is more serious than these of humans, with many of those that survive the high incidence of meconium ileus, dying soon after birth from malabsorption (Sun, 2010). Lastly, the CF ferrets also share many of the features of human pancreatic disease (Semaniakou et al., 2019).

1.9. Techniques to assess CFTR

1.9.1. ALI cultures

In order to mimic the airway epithelium <em>in vitro</em> and achieve CFTR expression, it is necessary to differentiate airway epithelial cells. Air liquid interface (ALI) cultures is the system used for this purpose and consists of permeable cell culture supports (inserts) placed in cell culture plates. Airway cells are plated on the inserts and allowed to expand until they form a monolayer. At this point, the medium at the apical side of the cells is removed and the expansion media at the basal side of the cells is switched to differentiation media. After 2-4 weeks, cells polarise and differentiate to a pseudostratified epithelium, which forms tight junctions and consists of ciliated, mucus and basal cells. As a result, ALI cultures develop transepithelial electrical resistance (TEER) and beating cilia and mucus can be observed (Castellani et al., 2018).

1.9.2. Ussing chambers

Polarised ALI culture inserts can be mounted in Ussing chambers, an apparatus which allows the measurement of resistance (R), current (I) and voltage (V). In Ussing chambers, the sample
(which apart from an ALI culture insert with differentiated cells can also be a small piece of epithelial tissue, like airway or colonic biopsy) is maintained between solutions which secure equal hydrostatic pressure on both sides. During the experiment, compounds can be added to one or both sides of the sample (Li et al., 2004).

Epithelial tissues display tightness and therefore transepithelial resistance, which can be calculated using Ohm’s law, after measuring voltage and current. Transepithelial voltage is present in epithelial tissue because of the movement of negative and positive charges from the apical to the basal side. Short circuit current is measured as the charge flow per time when the transepithelial voltage is zero (Li et al., 2004).

As epithelia contain a number of different apical and basolateral ion transporters, it can be difficult to distinguish the function of CFTR from this of other channels. For this reason, different types of drugs that inhibit other transporters or activate CFTR are used in Ussing chambers to study cAMP-mediated chloride and EnaC conductance. Measurements in Ussing chambers have demonstrated that CF epithelial cells have absence of Cl⁻ currents and greater percentage of inhibition of ENaC by amiloride.

1.9.3. Western blots

Protein lysate from ALI cultures can be used to capture CFTR via Western blot assay. Electrophoretic analysis has demonstrated that CFTR exists in three forms with different molecular weights. These weights are 127 kDa, 131 kDa and 160 kDa and are referred to as bands A, B and C. Band A represents the non-glycosylated CFTR, band B is the core glycosylated CFTR while band C is the mature CFTR with complex glycosylation. The ΔF508 mutation results in only bands A and B being present as the protein does not traffic successfully to the Golgi apparatus (O’Riordan et al., 2000). Western blotting for CFTR can however be elusive, due to the very low levels of protein in both tissues and differentiated cells (Farinha et al., 2004).

1.10. Current therapies: Ion channel modulators (potentiators and correctors)

In the last decade, novel types of drugs targeting the underlying genetic defect of CF have been explored and are either already approved for CF therapy or are the focus of major clinical trials. These drugs are targeting to correct the defect of CFTR cellular production and to potentiate the
These ion channel modulators can only target specific mutations because of the great heterogeneity of effect of different mutations on the CFTR (Kumar et al., 2014).

Ivacaftor (Kalydeco) is an oral potentiator, the only drug of this type which is licensed for clinical use in patients in most countries. The drug can treat class III mutations by increasing the time that the activated CFTR will stay open at the cell surface and has been shown to be particularly effective in patients with the G551D (Gly551Asp) mutation. Ivacaftor improves lung function ($\text{FEV}_1$) by 10%, reduces sweat chloride concentration, reduces the frequency of pulmonary exacerbations and generally improves the quality of life in these patients (Ramsey et al., 2011). Further clinical trials demonstrated clinical benefit from the use of Ivacaftor for patients with other partially functioning mutations and nowadays Ivacaftor is approved for use for patients 1 year or older who have one of a large number of gating, residual function, splice and conduction mutations. Since the approval of Ivacaftor, a number of other potentiators have entered clinical trials (Pranke et al., 2019).

Lumacaftor is a channel corrector which protects the ΔF508 CFTR from proteomic degradation and increases its trafficking to the surface of the cells (Ren et al., 2013). It has been shown to be effective in vitro in bronchial cells of ΔF508 homozygous patients by raising chloride secretion (Van Goor et al., 2011). A phase 2 trial in adult CF patients with the same gene mutation did not show significant improvement in CFTR function or lung function (Clancy et al., 2012).

A combination therapy of Ivacaftor and Lumacaftor (Orkambi) in homozygous ΔF508 patients, restores trafficking of the channel (corrector effect) and makes it functional (potentiator effect) (Van Goor et al., 2011). As a result, it improves $\text{FEV}_1$, reduces the frequency of pulmonary exacerbations and modestly improves the quality of life in patients. However, the improvement effects of the combination therapy are lower than these seen in G551D patients that are treated with Ivacaftor (Boyle et al., 2014). Orkambi was approved for patients homozygous for the ΔF508 mutation in 2015 in the United States (Antoniou and Elston, 2016) and in October 2019 in Scotland and in England.

A subsequent corrector, Tezacaftor, with optimised pharmacokinetic properties, increased chloride transport in bronchial cells of ΔF508 homozygous patients and in combination with lumacaftor showed even greater chloride transport increase (Pranke et al., 2017).
combination therapy, Symdeko/Symkevi, showed a 4% FEV\textsubscript{1} improvement in ΔF508 homozygous patients, decreased pulmonary exacerbations and did not demonstrate chest tightness and drug-drug interactions that were previously reported for Orkambi (Taylor-Cousar et al., 2017). Symdeko is approved in the United States for patients homozygous for the ΔF508 mutation or at least one of many residual function or splice mutations.

A number of other correctors are currently evaluated in clinical trials as are triple combination therapies, which include ivacaftor, tezacaftor and another corrector. Newer types of small molecule compounds include amplifiers, which enhance the expression of CFTR protein, and stabilisers, which target the protein instability and its degradation from the plasma membrane, and can be used in combination with correctors and potentiators. They are currently tested for treatment of Class V mutations that lead to decreased CFTR synthesis and Class II and VI mutations that present reduced half-life and instability of CFTR (Pranke et al., 2019).

Class I mutations, that result in no protein production, cannot be rescued by ion modulators. These can potentially be targeted by “read-through” agents such as Ataluren, a small molecule that allows ribosomes to read through premature stop codons. Ataluren was tested in clinical trials but showed no significant FEV\textsubscript{1} improvement (Kerem et al., 2014). Other compounds that could target premature termination codon mutations include Nonsense Mediated Decay (NMD) inhibitors.

1.11. New therapeutic approaches

Currently, the most promising new propositions for a CF therapy is the targeting of alternative ion channels on the epithelial surface of the cells and gene therapy, as both offer the prospect of a cure for every patient, irrespective of the type of CFTR mutation. Pharmacological targeting of alternative chloride channels such as the TMEM16A and the SLC26A9 or the sodium channel ENaC could restore the ion imbalance in CF through ion compensation (Mall and Galietta, 2015). Gene therapy for CF is an idea that is being considered for more than 25 years but has been proven more challenging than originally thought. However, recent advances in gene transfer and gene editing mean that gene therapy for CF and many other diseases could be possible in the near future.
1.12. Gene therapy

In gene therapy, a copy of a healthy gene is inserted into the cells of an individual to compensate for a disease-causing one and treat the disease. In order to deliver this therapeutic gene to the patient’s target cells, a carrier vector must be used, such as a virus. The vector releases the gene in the target cell either for transient expression or insertion in the genome (depending on the specific gene therapy strategy) and the subsequent generation of a functional protein can revert the cell to the normal state.

Gene therapy has been evolving for decades but has had limited successes in treating human diseases due to a number of challenges that had or still need to be overcome. Such challenges include the efficiency of the gene delivery, the immunogenicity of the delivery vectors and the possibility of disrupting important genes when the therapeutic gene is inserted in the genome. In 1999, a patient with ornithine transcarbamoylase (OTC) deficiency, died of an immune reaction after receiving the corrective OTC gene encased in an adeno-viral vector (Sibbald, 2001). Two incidences of leukemia in patients treated with gene therapy for X linked combined immune deficiency disorder (SCID) in the early 2000s, halted gene therapy trials for the disease (Check, 2002). Since then, gene therapy has cured patients from SCID and adenosine deaminase deficiency (ADA-SCID), another inherited immune disorder, without incidences of leukemia. With the use of adeno-associated viruses and lentiviruses, which are less immunogenic, diseases such as hemophilia and beta-thalassemia have been treated in clinical trials without side-effects. In Europe, the first two gene therapy products for treating genetic disorders that were approved for commercial use, were Glybera for Lipoprotein lipase deficiency (LPLD) and Strimvelis for ADA-SCID. However, Glybera was withdrawn in 2017 due to being commercially unsustainable as a result of the rarity of LPLD. The first gene therapy product for a genetic disorder approved in the United States was Luxturna which targets hereditary blindness (LCA - Leber congenital amaurosis) (Keeler et al., 2017). Since 2012, an increasing number of gene therapies are being approved not only for genetic disorders but also for many acquired diseases and injuries.

1.12.1. In vivo gene therapy for CF

An autosomal recessive disease, such as CF, should in theory be possible to cure by gene therapy with the insertion of one copy of the normal gene in the affected cells, independent of the class mutation of the patient (O'Sullivan and Freedman, 2009). Gene therapy should also be capable
of targeting the disease defect at the molecular level and therefore cure the disease permanently with one or a few treatment rounds. Additionally, the development of gene therapy does not require the complicated CF pathophysiology to be completely illuminated (Griesenbach et al., 2015).

Initial gene therapy trials for CF utilised adenovirus vectors to deliver the copy of the gene but the efficiency of insertion of DNA in epithelial cells was low and the vectors were immunogenic, thus preventing repeated administration (Crystal et al., 1994, Harvey et al., 1999). Following attempts including adeno-associated viruses (AAV), did not demonstrate an advantage for these vectors as they were less effective in crossing the cell membrane (Moss et al., 2007, Griesenbach and Alton, 2012b). Repeated dosing did not increase the level of chloride correction, either due to 1. inefficiency of the viral vector in transducing the epithelial cells via the apical membrane, 2. the promoter used for CFTR expression being too weak in vivo or 3. antibody production against the viral vector (Griesenbach et al., 2015).

Different types of RNA virus have been tested for airway epithelium transduction, including the human parainfluenza virus (hPIV) and the murine Sendai virus (SeV), with modest results (Zhang et al., 2017). A number of lentiviral vectors, including the human (HIV) and simian immunodeficiency (SIV) virus are being developed for CF gene therapy, however these viruses don’t have natural tropism for the lung. For this reason, they are pseudotyped with envelope proteins from other viruses such as the influenza and the SeV. An SIV virus pseudotyped with proteins from SeV, was able to successfully transduce airway epithelial cells in mice, to achieve sustained expression of the gene with a single dose and be repeatedly administered with no efficacy loss (Griesenbach et al., 2012a). An improved version of this virus is currently being developed for the first phase I clinical trial for CF gene therapy with a lentivirus (Alton et al., 2017).

Liposomal vectors are also suited for repeated dosing due to their low immunogenicity (Hyde et al., 2000), their level of transfection efficiency however, is lower than this of viral vectors. In clinical trials, they were found to cause inflammatory responses while showing only marginal stabilisation of lung function and no evidence of CFTR transgene expression (Alton et al., 2015). These issues have been addressed by improved versions of these vectors and further studies, making nonviral gene transfer agents a possible candidate for a future CF gene therapy.
1.13. New era for gene therapy: CRISPR/Cas9 genome editing

In 2013, CRISPR/Cas9, a new tool which allows precise and targeted changes of genetic sequences in eukaryotic cells was created by adapting the bacterial CRISPR-associated protein-9 (Cong et al., 2013). This protein is a nuclease whose original function in the immune system of bacteria is to enable the memory and destruction of phages (Barrangou et al., 2007). In short, Cas9 nuclease from Streptococcus pyogenes (SpCas9), when directed by an sgRNA, is able to recognise a 20-nucleotide sequence which is complementary to the sgRNA sequence and adjacent to an NGG sequence (PAM - protospacer adjacent motif) and subsequently create a double strand break (DSB) at the target site (Figure 1.3). Following the creation of a DSB, the sequence can be repaired with either of two DNA repair pathways: the non-homologous end joining pathway (NHEJ) or the homology directed repair pathway (HDR).

Without a repair template the ends created by the DSB will be rejoined via NHEJ, a relatively error-prone process which can result in insertion-deletion (indel) mutations at the joining site. Within a coding region, these indels can result in a frameshift and/or the creation of a premature stop codon and lead to the creation of a gene knockout. In presence of a repair template, the high-fidelity HDR pathway can lead to precise gene editing (Ran et al., 2013b) (Figure 1.4).

Both NHEJ and HDR can be used for genome engineering, though HDR is significantly less efficient, as it is active only in the late S and G2 phases of the cell cycle when the sister chromatids are available as repair templates. HDR also depends greatly on cell type and state, genomic target and repair template (Saleh-Gohari and Helleday, 2004). However, it is the HDR pathway that has mainly been proposed for gene therapy since it can be used for both the correction of small point mutations and the insertion of longer gene fragments with the use of repair templates consisting of the correct gene sequence, flanked with long homology arms on either side of the DSB (Sander and Joung, 2014).
Figure 1.3. Schematic of Cas9 nuclease DNA targeting. *S. pyogenes* Cas9 (SpCas9), guided by an sgRNA of 20 nucleotides (blue) and an RNA scaffold (red), targets genomic DNA. The sgRNA sequence pairs with the DNA upstream of a PAM (NGG) site (pink). The Double Strand Break is created 3 bp upstream of the PAM (triangle) (Ran et al., 2013b).

Figure 1.4. Gene editing is promoted by DSB repair. Cas9 induced DSBs can be repaired with either of two ways. The error-prone NHEJ pathway results in the rejoining of the ends of the DSB which often creates random indel mutations at the junction site. Within the coding region of a gene, indel mutations can result in frameshifts and premature stop codon creation, leading to a gene knockout. Alternatively, a repair template can leverage the HDR pathway for precise gene editing (Ran et al., 2013b).
Compared to earlier protein-based genome engineering techniques like ZNFs and TALENs, the CRISPR/Cas9 system is much simpler and very easy to reprogram. The generation of a new, specially targeted molecule only requires a different RNA oligonucleotide and is therefore cost effective and quick (Ran et al., 2013b). A limitation of the CRISPR/Cas9 system is related to the requirement of a neighbouring PAM sequence at the desired target site. This has been addressed with the use of Cas9 nucleases of different bacterial species (Feng Zang lab, unpublished, from Addgene) and S. pyogenes engineered variants (Kleinstiver et al., 2015) which recognise different PAM sequences and allow more flexibility when designing an sgRNA.

Risks associated with the CRISPR/Cas9 systems which are directly relevant to its use for gene therapy, include the potential of creating mutations at off-target sites, undesirable on-target mutations, and permanent integration of the CRISPR/Cas9 components into the host genome (Cox et al., 2015). Off-target effects can be reduced by modifying the Cas9 nuclease to make variants with decreased off-target activity (eg. Cas9 nickase) (Ran et al., 2013a), careful selection of target sequences (Cho et al., 2014), adaptation of sgRNA molecules (Fu et al., 2014) and right titration of enzyme and RNA, as increased quantities are believed to be associated with higher off-target rates (Hsu et al., 2013). Unintended on-site mutations could be a result of insufficient donor template which will favour NHEJ repair mechanisms over HDR (Cox et al., 2015). Lastly, integration of CRISPR/Cas9 components can be avoided by the direct use of Cas9/sgRNA ribonucleoproteins (RNPs) instead of plasmid-based expressed Cas9 and sgRNA. These RNPs demonstrate similar efficiency to the plasmid-based form while being present at high levels in the cell shortly after transfection and getting quickly cleared away from the cell by protein degradation pathways. As a result, they may also decrease off-target cleavage by reducing the time the Cas9 is available in the cell (from Addgene).

1.13.1. CRISPR/Cas9 for CF gene therapy

Despite the above risks, CRISPR/Cas9 correction of gene defects can be more precise and potentially have fewer problems than classic gene therapy, where the correct gene copy can be inserted anywhere in the genome and cause gene disruption or even malignancies. That being said, in vivo CRISPR/Cas9 gene therapy, where the therapeutic agents are directly delivered to the airways, still needs to overcome the obstacle of a delivery vehicle that would efficiently and
safely target airway epithelial cells \textit{in vivo} (Colemeadow et al., 2016). Additionally, even by taking measures to reduce the risks associated with CRISPR/Cas9 genome editing such events could still occur with unpredictable consequences.

An alternative to \textit{in vivo} gene therapy for CF is the \textit{ex vivo} correction of the CFTR in CF patient stem cells of the respiratory epithelium. These cells would subsequently be administered back to the patient as cell therapy. The corrected cells would have the advantage of being patient specific, thus avoiding immune responses and rejection and could additionally be tested for undesirable off- and on-target mutations before being delivered to the airways for repopulation.

In 2013, CRISPR/Cas9 was used to correct the ΔF508 CFTR mutation in adult intestinal stem cells from pediatric CF patients which were cultured to form intestinal organoids. Analyses showed site-specific integration of the donor sequence leading to genotypic repair and only a single off-target mutation within a coding region (Schwank et al., 2013). More recently, the ΔF508 mutation was corrected in induced pluripotent stem cells (iPSCs) developed from CF patient fibroblasts, with no mutations or random integration events found elsewhere in the genome. The corrected iPSCs were subsequently differentiated into airway epithelial cells (Firth et al., 2015).

\section*{1.14. Epithelial tissues}

The cell layer that lines the outer and inner body surfaces is called epithelium. Cells of epithelia, have one free surface exposed to the external environment (apical side) and are involved in a number of functions including protection, absorption, excretion, secretion and sometimes sensory reception and contraction. Common characteristics of epithelia are the expression of keratins as intermediate filament proteins and the existence of intercellular junctions. Tight junctions create tight seals between epithelial cells, while gap junctions allow the flow of ions between them. Lastly, epithelia are avascular and are supplied with nutrients by the vasculature of tissues bellow the epithelium basement membrane (basal surface).

There are different types of epithelia depending on the organ they line and are classified by cell layering and cell shape. Simple epithelia have one cell layer, pseudostratified epithelia have a single cell layer that appears as two or more layers and stratified epithelia have several cell layers. Squamous epithelia are comprised of flattened cells, cuboidal epithelia are comprised of
cells with equal height and width and columnar epithelia are comprised of cells that are greater in height than width. In the transitional epithelium, the shape of the cells changes depending on its stretch degree (Bergman et al., 2005; Clark, 2015) (Figure 1.5).

Cell markers such as cytokeratins 5 and 14, tight junction proteins (claudins, occludin, zonula occludens-1) and cell adhesion molecules E-cadherin and EpCAM are universally expressed in all epithelia and can be used for epithelial cell identification. Transcription factor p63 is expressed by epithelial stem cells and functions to maintain their extraordinary proliferative capacity (Senoo et al., 2007).
<table>
<thead>
<tr>
<th>Cells</th>
<th>Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple squamous epithelium</td>
<td>Air sacs of lungs and the lining of the heart, blood vessels, and lymphatic vessels</td>
<td>Allows materials to pass through by diffusion and filtration, and secretes lubricating substance</td>
</tr>
<tr>
<td>Simple cuboidal epithelium</td>
<td>In ducts and secretory portions of small glands and in kidney tubules</td>
<td>Secretes and absorbs</td>
</tr>
<tr>
<td>Simple columnar epithelium</td>
<td>Ciliated tissues are in bronchi, uterine tubes, and uterus; smooth (nonciliated tissues) are in the digestive tract, bladder</td>
<td>Absorbs; it also secretes mucous and enzymes</td>
</tr>
<tr>
<td>Pseudostratified columnar epithelium</td>
<td>Ciliated tissue lines the trachea and much of the upper respiratory tract</td>
<td>Secretes mucus; ciliated tissue moves mucus</td>
</tr>
<tr>
<td>Stratified squamous epithelium</td>
<td>Lines the esophagus, mouth, and vagina</td>
<td>Protects against abrasion</td>
</tr>
<tr>
<td>Stratified cuboidal epithelium</td>
<td>Sweat glands, salivary glands, and the mammary glands</td>
<td>Protective tissue</td>
</tr>
<tr>
<td>Stratified columnar epithelium</td>
<td>The male urethra and the ducts of some glands</td>
<td>Secretes and protects</td>
</tr>
<tr>
<td>Transitional epithelium</td>
<td>Lines the bladder, urethra, and the ureters</td>
<td>Allows the urinary organs to expand and stretch</td>
</tr>
</tbody>
</table>
Figure 1.5. Classifications of epithelia. Schematics of the different type of epithelia, their location and their function (OpenStax College - Anatomy & Physiology, Connexions Web site).

1.14.1. The respiratory system

The respiratory system can be split into two major components: the conducting portion whose function is to condition the air by humidifying, warming and filtering it, and the respiratory portion whose function is the passive exchange of gases between the blood and the atmosphere. The conducting portion is consisted of the nasal cavities, the nasopharynx, the larynx, the trachea, the bronchi and the bronchioles, while the respiratory portion is consisted of the respiratory bronchioles, the alveolar ducts, the alveolar sacs and the alveoli (Figure 1.6).

Figure 1.6. The respiratory system. The figure shows the different components of the respiratory system and their location in the human body (From: Pocket Dentistry, Access Jan 2018)

The respiratory system is comprised of four main layers: the respiratory mucosa (consisted of the epithelium and the supporting lamina propria), the submucosa, the cartilage and/or muscle layer and finally, the adventita (Figure 1.7).
1.14.2. The respiratory epithelium

The epithelium of the respiratory system at the upper (proximal) airways is a pseudostratified epithelium which contains ciliated cells, goblet cells, club cells and basal cells. Ciliated cells are cells with tiny hair-like structures on their apical surface whose function is to move and sweep particles out of the body, while goblet cells are the cells that produce mucus. In the lower (distal) airways, the epithelium begins as simple columnar, comprised of ciliated cells in the larger bronchioles and progresses to simple cuboidal, with non-ciliated cells, in smaller bronchioles. In the distal airways instead of goblet cells, there are Clara cells, a type of secretory cells responsible for secreting one of the components of surfactant. Lastly, the epithelium of the alveoli is a simple squamous epithelium, comprised of type I (ATI) and type II (ATII) pneumonocytes.

In 2018, two separate groups discovered a new type of lung cell (Plasschaert et al., 2018; Montoro et al., 2018). These rare and previously uncharacterised cells were named pulmonary ionocytes because of their similarities with a primitive cell type in the larval skin of zebrafish and Xenopus, the ionocyte. Around 1% of airway epithelial cells are pulmonary ionocytes and they are characterised by transcription factor FOXI1 and genes encoding the V-ATPase. Pulmonary ionocytes were identified both among mouse airway epithelial tissue and among human cells.
and are descendent from basal cells. Importantly, pulmonary ionocytes express CFTR at much higher levels that any other cell type in the lung and therefore could be of great significance for CF. However, with a plethora of earlier studies suggesting that CFTR expression is present at more common types of airway cells, it might be premature to accept these new findings without further investigation on their relevance to CF pathology and future therapies (Hawkins and Kotton, 2018).

1.15. Stem cells in the lung

Defining characteristics of stem cells include their capacity for self-renewal, extensive proliferation and daughter cell production. Generally, stem cells have a slow turn over and are undifferentiated. Transient amplifying cells are the early descendants of stem cells which retain a significant growth. They eventually become terminally differentiated and incapable of further proliferation. Much of the cell number increase occurs most probably at the transient amplifying population stage, as stem cells cycle slowly. Stem cells are recruited as demanded for tissue turnover and enable the regeneration and repair of the damaged epithelial tissue (Piro et al., 2008).

Stem cells have been identified throughout the airways and are capable of giving rise to both transient amplifying cells and terminally differentiated cells. In the upper airways, the basal cells (positive for keratin 5/14 and p63), show progenitor/stem cell characteristics (Hajj et al., 2007), while in the lower airways Clara (Club) cells are probably the progenitors of ciliated cells and other Clara cells (Hong et al., 2001). In the distal alveoli, there is evidence showing that type II cells have stem cell capacity and are able to renew type I cells and themselves (Reynolds et al., 2004). Finally, a further stem cell niche was identified between the conducting and the respiratory epithelium (Giangreco et al., 2002). The cells in this zone, called bronchioalveolar stem cells, coexpress secretoglobin 1A1, a marker of Clara cells, the surfactant marker protein of type II cells (Sp)-C, CD34 and stem cell antigen (Sca)-1 (Kim et al., 2005) (Figure 1.8).
Concluding, in the upper airways, where the majority of the CF lung pathophysiology has been observed, basal cells function as the multipotent, tissue-specific cells of the airways. The airway epithelium is believed to be maintained by an equipotent basal progenitor cell population through ongoing stochastic loss and replacement (Texeira et al., 2013). However, it is possible, that the heterogeneity of basal cells is not yet fully understood, with different subsets of basal cells potentially being of particular importance in the regeneration of the human lung.

### 1.16. Maintenance of epithelial stem cells *in vitro*

The successful long-term *ex vivo* expansion of human epithelial (epidermal) cells has been possible since the mid-1970s in a co-culture system with a mitotically inactivated mouse embryonic fibroblast feeder layer. In contrast to when cultured in a plastic dish where the epithelial cells are poorly proliferative, the co-culture creates growing and strongly adhesive cell
colonies. These cells push away the feeder layer, display partial stratification and resemble basal cells.

Throughout the years, both the feeder cells and the culture media for human keratinocyte culture have been optimised. Nowadays, 3T3-J2 cells, a subclone of the original feeder cells, which is more supportive for keratinocyte culture and a medium consisting of 3:1 ratio of DMEM and Ham’s F12 supplemented with fetal bovine serum (FBS), hydrocortisone, adenine, cholera toxin, insulin, triiodothyronine and epidermal growth factor (EGF) are routinely used for this co-culture system (Hynds et al., 2018). More recently, many studies have included the Rho-associated protein kinase (ROCK) inhibitor, Y-27632, in the co-culture system medium for growing keratinocytes and other epithelial cells, suggesting that it increases cell proliferation (Chapman et al., 2010).

The long-term expansion of epidermal cells in the co-culture system is enabled by the maintenance of stem cells (Barrandon and Green, 1987) and the epithelial cells exhibit upregulated expression of stem cell markers (Suprynowicz et al., 2012). Retrospective identification of stem cells is possible through the analysis of the growth capacity of the epidermal cells in vitro. Cells from an individual colony plated into secondary cultures, form different clonal types, called holoclones, meroclones and paraclones. The holoclones, are capable of the greatest further expansion with 95% of the secondary culture colonies being large and containing small highly proliferative cells, while the paraclones giving secondary culture colonies that are small and soon become terminally differentiated. The secondary culture of meroclones contains both types of colonies (Hynds et al., 2018). Holoclones are derived from stem cells, paraclones are derived from differentiated cells and meroclones are generated by a progenitor with intermediate potency (transient-amplyfying cells) (Beaver et al., 2014). This heterogeneous ability of epithelial cells to form clones in culture is termed clonogenicity and clonogenic assays are used to test an epithelial cell population’s ability to undergo “unlimited” division.

Butler et al. (Butler et al., 2016), as well as others, have demonstrated that the co-culture method is also suitable for rapid and efficient expansion of primary human airway epithelial cells. The cells express airway basal stem cell markers and the technique can generate quantities of functional epithelial cells required for personalised and regenerative medicine applications. These cultures are additionally not associated with pitfalls of iPSC cultures such as karyotype-
instability, tumorigenicity and altered antigenicity (Suprynowicz et al., 2012). Downstream of the co-culture system, the proliferative state can be reverted by the removal of the feeder layer and the ROCK inhibitor and the process of basal cell differentiation into ciliated and mucus cells can be recapitulated experimentally on air-liquid interface (ALI) cultures.

For airway epithelial cells, it is still unclear whether the co-culture system with the addition of the ROCK inhibitor conditionally reprograms cells to a highly proliferative, “immortalised” state (Chapman et al., 2010; Suprynowicz et al., 2012; Liu et al., 2012; Liu et al., 2017), or alternatively is truly able to maintain the bona fide stem cells (holoclones) of the tissue which contribute to “unlimited” proliferation (Rheinwald and Green, 1975; Barrandon and Green, 1987; Pellegrini et al., 1999).

1.17. Transplantation of stem cells into the lung

As mentioned earlier, currently, the only treatment for end-stage lung disease are lung transplants. Considering, however, the shortage of donor organs, stem cell therapies for lung diseases can provide an alternative through which the damaged or dysfunctional respiratory epithelium of a patient can be replaced without the need of a full organ transplantation or the associated risks. Mouse studies and clinical trials have so far focused mainly on the transplantation of mesenchymal cells in the airway (Weiss et al., 2011), but it is believed that benefits from this type of treatment come from paracrine or immunomodulatory effects instead of lung regeneration through engraftment and transformation of the mesenchymal donor cells to epithelium (transdifferentiation) (Lama et al., 2007). True regeneration poses a major challenge due to the very complex structure of the lung.

Hematopoietic stem cell transplantation studies were proven to have exaggerated the level of engraftment of airway and alveolar epithelium that could be achieved with this method (Piro et al., 2008). However, they highlighted the role of niche occupancy as a challenge for engraftment of donor cells and parallel to this, stem cell competition in the lung niches being a major barrier for engraftment of transplanted stem cells. Repair of the lung can therefore only be achieved after pre-clearing of host lung progenitors from the niche by preconditioning. The studies of the Reisner lab (Rosen et al., 2015; Milman Krentsis et al., 2018) were the first to demonstrate that both fetal and adult progenitor cells harvested from lungs can successfully repair lung injury if
the stem cell niche has been vacated by a combination of naphthalene and irradiation preconditioning. Different ways of epithelial injury have been used for stem cell niche clearing and include treatment with naphthalene (Van Winkle et al., 1995), sublethal irradiation (Theise et al., 2002), bleomycin (Bigby et al., 1985) and polidocanol (Suzuki et al., 2000). The drawback of this approach is that these agents cannot be used clinically, making the investigation for clinically approved compounds for airway injury crucial. Compounds for human lung injury could include the influenza virus, which is a biological agent that has been shown to severely damage the airway epithelium, different types of mechanical injury like nasal and bronchial brushings, bronchial thermoplasty or liquid nitrogen cryosprays (Quantius et al., 2016).

1.18. CF cell therapy

Early studies on transplantation of endogenous lung stem cells show an advantage of this type of cells for lung cell therapy (Vaughan et al., 2015; Rosen et al., 2015; Ghosh et al., 2017; Nichane et al., 2017; Milman Krentsis et al., 2018). Basal cells self-renew and replace all lung cell types, including the potentially CF-relevant pulmonary ionocyte, making these cells ideal for a long lasting therapy. Additionally, the paradigms of other epithelial cell therapies, as are the development of skin from epidermal keratinocytes for burn treatment and the transplantation of corneal limbal stem cells for eye damage, have demonstrated the feasibility of a cell therapy from autologous, tissue specific, adult, epithelial stem cells (Green et al., 1979; Rama et al., 2017).

For a successful CF lung cell therapy via epithelial stem cell transplantation, many challenges need to be addressed. An important issue is the inflammation present in the CF lung and how this will affect cell engraftment. Secondly, the number of engrafted cells should be sufficient to restore the ion transport and improve lung disease CF patients. Here, mouse models not developing lung disease is an obvious limitation for assessing the functional restoration of the epithelium after engraftment and therefore different in vivo CF models need to be used. Other challenges that need to be considered include the methods for obtaining and sufficiently expanding airway epithelial cells as well as the method for CFTR expression corection for each patient. Lastly, as already mentioned, clinically appropriate compounds for lung injury and ways for subsequent cell delivery in the patients’ airways need to be developed (Figure 1.9).
1.19. Decellularised organs as a model for cell engraftment

With lung transplantation being the only treatment for end-stage lung disease and insufficient donor organ availability, many researchers propose the development of bioengineered lungs as an alternative way to treat CF.

There are three important considerations for the construction of bioengineered organs: the cell type, the origin of the scaffold and the properties of the extra cellular matrix (ECM) (Schilders et al., 2016). Usually, different cell types need to be combined to recapitulate the complex cell interactions and scaffolds can be either synthetic or biological (Murphy and Atala, 2015). Additional support is provided to the construct in the form of mechanical or biochemical cues (Schilders et al., 2016).

Compared to synthetic scaffolds, biological scaffolds have many advantages for tissue engineering, including being more similar to the tissue and organ for which they are providing the matrix. Decellularised organs, provide a biological scaffold which, after the removal of the
endogenous cells, can be recellularised with cells such as embryonic stem cells, adult stem cells or iPSCs. Methods for decellularisation include chemical, physical and enzymatic methods and aim in the best possible preservation of the ECM components (Schilders et al., 2016).

Trachea bioengineering efforts have faced great controversy after the scandalous Macchiarini transplantations, which resulted in the deaths of a number of patients. Investigations on these incidents concluded that both the trachea scaffolds were of poor quality and preparation and the seeded cells were not fit for this purpose. These failures, have highlighted the need for vigorous preclinical testing, as well as the necessity to find the most appropriate cell sources and decellularisation methods for tissue engineering. Whole lung bioengineering is even more challenging than this of the trachea due to the higher complexity of the lung. Nevertheless, much progress has been made in the form of lung decellularisation protocols and cell engraftment (Doi et al., 2017; Skolasinki et al., 2018; LaRanger et al., 2018).

With regenerative medicine still being in the early stages, off-the shelf bioengineered lungs for transplantation in CF patients might not be available soon. However, there are valuable lessons to be learned from this field in terms of cell seeding and cell differentiation. Such information can be valuable for in vivo cell transplantation for the development of CF cell therapies.
1.20. Aims

The aim of this project is to combine ex vivo gene and cell therapy as proof of principle for a possible treatment for cystic fibrosis (CF). The specific objectives of the study include:

a. Cultivation and characterisation of epithelial cells from a variety of sources (including cells from CF patients and healthy donors),
b. correction of the CFTR mutation in CF patient cells via the CRISPR/Cas9 HDR pathway,
c. creation of isogenic CFTR knockouts in healthy donor cells with CRISPR/Cas9 technology,
d. development of a bioengineered system based on decellularised airway scaffolds, where engraftment capacity and functionality of cultivated epithelial cells can be assessed in vitro,
e. delivery of cells to the airways of small animal models to assess in vivo engraftment.
CHAPTER 2. MATERIALS AND METHODS
2.1. Isolation of Human Fetal Tracheal Epithelial Cells

Human fetal tracheal epithelial cells (HuFeTRC) were isolated from fetal tracheas collected from 13 to 20 post conception week fetuses.

The human fetal tracheas were cleaned from extra tissue and muscle and the tube of the trachea was slit open. The tissue was washed with RPMI 1640 medium (Gibco) and then incubated with 1 U/mL Dispase II in RPMI at 37°C for 30 minutes. Following the incubation, the epithelial layer was removed from the trachea using forceps. The epithelium was then incubated with 0.4 mg/mL Collagenase D in RPMI at 37°C for 30 minutes, while being further dissociated with pipetting every few minutes. Dissociated cells were centrifuged at 1200 rpm for 5 minutes and resuspended in cFAD medium. Finally, the cell suspension was plated in a T75 flask already containing a lethally irradiated 3T3-J2 mouse embryonic fibroblast layer.

Isolation of HuFeTRC01 was performed by Paola Bonfanti.

2.2. Airway Epithelial cell culture

Fetal and adult cells were plated on a layer of lethally irradiated mouse 3T3-J2 cells (Rheinwald and Green, 1975) with keratinocyte culture medium cFAD, unless otherwise indicated. cFAD medium consists of 3:1 DMEM (Gibco) to F-12 Nut Mix (Ham) (Gibco), 10% Fetal Bovine Serum (Sigma), 1% Penicillin-Streptomycin (100X, Sigma), 0.4 μg/mL Hydrocortisone (Calbiochem, USA), 5 μg/ml Insulin (various), 10^{-10} Cholera Toxin (Sigma) and 2x10^{-9} Triodothyronine (Sigma). cFAD medium is supplemented with 10 ng/mL human epidermal growth factor (hEGF) (PeproTech, USA) for the feeding (usually day 4 in 7-day passaging). Where indicated, cFAD medium was additionally supplemented with 10 nM Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Sigma). Cultures were incubated at 37°C with 5% CO₂.

Where the medium is indicated as BEGM, the cells were grown with Bronchial Epithelial Cell Growth Medium (BEGM) (consists of: BEBM + BulletKit, Lonza) on flasks not containing a feeder layer.

Cells at subconfluence were either trypsinised with 0.25% Trypsin/EDTA (Sigma) and passaged, or frozen down with 10% glycerol in expansion medium.
The cumulative cell number at the end of culture was calculated with \( n = T^*\left(\frac{F}{I}\right) \), with \( T \) = the cumulative cell number at the end of the previous passage, \( F \) = the number of cells harvested at the given passage and \( I \) = the number of cells plated at the beginning of given passage.

Population doublings were calculated with \( PD = \log_2 \left( \frac{n}{s} \right) \) with \( n \) = the cumulative cell number at the end of a given passage and \( s \) = the starting number of cells of the culture.

2.3. Single cell cloning of Airway Epithelial Cells

At the end of passage, cells were trypsinised and collected. \( 10^5 \) cells were resuspended in 1mL cFAD medium and were serially diluted 1:10 three times to achieve a final dilution of 100 cells/mL. 480 mL (48 cells) were further diluted in 14.4mL cFAD supplemented with 10 nM Y-2763. 300μl of the suspension were plated in each of the wells of a 48-well plate (preplated with lethally irradiated mouse 3T3-J2 cells), resulting in approximately one epithelial cell/well. The cells were fed at 48 hours and 96 hours after plating, with cFAD supplemented with 10 nM Y-2763 and 10 ng/mL hEGF. After approximately 11 days, each visible colony was transferred to a well of a 12-well plate or directly to a T25 flask. Further expansion and freezing down of clones was performed where possible.

2.4. 3T3 culture

3T3-J2 mouse embryonic fibroblasts (gifted by Howard Green, Harvard Medical School, Boston) were cultured in DMEM medium with 10% Fetal Bovine Serum (FBS) (HyClone) and 1% Penicillin-Streptomycin. Cells at subconfluence were trypsinised with 0.25% Trypsin/EDTA and γ-irradiated at 60 Gy. Irradiated cells were plated on cell culture flasks and dishes and were used as feeder layer for the airway epithelial cell culture.

2.5. HEK293 culture

HEK293 cells were cultured either with DMEM medium with 10% FBS and 1% Penicillin-Streptomycin or Minimum Essential Medium Eagle with 10% FBS, 2mM L-Glutamine, 0.1mM non essential amino acids and 1% Penicillin-Streptomycin. Cells at subconfluence were trypsinised with 0.25% Trypsin/EDTA and passaged, or frozen down with 10% DMSO in expansion medium.
2.6. Air Liquid Interface (ALI) culture of Airway Epithelial Cells

After expansion in a submerged culture, cells were harvested and plated at a density of $10^5/cm^2$ on 0.4 μm pore PET cell culture inserts (Greiner) containing or not a layer of irradiated 3T3 feeder cells. The insert was supplemented with expansion medium (cFAD or BEGM) on the top and bottom for 48 hours (Figure 2.1, Panel A), after which, medium was removed from the top of the culture (exposure) and the culture was supplemented with fresh ALI medium three times a week, only from the bottom (Figure 2.1, Panel B). Inserts for 12-well plates were used, with the exception of Ussing Chamber experiments where special inserts, Snapwells™ (Costar) for 6-well plates are necessary. ALI cultures lasted approximately 2 to 5 weeks, until cell ciliation was observed.

![Figure 2.1. Air Liquid Interface (ALI) Culture Airway Epithelial Cells. Schematic of: (A) Expansion phase of ALI culture; (B) Exposure phase of ALI culture. Picture adapted from stemcell.com](image)

Medium used for ALI cultures was either cFAD supplemented with 100 nM Retinoic Acid, 1:1 BEBM to DMEM supplemented with BulletKit and 100 nm Retinoic Acid, or PneumaCult™- ALI Medium (Stemcell), as indicated.

2.7. Histological analysis of human fetal tracheas, decellularised rat trachea scaffolds and mouse lungs
Tissue was fixed and stored in 4% Paraformaldehyde (PFA) until further processing, when the PFA was removed with PBS washes. The samples were subsequently dehydrated with ethanol and infiltrated with paraffin in a tissue processor for subsequent paraffin embedding. Sections (5 μm thickness) of paraffin embedded tissue were stained with Hematoxylin & Eosin (H&E staining, in house), Masson’s Trichrome (RAL diagnostics), Picro-Sirius Red (Abcam), Elastica van Gieson (Sigma) or immunological stains and imaged with Brightfield microscopy (Zeiss Axioplan2), Whole Slide Imaging (NanoZoomer) or a confocal microscope (Zeiss LSM 710).

2.8. Immunostaining

Cells from expansion cultures were plated on 0.13 μm coverslips with an irradiated 3T3 feeder layer and cFAD medium. At the given time point for analysis, cells were fixed for 10 minutes with 4% PFA and subsequently washed with PBS. The cells were permeabilised and blocked with 0.5% Triton X (Bio-Rad) in 5% Donkey Serum (Jackson Immuno Research, USA) for 20-25 minutes. Primary antibodies were prepared in 0.01% Triton X in 5% Donkey Serum and the fixed coverslips were incubated with the antibodies overnight at 4°C (Table 2.1). The primary antibodies were washed off with PBS on the following day and the secondary antibodies were prepared in 0.01% Triton X in 5% Donkey Serum and the fixed coverslips were incubated with the antibodies overnight at 4°C (Table 2.1). The coverslips were incubated with the secondary antibodies for 1 hour and subsequently washed with PBS. Mounting medium with DAPI (Abcam) was used to mount the coverslips on slides and imaging was performed with a confocal microscope (Zeiss LSM 710). No primary antibody negative controls were performed for every immunostaining.

In the case of ALI cultures, the culture insert was removed from the plastic ring and was either divided into sections for different staining or used as a whole. Fixation and staining protocols were the same used for cells on coverslips. When mounting, the ALI insert was placed on a slide with the cells facing upwards, mounting medium with DAPI was applied and a 0.13 μm rectangular coverslip was placed on top of the ALI insert.

In the case of paraffin sections, dewaxing and antigen retrieval in Citrate Buffer pH 6 was performed before staining as mentioned previously. For the antigen retrieval, the sections were boiled for 23 minutes in a 900W microwave oven, submerged in a 100mM solution of the buffer and then allowed to cool under a running tap.
2.9. Immunohistochemistry of mouse lungs

Paraffin sections of mouse lungs were dewaxed and antigen retrieval was performed in Citrate Buffer pH 6 in a microwave oven. After cooling and washing in TBS, sections were incubated with 3% hydrogen peroxide. Following, sections were blocked with ImmPRESS 2.5% normal horse serum (Vector) and incubated overnight with primary antibodies. After washing in TBS, ImmPRESS Peroxidase Anti-Rabbit or Anti-Mouse Ig Reagent (Vector) was applied to sections for 30 mins. New TBS washing was followed by incubation with ImmPACT NovaRed substrate (Vector) until colour development. Sections were then counterstained with Hematoxylin.

Table 2.1. Primary and secondary antibody information. The table shows the names of the antibodies used, the host species, the dilution and the manufacturer.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin 5/14</td>
<td>Rabbit</td>
<td>1:800</td>
<td>BioLegend, USA</td>
</tr>
<tr>
<td>p63</td>
<td>Mouse</td>
<td>1:50</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Mouse</td>
<td>1:100</td>
<td>BD Transduction Laboratories, USA</td>
</tr>
<tr>
<td>ki67</td>
<td>Rabbit</td>
<td>1:400</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>Mouse</td>
<td>1:1000</td>
<td>EMD Millpore Merck, USA</td>
</tr>
<tr>
<td>Acetylated - tubulin</td>
<td>Mouse</td>
<td>1:500</td>
<td>Sigma</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Life Technologies, USA</td>
</tr>
<tr>
<td>Cytokeratin 8</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>FOXI1</td>
<td>Mouse</td>
<td>1:150</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>CFTR 596</td>
<td>Mouse</td>
<td>1:100</td>
<td>Cystic Fibrosis Foundation, University of North Carolina at Chapel Hill</td>
</tr>
<tr>
<td>mCherry</td>
<td>Rabbit</td>
<td>1:300</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>STEM121</td>
<td>Mouse</td>
<td>1:500</td>
<td>Takara, Japan</td>
</tr>
<tr>
<td>Anti-Nuclei</td>
<td>Mouse</td>
<td>1:100</td>
<td>Sigma</td>
</tr>
<tr>
<td>EpCAM eFluor 660</td>
<td>Mouse</td>
<td>1:50</td>
<td>eBioscience, USA</td>
</tr>
<tr>
<td>Alexa Fluor 488 Anti-mouse</td>
<td>Donkey</td>
<td>1:500</td>
<td>Jackson Immuno Research, USA</td>
</tr>
<tr>
<td>Cy3 Anti-rabbit</td>
<td>Donkey</td>
<td>1:500</td>
<td>Jackson Immuno Research, USA</td>
</tr>
<tr>
<td>Alexa Fluor 594 Anti-rabbit</td>
<td>Donkey</td>
<td>1:500</td>
<td>Jackson Immuno Research, USA</td>
</tr>
</tbody>
</table>
### 2.10. Ussing chambers

Snapwells with ciliated (differentiated) cells were mounted on Ussing chambers to measure short circuit current ($I_{sc}$). The cell monolayer was exposed to a physiological salt solution (117 mM NaCl, 25 mM NaHCO$_3$, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 2.5 CaCl$_2$, 11 mM D-glucose, equilibrated to pH 7.3-7.4 with 5% CO$_2$). The solution was maintained at 37°C, bubbled with 5% CO$_2$ and 21% O$_2$ and continuously circulated for the duration of the experiment. The cell monolayer was clamped with voltage ($V_{te}$) at 0 mV using a DVC-4000 voltage/current clamp and the $I_{sc}$ required to maintain this condition was measured with PowerLab computer interface. 2 mV were passed through the chamber every 30 seconds, to measure spontaneous current and calculate transepithelial resistance ($R_{te}$).

Amiloride-sensitive transepithelial Na$^+$ $I_{sc}$ ($I_{amiloride}$) was measured with the addition of 10 μM amiloride to perfusing solution at the apical side of the cell monolayer. CFTR Cl$^-$ $I_{sc}$ ($I_{fSk+IBMX}$) was measured by activation of cAMP with 10 μM forskolin in combination with 10 μM IBMX to prevent cAMP degradation by inhibition of phosphodiesterase. When peak current was achieved, 10 μM of CFTR inhibitor 172 was added to the apical side of the cell monolayer to block CFTR on the apical cell membrane. The CFTR inhibitable transepithelial Cl$^-$ $I_{sc}$ ($I_{CFTR}$) was calculated as the difference in $I_{sc}$ before and after CFTR inhibitor-172. Basolateral ouabain-sensitive $I_{sc}$ ($I_{ouabain}$) was calculated as the difference in $I_{sc}$ before and after the application of 1mM of ouabain at the basolateral compartment to inhibit Na$^+$K$^+$ATPase activity.

All calculations were made according to Ohm’s law: $R_{te} = \Delta V_{te}/\Delta I$.

Ussing chamber measurements were performed by Maximillian Woodall (St George’s University, London).

### 2.11. Transfections with lipofectamine

pEGFP-N1 transfections of HuFeTRC with Lipofectamine 3000 (ThermoFisher) were performed according to the standard protocol of the reagent. Lipofectamine was diluted in Opti-MEM medium (Gibco) and separately a master mix of GFP plasmid, Opti-MEM medium and P3000 Reagent-Mix was prepared. The two mixes were combined at a 1:1 ratio and incubated for 15
minutes. The DNA-lipid complexes were then added to HuFeTRC at plating. For each reaction at a 12-well plate, 2 μg of GFP and 1.5-3 μl of Lipofectamine was used. The cells were analysed with Fluorescence Activated Cell Sorting (FACS) 24 hours post transfection.

2.12. Nucleofections

Plasmid (pmaxGFP, pEGFP-N1, px330-g7 or Excision only piggyBac Transposase expression vector) nucleofections of Airway Epithelial Cells were prepared using the standard protocol of the Neon Transfection System (Life Technologies). Cells were harvested, washed with Hank’s Balanced Salt Solution (HBSS) (Sigma) and resuspended in buffer R. 5μg of plasmid per 4x10^5 cells (5-15μg per 10^6 cells for piggyBac excision transposase) was added to the cell suspension. The Neon was set with pulse length at 20 ms, pulse number at 2 and pulse voltage at 1100 mV-1500 mV. These settings were indicated by Ileana Guerrini (GOS Institute of Child Health UCL, London) who had previously optimised the nucleofection protocol for NHBE cells transduced with the BMI-1 proto-oncogene. After nucleofection the cells were plated for expansion. Depending on the experiment, cells were analysed with FACS or genotyped.

2.13. Preparation of cells for Fluorescence Activated Cell Sorting (FACS) analysis and sorting

Cells were harvested and centrifuged for 5 minutes at 1200 rpm, then resuspended with 2% FBS (Gibco) in HBSS. When cell staining was required, cells were centrifuged again and resuspended with antibody solution in 2% FBS in HBSS. Epithelial cells were stained with EpCAM Alexa Fluor 660 from eBiosciences (1:100) or alternatively 3T3 cells were stained with anti-feeder PE from Miltenyi Biotec (1:10). Cells were incubated with the antibodies for 20 minutes in the dark and on ice. When cells had to be fixed because of facility requirements, they were centrifuged and resuspended in 1% PFA for 20 minutes and then washed with 2% FBS in HBSS. Cells were analysed at the BD LSRII or the CytoFLEX flow cytometers 48-72 hours post transfection and results were analysed with the software FlowJo.

Where (non-fixed) cells were sorted for subsequent culture, they were collected in polypropylene tubes pre-coated and filled with cFAD medium. The cells were centrifuged and resuspended in new cFAD medium before plating.
2.14. Selection of a CRISPR/Cas9 guide target for creating a CFTR knockout in NHBE

The site of guide 1 for the creation of CFTR knockouts in NHBE was designed with the help of the Synthego website knockout guide designer. The guide was selected to target exon 2, which is present in all three described CFTR isoforms. As it targets an early exon, it is likely to disrupt the reading frame and create a stop codon towards the start of the mRNA, therefore avoiding being translated into a protein with residual function. It was also calculated to have high on target and low off-target activity.

2.15. Preparation of sgRNA reagents

Three different guide RNA reagents were used, plasmid pX330, and sgRNAs from Sigma and Synthego.

Plasmid pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid 42230) expresses human codon-optimised SpCas9 and is a chimeric guide RNA expression plasmid (Figure 2.2). It was acquired from Ahmad Aldossary (GOS Institute of Child Health UCL, London) and already included annealed oligos of guide 7.
Figure 2.2. pX330-U6-Chimeric_BB-CBh-hSpCas9 vector. The plasmid expresses human codon-optimised SpCas9 under a CBh promoter and chimeric guide RNA.

The sgRNAs from Sigma were received as separate tracrRNA and crRNA and were subsequently resuspended in 0.1 M Trizma buffer for stock concentration of 100 μM. Resuspended tracrRNAs and crRNAs were prepared in a 20 μM final concentration with 0.1 M Trizma buffer and heated at 95°C. Aliquots were stored at -20°C until use.

SgRNAs from Synthego were received as pre-annealed synthetic sgRNAs and were resuspended in TE buffer for a stock concentration of 100 μM. Resuspended sgRNAs were diluted in water for a stock concentration of 30 μM and were stored at -20°C until use (Table 2.2).
Table 2.2. sgRNA information. The table shows the different sgRNAs used, their brand, their CFTR exon target and their sequence.

<table>
<thead>
<tr>
<th>sgRNA and Brand</th>
<th>CFTR Exon target</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma - guide 7</td>
<td>10</td>
<td>GAGGGUAAAAUUAAGGCAGGCUUUUAGAGACUGCUGUUUUG</td>
</tr>
<tr>
<td>Synthego - guide 7</td>
<td>10</td>
<td>GAGGGUAAAAUUAAGGCACAG</td>
</tr>
<tr>
<td>Synthego - guide 1</td>
<td>2</td>
<td>GCUGGACCAGACCAAUUUUUG</td>
</tr>
</tbody>
</table>

2.16. CRISPR/Cas9 nucleofections

Nucleofections of sgRNA/Cas9 complexes were performed by optimisation and adaption of the nucleofection protocol for plasmids. The Neon Transfection System was used set at 20 ms pulse length, 2 pulses and 1200 mV pulse voltage and the cells were prepared as mentioned previously. sgRNA/Cas9 complexes were mixed at various molar ratios (with 1-1.6 μg of Cas9 per 2x10^5 cells) and incubated at room temperature for 5 minutes before being added to the cells. Where a repair template was used, this was added to the sgRNA/Cas9 mix after the 5-minute incubation at a concentration of 15 μg per 10^6 cells.

Three different Cas9 reagents were used, plasmid pX330, EnGen Cas9 NLS (NEB) at 20 μM and TrueCut™ Cas9 Protein v2 (ThermoFisher Scientific) at 30 μM.

Alt-R HDR Enhancer (IDT) at 30 μM was included in the culture immediately after nucleofection in one HDR experiment.

2.17. Assessment of Double Strand Breaks

The efficiency of the sgRNA/Cas9 complexes to create Double Strand Breaks (DSBs) was assessed by genomic PCR around the target site of the sgRNA, subsequent Sanger sequencing of the PCR product and analysis of the sequence with the TIDE (Tracking of Indels by Decomposition) software (Table 2.3). TIDE allows to infer the percentage of double strand breaks in the cell population by capturing the indel percentage in the mixed cell population after gene editing. TIDE decomposes the Sanger sequencing chromatogram of a mixed cell population based on a
reference Sanger sequence and subsequently calculates the percentage of indels. A higher percentage of indels indicates a higher percentage of double strand breaks.

Table 2.3. Primers and PCR conditions used for the assessment of Double Strand Breaks. The table shows the sgRNA, the sequence of the primers, the primer annealing temperature, the extension time for every PCR performed and the sequencing primer used for TIDE.

<table>
<thead>
<tr>
<th>sgRNA</th>
<th>DSB PCR Primers</th>
<th>Annealing Temperature PCR</th>
<th>Extension Time PCR</th>
<th>Product Length</th>
<th>Sequencing Primer for TIDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>guide 7</td>
<td>F - CTTCCTCTGCTACC</td>
<td>66°C</td>
<td>1 min</td>
<td>1965 bp</td>
<td>F - T1 - GTGCATAGCAGAGTA CCTG R - T2 - CCGATTGAATATGGA GCC</td>
</tr>
<tr>
<td></td>
<td>TCTTTTCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R - TCTAATCCACGGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TTGCCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>guide 1</td>
<td>F - CAATCCCTTTTGAC</td>
<td>64°C</td>
<td>30 sec</td>
<td>789 bp</td>
<td>GTGACAGTACATTA GTTCAGAGA</td>
</tr>
<tr>
<td></td>
<td>AAAGTGACAGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R - TGATTTTACGCAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CATTTCAGTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.18. CRISPR/Cas9 for the correction of the ΔF508 CFTR mutation

SpCas9 PAM sites around the ΔF508 mutation were identified *in silico* with the software Benchling. After consideration of the off-target (specificity) and on target (efficiency) scores, a PAM site 65 bp upstream of the mutation was selected. This PAM site has been selected in the past by multiple teams including Schwank et al. (Schwank et al., 2013), since it results in the only sgRNA with acceptable scores in good proximity of the mutation. A higher score is better for both specificity and efficiency (Table 2.4).
Table 2.4. PAM sites and respective sgRNA sequences 130 bp upstream and 130 bp downstream of the ΔF508 mutation. The table shows the sgRNA sequence, the respective PAM site, the DNA strand of the sgRNA, the specificity score and the efficiency score.

<table>
<thead>
<tr>
<th>Strand</th>
<th>Sequence</th>
<th>PAM</th>
<th>Specificity Score (Hsu et al., 2013)</th>
<th>Efficiency Score (Doench et al., 2016)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TTCACTCTTAATGATGATTA</td>
<td>TGG</td>
<td>49.663577</td>
<td>29.28429065</td>
</tr>
<tr>
<td>1</td>
<td>TCATTCTTAATGATGATTAT</td>
<td>GGG</td>
<td>46.8115378</td>
<td>34.5387229</td>
</tr>
<tr>
<td>1</td>
<td>AATGATGATTATGGAGAAC</td>
<td>TGG</td>
<td>34.6297506</td>
<td>37.66692553</td>
</tr>
<tr>
<td>1</td>
<td>GGGAGAACGAGGCCCTTCA</td>
<td>AGG</td>
<td>36.2514042</td>
<td>59.40073269</td>
</tr>
<tr>
<td>1</td>
<td>GGGAGAACGAGGCCCTTCA</td>
<td>GGG</td>
<td>34.6930659</td>
<td>63.36698225</td>
</tr>
<tr>
<td>-1</td>
<td>TGCTTAATTTACCCCTCTGA</td>
<td>AGG</td>
<td>37.0694136</td>
<td>49.02386694</td>
</tr>
<tr>
<td>1</td>
<td>GAGGGTTAAAAATTAAGCACAG</td>
<td>TGG</td>
<td>36.4849428</td>
<td>69.36750179</td>
</tr>
<tr>
<td>1</td>
<td>CATTCTGTTCATGTTCATCCCC</td>
<td>TGG</td>
<td>30.8771823</td>
<td>23.82357169</td>
</tr>
<tr>
<td>-1</td>
<td>AATGGTGCCAGCCATAATCC</td>
<td>AGG</td>
<td>42.0996827</td>
<td>54.07076945</td>
</tr>
<tr>
<td>1</td>
<td>CAGTTTTCTGGATTATGCCC</td>
<td>AGG</td>
<td>41.4501842</td>
<td>41.95857719</td>
</tr>
<tr>
<td>-1</td>
<td>ATATTTCTTTAATGGTGCC</td>
<td>AGG</td>
<td>37.9568625</td>
<td>46.10411413</td>
</tr>
<tr>
<td>-1</td>
<td>ACCAATGATATTTTCTTTAA</td>
<td>TGG</td>
<td>37.1190731</td>
<td>1.315752767</td>
</tr>
<tr>
<td>1</td>
<td>ACCATTAAAGAAAATATCAT</td>
<td>TGG</td>
<td>39.7635327</td>
<td>47.38347549</td>
</tr>
<tr>
<td>-1</td>
<td>TCTGTATCTATATTCAATCAT</td>
<td>AGG</td>
<td>33.9304131</td>
<td>53.23183464</td>
</tr>
<tr>
<td>-1</td>
<td>AGTTTTTCTACCTCTCTAGT</td>
<td>TGG</td>
<td>37.334661</td>
<td>43.90383453</td>
</tr>
<tr>
<td>1</td>
<td>CAAAGCATGCCAACCTAGAAG</td>
<td>AGG</td>
<td>41.2009261</td>
<td>50.33071741</td>
</tr>
<tr>
<td>-1</td>
<td>TATAATTTGGTATGGTGAA</td>
<td>GGG</td>
<td>37.5495054</td>
<td>51.41227562</td>
</tr>
<tr>
<td>-1</td>
<td>ATATATTGGTAGTGTAAG</td>
<td>AGG</td>
<td>37.2857405</td>
<td>47.34347698</td>
</tr>
<tr>
<td>1</td>
<td>CACTACCCAAATTATATATT</td>
<td>TGG</td>
<td>35.8454794</td>
<td>18.22993783</td>
</tr>
</tbody>
</table>

Genomic DNA from ΔF508 NECs was extracted with the DNeasy Blood & Tissue Kit (Qiagen). The genomic DNA approximately 1000 bp upstream and 1000 bp downstream of the ΔF508 mutation locus was amplified by PCR reaction. The PCR product was used for Sanger sequencing in order
to molecularly confirm the genotype of the cells and acquire the specific sequence to build a fully homologous repair template (Table 2.5).

Table 2.5. Primers used for PCR amplification and Sanger sequencing of a region 1000 bp upstream and 1000 bp downstream of the ΔF508 mutation. The table shows the name of the primer, the sequence and the application for which it was used.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Used for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>del508flankPCR-F</td>
<td>CTTCCTCTGCTACCTCCTTTCC</td>
<td>PCR &amp; Sequencing</td>
</tr>
<tr>
<td>del508flankPCR-R</td>
<td>TCTAATCCACGGTTTGCCC</td>
<td>PCR &amp; Sequencing</td>
</tr>
<tr>
<td>del508seq-F2</td>
<td>GAACAGCACTCGACACAGAGT</td>
<td>Sequencing</td>
</tr>
<tr>
<td>del508seq-F3</td>
<td>TGAACCTTCACACTACCCA</td>
<td>Sequencing</td>
</tr>
<tr>
<td>del508seq-F4</td>
<td>GCCAGCAGAGAATTAGAGGGG</td>
<td>Sequencing</td>
</tr>
<tr>
<td>del508seq-F5</td>
<td>GCACTTTGGCAACTGTTAGCTG</td>
<td>Sequencing</td>
</tr>
</tbody>
</table>

Q5 high fidelity polymerase (NEB) was used for this PCR and all following PCR reactions with the standard protocol of the reagent. Annealing temperature for this primer pair was calculated with the online NEB Tm calculator and was set at 66°C. The extension time was set at 30 seconds.

A selection cassette with the Puromycin and Thymidine Kinase (puromycin/TK cassette) genes under an EF1a promoter flanked by ITR sites (piggyBac recognition sites) was ordered as an empty multivector from Transposagen (product code SGK-007) (Figure 2.3).
Figure 2.3. SGK-007 Multivector from Transposagen. Empty multivector containing Puromycin and Thymidine Kinase selection cassette under an EF1α promoter (MV-EF1α-Puro-TK). PiggyBac ITRs flank the selection cassette and the vector contains an Ampicillin resistance gene.

Two gBLOCKs were ordered from IDT, one for the left homology arm (LHA) and one for the right homology arm (RHA). Both homology arms were approximately 800 bp long and identical to the native sequence, except for the addition of the missing triplet (TCT) coding for the Phenylalanine amino acid on the RHA. The repair template was divided in LHA and RHA at position 14 of the sgRNA sequence for two reasons: 1. Disrupting the sgRNA site with the insertion of the cassette
avoids further Cas9 cutting caused by lingering sgRNA and protein; 2. Cassette needs to be inserted after an endogenous TTAA site, because the piggyBac excision transposase recognises a TTAA site followed by the 3’-ITR sequence and 3. Leaving the sgRNA site intact in the repair template could cause cutting of the template itself by the Cas9. Lastly, an extra TTAA site was added at the beginning of the RHA arm, which needs to follow the 5’-ITR site for piggyBac recognition but will be removed by the piggyBac transposase upon excision (Table 2.6 and Figure 2.4).

Table 2.6. Sequence of the Homology Arms used for the construction of the repair template. The table shows the sequence of the Left Homology Arm (LHA) and the Right Homology Arm (RHA). In bold are shown the native and added TTAA sites used for piggyBac recognition at excision.

<table>
<thead>
<tr>
<th>Homology Arm</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHA</td>
<td>TTTAATGATACCTTTTGA CTTTTCAGCTTTTCTTTAATAAACGCAATCGAAAT TTTCCCTT</td>
</tr>
<tr>
<td></td>
<td>TACACTCCACACATTACCCCCATTTCCCTTTTGTGGTTTTTGTTTTACTCTAACCTT</td>
</tr>
<tr>
<td></td>
<td>TTCTTTATGTCAGGACATATAACATATTAAAAAACCTTTTTTCCAATCGAAT CTTGCA</td>
</tr>
<tr>
<td></td>
<td>TTGTTTTTTAAATTTTTGTCACAGTTATAAACATTTGTTTTACTCTTGACTAGTCCTTTTTGTA</td>
</tr>
<tr>
<td></td>
<td>CTATCATCTCTTTATGACCTTTATACCTCAGAAAGGAACATCGGCAATATICAATTACTT</td>
</tr>
<tr>
<td></td>
<td>GAAATATGTCTCTATTCTTAAAATGCTCTAATAATAATGGAAGTTAATCTACTTTTGAG</td>
</tr>
<tr>
<td></td>
<td>GATTTTTGCTGAAGATTAATAAATAAATATATGATTAAGACACATAGAACAGCAGCTCA</td>
</tr>
<tr>
<td></td>
<td>CACAGAGTGAGCCTGGCCACTGGTTAGCTGTACTAAACCTTTCCCCATTTCTCCCA</td>
</tr>
<tr>
<td></td>
<td>AACTATTCACACTATCTGAAATCTGTGCCCCTTTCTCTGTGAACCTCTATACAACT</td>
</tr>
<tr>
<td></td>
<td>TGTCACACTGTATTGTGAAATCTCTTTACTTTACTTTCCCTTGATCTTTGTGCATACGAG</td>
</tr>
<tr>
<td></td>
<td>AGTACCTGAAACAGGAAGATTTTTAATAATTTGGAATCAATAGGTAATAGAATCT</td>
</tr>
<tr>
<td></td>
<td>TTACCAAAATGAAATACACTTCTCTGCTTAGTAGAATTTGGGGCAATGAGG</td>
</tr>
<tr>
<td></td>
<td>GAGCGTGATTGGTAAATGGACCTAATAATAGATGATGTTTTTATTTCCCAATCGACTTCTCA</td>
</tr>
<tr>
<td></td>
<td>ATGATGATATTATGGAAGAATGGGAGCCCTCAGAGGTAAATTTTTTTAATTTTTT</td>
</tr>
</tbody>
</table>

RHA | TTAA GCACAGTGGAAAGAATTTTCCCTCGCTCAGCTTTTTTCGGATTATGCCCTGGCAC |
|     | CATTAAAGAAATATCATCTTTTGTTTTCTCTATGGAATATAGATACAGAGCCTG |
|     | CATAGGCTGAATCAGGGAAGATTTGAAAATATGTGGAACCTTTTTGATTATG |
|     | CATATGAAACCTTTACACTACCACAAATTTATAATATATGATGCTCCTCATACTCAGGTAG |
Two different constructs were created, in one of which, the whole repair template was flanked by two sgRNA cut sites with the same sequence as the endogenous sgRNA cut site which is used to create the Double Strand Break (DSB), as this can increase the efficiency of HDR (Zhang et al., 2017) (Figure 2.4).

Figure 2.4. Schematic of plasmid assembly design for the correction of the ΔF508 mutation in ΔF508 Nasal Epithelial Cells. The sgRNA sequence is shown in green and is interrupted by the insertion of the selection cassette. The sgRNA sequence contains an endogenous TTAA site which is recognised by the piggyBac excision transposase and an additional TTAA site needs to be added at the other side of the cassette, after the 5'-'ITR. The sgRNA sites at either end of the construct are optional and only used for one of the two repair plasmids.
Due to problems with the gBLOCK synthesis at the Integrated DNA Technologies (IDT, Belgium) facility, secondary products were present in addition to the RHA. For this reason, the RHA had to be cloned and some of the recognition sequences in both homology arms were added later by PCR primers with overhangs. These primers were designed to also add the overlapping fragments required for Gibson assembly (NEB). Table 2.7 shows the primers used and the respective PCR conditions.

In order to acquire pure RHA, the RHA received was A-tailed and then cloned in the pGEM-T easy system (Promega), according to the standard protocol of the reagent (Figure 2.5). JM109 competent cells (Promega) were transformed with the pGEM-T easy plasmid containing the RHA and the cells were plated on 100µg/ml Ampicillin, 0.5mM IPTG and 80µg/ml X-Gal agar plates. White colonies (indicative of lacZ gene disruption and successful cloning) were selected to inoculate mini plasmid preparations (minipreps) in LB broth with 100 µg/mL Ampicillin. The miniprep DNA was extracted with the Plasmid Miniprep Kit (Qiagen) and the plasmid DNA was sequenced with Sanger sequencing for the identification of a plasmid with the desired RHA sequence.

Table 2.7. Primers and PCR conditions used for the addition of overlapping fragments required by Gibson Assembly. The table shows the PCR product and the expected length, the sequence of the primers, the DNA template, the primer annealing temperature and the extension time for every PCR performed.

<table>
<thead>
<tr>
<th>PCR Product and Length</th>
<th>Primers</th>
<th>Template</th>
<th>Annealing Temperature PCR</th>
<th>Extension Time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassette - 3069 bp</td>
<td>F-CTTCAGAGGGTAAAATTAACCCTAGAAAGATAATCATATTGA</td>
<td>SGK-007 Multivector</td>
<td>60°C</td>
<td>1 min 30 sec</td>
</tr>
<tr>
<td></td>
<td>R-AATGAAATTCTTCCACTGTGTTAACCCCTAGAAAGATAGCTCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LHA (without flanking sgRNA cut sites) - 843 bp</td>
<td>RHA (without flanking sgRNA cut sites) - 904 bp</td>
<td>LHA (with flanking sgRNA cut sites)</td>
<td>RHA (with flanking sgRNA cut sites) - 866 bp</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-----------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td><strong>Primers</strong></td>
<td>F- CCGGTACCGAGCTGAATTTTTAATGATACCTTTTTGACCTTGAGCTTTTCTTAATAAAAGCA</td>
<td>F- CGCAGACTATCTTTTCTAGGGT TAAGCACAAGCTTCAGGAATTTTAATCTGTTTCTCAGT</td>
<td>NO PCR needed, original gBLOCK used</td>
<td>F- CGCAGACATCTTTTTCTAGGGT TAAGCACAAGCTTCAGGAATTTTAATCTGTTTCTCAGT</td>
</tr>
<tr>
<td></td>
<td>R- ATATGATTATCTTTTCTAGGGTT AATTATACCATCTGGGAATTTTAATCTGTTTCTCAGT</td>
<td>R- AAAACGACGGCCAGTGGAATTTTAATCTGTTTCTCAGT</td>
<td>Cloned RHA in pGEM-T easy</td>
<td>R- AAAACGACGGCCAGTGGAATTTTAATCTGTTTCTCAGT</td>
</tr>
<tr>
<td><strong>Annealing Temperature</strong></td>
<td>65°C</td>
<td>69°C</td>
<td>30 sec</td>
<td>69°C</td>
</tr>
</tbody>
</table>
Figure 2.5. pGEM-T easy vector. Vector system for cloning PCR products generated by certain thermostable polymerases. The polymerases add deoxyadenosines to the 3’ ends of the PCR products while the vector contains 3’ T overhangs at the insertion site. The vector contains an Ampicillin resistance gene and β-galactosidase for the detection of successful cloning.

The construct which was not flanked by the two sgRNA cut sites was assembled with the pUC19 plasmid (NEB) as a backbone, which was linearised at the multiple cloning site by EcoRI restriction endonuclease (NEB), according to the standard protocol of the reagent (Figure 2.6). EcoRI was inactivated at 65°C for 20 minutes.
The 4 fragments for each of the two repair plasmids were assembled with Gibson Assembly cloning method using the following concentrations: 25 ng of linearised pUC19, 50 ng of cassette, 50 ng of LHA and 50 ng of RHA. 5-alpha competent cells (NEB) were transformed with the plasmids and cells were plated on Ampicillin/IPTG/X-gal agar plates. The plasmids were prepared as described earlier. DNA from ten minipreps per construct was digested with EcoRI and the products of the digestion were separated by agarose gel electrophoresis in a 1% gel. DNA from minipreps with the expected fragment sizes after digestion were sequenced with Sanger sequencing using the primers on Table 2.8 for confirmation of the desired sequence.
Table 2.8. Primers used for confirmation of construct sequence by Sanger sequencing. The table shows the primer name and the sequence.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOR1</td>
<td>TATACTCAAAGAAAGGCTCATGG</td>
</tr>
<tr>
<td>FOR2</td>
<td>TACACTTCTGCTTAGGATGATAATTG</td>
</tr>
<tr>
<td>FOR4</td>
<td>CTGTGGTGCCCTCTGAACTG</td>
</tr>
<tr>
<td>FOR7</td>
<td>GGCCTCACCCCTCATCTTT</td>
</tr>
<tr>
<td>FOR8</td>
<td>TTATTTACCCCTTTCCGGGC</td>
</tr>
<tr>
<td>FOR9</td>
<td>CATGTCTGCAATTGATAACTTCG</td>
</tr>
<tr>
<td>FOR10</td>
<td>CATTCTGTTCAGTTTTTCCTGG</td>
</tr>
<tr>
<td>FOR11</td>
<td>GAGACAAACGTCTCAATGG</td>
</tr>
<tr>
<td>REV2</td>
<td>AGTCTGGAAATAAAACCCATCA</td>
</tr>
<tr>
<td>REV4</td>
<td>TCTAGGTAGGCTCCAAGGGA</td>
</tr>
<tr>
<td>REV6</td>
<td>AGTAGCGTGGGCGATTGAT</td>
</tr>
<tr>
<td>REV7</td>
<td>TCATGCTGCCATAAGGTATC</td>
</tr>
<tr>
<td>REV8</td>
<td>GTTTGGCAAGACGTCCA</td>
</tr>
<tr>
<td>REV10</td>
<td>TGACGCTTCTGTATCTATTTCACTCAT</td>
</tr>
<tr>
<td>REV11</td>
<td>AAGATGCATATTTTGCGATATTCTTT</td>
</tr>
</tbody>
</table>

After multiple unsuccessful attempts to assemble the components of the construct flanked by sgRNA cut sites (Table 2.7) with the pUC19 plasmid as described above, this was instead assembled with the pGEM-T easy plasmid (Figure 2.5). A PCR was performed on the construct not flanked by the sgRNA sites, to acquire a single fragment consisting of the LHA followed by the cassette and by the RHA. The primers used for this PCR included overhangs in order to add the sgRNA cut sites on either side of the fragment (Table 2.9). The fragment was A-tailed and cloned to the pGEM-T easy system as described above (Figure 2.5). The DNA of twelve minipreps was digested by EcoRI and agarose gel electrophoresis was performed in a 1% gel. DNA from one miniprep with the expected fragment sizes after digestion was sequenced with Sanger sequencing using the primers on Table 2.8 for confirmation of the desired sequence.
Table 2.9. Primers and PCR conditions used for the creation of the fragment required for the construct with the sgRNA sites. The table shows the PCR product and the expected length, the sequence of the primers, the DNA template, the primer annealing temperature and the extension time for the PCR performed.

<table>
<thead>
<tr>
<th>PCR Product and Length</th>
<th>Primers</th>
<th>Template</th>
<th>Annealing Temperature PCR</th>
<th>Extension Time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>sgRNA-LHA-cassette-RHA-sgRNA</td>
<td>F-GAGGGTAAAAATTAAGCACA GTGGTTTAAATGATACCTTTT GACTTTTC R-CCACTGTGCTTAATTTCATCC TCAGTGAACACAGCACAAGATCATATAAC</td>
<td>Construct without sgRNA sites</td>
<td>56°C</td>
<td>2 min 30 sec</td>
</tr>
</tbody>
</table>

Both constructs were prepared with the Plasmid plus MAXI kit (Qiagen) for a high yield endotoxin free preparation, necessary for downstream transfection applications.

For the correction of the ΔF508 mutation, cells were nucleofected with TrueCut Cas9 protein, sgRNAs (Synthego) and a repair construct. After 48 hours, cells were selected with 1 μg/mL puromycin and allowed to expand.

Successful HDR, which results in the incorporation of the puromycin/TK cassette and the correction of the ΔF508 mutation was confirmed with in-out genomic PCRs on either side of the selection cassette. For these PCRs, one primer is located on the endogenous genomic sequence flanking the cassette (further out than the homology arm) while the second primer is located inside the cassette (Figure 2.7 and Table 2.10). After agarose electrophoresis indicated bands of the expected size, the PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced with Sanger sequencing.
Figure 2.7. Position of screening primers for successful HDR with the puromycin/TK selection cassette. For the detection of the incorporation of the puromycin cassette and the correction of the ΔF508 mutation, in-out genomic PCRs are performed as shown. For the 3’ end of the selection cassette, the forward primer (del508flankPCR-F) is located on the endogenous genomic sequence flanking the cassette (further upstream than the homology arm) while the reverse primer (3-ITR-cas-R) is located inside the cassette, close to the 3’ end. For the 5’ end of the selection cassette, the forward primer (FOR9) is located inside the cassette, close to the 5’ end, while the reverse primer (Rflank) is located on the endogenous genomic sequence flanking the cassette (further downstream than the homology arm).

Table 2.10. Primers used for in-out PCR’s for the confirmation of the incorporation of the puromycin/TK cassette and the correction of the ΔF508 mutation. The table shows the name of the primer, the PCR product and length, the sequence of the primer, the annealing temperature and extension time for the PCR and the primers used for Sanger Sequencing.

<table>
<thead>
<tr>
<th>PCR Product and Length</th>
<th>Primers</th>
<th>Annealing Temperature PCR</th>
<th>Extension Time PCR</th>
<th>Sequencing Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-out 3’ – 1001 bp</td>
<td>del508flankPCR-F-CTTCCTCTGCTACCTCCTTTCC 3-ITR-cas-R-CCGATAAAAACACATGCGTCA</td>
<td>64°C</td>
<td>30 sec</td>
<td>TIDE 1 - GTGCATAG CAGAGTAC CTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FOR9 &amp; REV11</td>
</tr>
<tr>
<td>In-out 5’ – 1435 bp</td>
<td>FOR9-CATGTCTGCAATTGATAACTTTCG Rflank-TCATGTAAGCATGCAACCCTTT</td>
<td>63°C</td>
<td>2 min</td>
<td></td>
</tr>
</tbody>
</table>

For excision of the puromycin/TK selection cassette, cells were nucleofected with the piggybac excision only transposase (Cambridge Bioscience) with a ratio of 15 μg per 10^6 cells. After transfection, cells were selected with 3 μg/mL of ganciclovir (Sigma). Successful excision was
confirmed with the genomic PCR of Table 2.5 and subsequent TIDE analysis. Unsuccessful excision was indicated by the in-out PCRs on Table 2.10.

Additionally to the cloned constructs, a third construct, CFTR Donor-G, was kindly provided by Brian Davis (University of Texas Health Science Center, Texas) as referenced in Crane et al., 2015. The plasmid was prepared with Plasmid Plus MAXI kit and successful HDR was confirmed with in-out genomic PCRs (Table 2.11) and Sanger sequencing.

Table 2.11. Primers used for in-out PCR’s for the confirmation of the incorporation of the CFTR-Donor G cassette and the correction of the ΔF508 mutation. The table shows the name of the primer, the PCR product and length, the sequence of the primer, the annealing temperature and extension time for the PCR and the primers used for Sanger sequencing.

<table>
<thead>
<tr>
<th>PCR Product and Length</th>
<th>Primers</th>
<th>Annealing Temperature PCR</th>
<th>Extension Time PCR</th>
<th>Sequencing Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-out 3’ – 923 bp</td>
<td>3’ F - CCTGCAGCCCGGGGATCTAT</td>
<td>68 °C</td>
<td>30 sec</td>
<td>3’F &amp; 3’R</td>
</tr>
<tr>
<td></td>
<td>3’ R - GCCATTCATGTAAGCATGCAACCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-out 5’ – 1785 bp</td>
<td>5’ F - AAATCAGTGCTTTTTGAGGTTAGGAG</td>
<td>66 °C</td>
<td>1 min</td>
<td>5’ F &amp; 3’ R</td>
</tr>
<tr>
<td></td>
<td>5’ R - CGACTGTGGCTTTCTAGTTGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.19. Puromycin kill optimisation

Cells were plated on a 6-well plate and different concentrations of puromycin (0 μg/mL, 1 μg/mL, 3 μg/mL, 6 μg/mL and 10 μg/mL) were added to the culture after 48 hours. Fresh medium with puromycin was added to the culture every 48 hours. Cells were monitored via microscopy for 7 days, to determine the concentration of puromycin which kills 100% of the cells in this timeframe.

2.20. Assessment of puromycin/TK selection cassette excision
Cells were genotyped with the in-out PCRs of Table 2.10 and the genomic PCR of Table 2.5. Sanger sequencing results of the genomic PCR were analysed by ICE (Inference of CRISPR Edits) software (Hsiau et al., 2018). ICE works similarly to TIDE but additionally provides details on the genotype of the resolved sequences.

2.21. Lentivirus production

LB broth was inoculated by a bacterial stab of pSicoR-EF1a-mCh-Puro plasmid (Addgene plasmid 31845), (Salomonis et al., 2010) which contains mCherry followed by an in-frame T2A puromycin resistance gene cassette under a EF1a promoter (Figure 2.8), and plated on 100 µg/mL Ampicillin agar plates. The plates were incubated overnight at 37°C. Colonies were selected to inoculate maxi plasmid preparations (maxipreps) in LB broth with 100 µg/mL Ampicillin. The maxiprep DNA was extracted with the Plasmid plus MAXI kit (Qiagen).
Figure 2.8. pSicoR-EF1a-mCh-Puro vector. Lentiviral construct with mCherry followed by an in-frame T2A puromycin resistance gene cassette under an EF1a promoter.

A DNA mix of 40 μg of the pSicoR-EF1a-mCh-Puro plasmid, 30 μg of pCMVdR874 and 10 μg of pMD2G (provided by John Counsell, GOS Institute of Child Health, UCL, London) was prepared in 5 mL of OptiMEM and filtered through a 0.22 μM filter. 1μl PEI was added to 5 mL of OptiMEM and filtered. The two mixes were combined and incubated at room temperature for 20 minutes. The DNA-PEI mix was added to a T175 flask of HEK293T cells (1.8 x 10^7 cells seeded the previous day), after the removal of the culture medium. The DNA-PEI mix was removed from the flask after 4 hours and fresh medium was added to the culture. 48 and 72 hours after the transfection, the medium was harvested from the flask and centrifuged at 500 rpm for 5 minutes. The
supernatant was filtered and centrifuged for 2 hours at 23000 rpm. The supernatant of the super centrifugation was disposed and after drying the tube, 50 μl OptiMEM was added. The tube was incubated on ice for 1 hour after which the viral vector pellet was resupended, aliquoted and stored at -80°C until use. The above protocol can be scaled up for higher concentration of viral vector.

2.22. Lentivirus titration and optimisation

Different dilutions of lentivirus (1 μl, 1:5 μl, 1:25 μl, 1:125 μl and 1:625 μl) with 10 μg/mL polybrene were added to different wells of a 6-well plate with subconfluent HEK293. One well was trypsinised to count the number of cells at transduction. 48 hours after transduction, the cells were prepared for FACS as described previously. The TU/mL was calculated by:

\[ \text{TU/mL} = \text{number of cells at transduction} \times \text{percentage of mCherry+ cells} \div \text{volume of viral vector}. \]

This protocol was used to optimise the transduction and calculate the TU/mL for both Epithelial Airway Cells and 3T3-J2 cells.

2.23. Lentivirus transduction

After the selection of the appropriate amount of viral vector for the transduction of Epithelial Airway Cells and 3T3-J2 cells with an MOI of 0.3, transductions were repeated in a large number of these cells. 72 hours after transduction, 3T3-J2 were selected with 1μg/mL puromycin for 7 days while Airway Epithelial Cells were sorted for mCherry+. Successfully transduced cells were frozen for stock.

2.24. RNA extraction

RNA was extracted from ALI culture inserts with differentiated cells using the ReliaPrep™ RNA Miniprep System (Promega).

2.25. qRT-PCR

cDNA synthesis and qRT-PCR was performed with the SensiFAST Probe Hi-ROX One-Step Kit (Bioline). The probes used were TaqMan™Gene Expression Assay (FAM) (ThermoScientific), Hs00357011_m1 for CFTR and Hs01060665_g1 for beta-actin. Data was analysed with the ddCt method.
2.26. Protein extraction

Protein was harvested from ALI culture inserts with differentiated cells. The cells were scraped from the insert in PBS, transferred to a microcentrifuge tube and pelleted. The pellet was resuspended in pre-chilled Pierce Lysis buffer (Thermo Scientific) with complete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail (Sigma) and incubated on ice for 20 minutes. After incubation, the protein lysates were centrifuged and the supernatant was aliquoted and stored at -80°C until use.

2.27. Western Blots

Two different Western Blot protocols were performed.

For protocol 1, protein lysates were combined with 6x sample buffer (20 μl lysate to 4 μl buffer) and incubated at 37°C for 30 minutes. The samples and pre-stained protein ladder (Precision Plus Protein™ Dual Color Standards, Bio-rad) were loaded on a 4-12% NuPAGE™ Bis-Tris protein gel (Thermo Scientific) and run with SDS running buffer on the XCell SureLock™ Mini-Cell (Thermo Scientific) for 70 minutes at 200 V and 78 mA. Protein was transferred to an Imobilon-P PVDF membrane (Sigma) with NuPAGE™ transfer buffer (Thermo Scientific) for 1 hour at 140 V. The membrane was blocked for 1 hour in 5% milk in PBS-T. After blocking, the membrane was incubated with the primary antibody (CFTR 596) at 1:1000 dilution in blocking solution overnight at 4°C. After incubation, the membrane was washed with PBS-T. Secondary antibody HRP-conjugated goat anti-mouse (Dako) was incubated with the membrane at 1:1000 dilution for 1 hour at room temperature. After washing with PBS-T, the membrane was incubated with chemiluminescent substrate SuperSignal West Femto Maximum Sensitivity (Thermo Scientific) for 4 min. The membrane was imaged on ChemiDoc™ MP Imaging System (Bio-Rad) and processed on Image Lab (Bio-Rad). Total protein content was measured by imaging after staining with Coomassie stain (0.1% Coomassie brilliant blue, 50% ethanol, 50% UltraPure water) and destaining (50% methanol, 50% UltraPure water, 10% Acetic Acid). The loading control antibody was Anti-GAPDH rabbit polyclonal (Thermo Scientific).

For protocol 2, only differences from protocol 1 are reported.

Protein lysates were combined with XT sample buffer and XT reducing agent (Bio-rad) (28 μl sample to 12 μl buffer). Samples were ran on a Criterion XT Tris-Acetate protein gel (Bio-rad) with XT Tricine Running Buffer (Bio-rad) in a Criterion cell tank (Bio-rad). Transfer was performed
with the Immun-Blot® PVDF/Filter Paper Sandwiches (Bio-rad) in the Trans-Blot Turbo Transfer system (Bio-rad) at 25 V for 30 minutes. The loading control antibody was Anti-Na+/K+-ATPase mouse (Santa Cruz Biotechnology).

Western Blots were performed by Martin Attwood or Amy Walker, GOS Institute of Child Health, UCL, London.

2.28. Decellularisation of rat tracheas

Fresh rat tracheas were decellularised with detergent-enzymatic treatment consisting of deionised water, 4% sodium deoxycholate (SDC) (Sigma) or 1% sodium dodecyl sulfate (SDS) (Sigma), PBS and 0.025 mg/mL Deoxyribonuclease-I (DNase-I) (Sigma) in 1 M NaCl (Sigma) or in 2.5 mM MgCl₂ (Sigma). A series of different protocols were attempted. All protocols, apart from protocol 6, included the delivery of solutions through the trachea lumen with the use of a variable speed roller pump (iPump). The rat tracheas were attached to the pump through a cannula; cannula widths of 0.7 mm and 1.5 mm were used. Protocol 6 included the submersion and stirring of the trachea in the treatment solutions using a glass bottle on a magnetic stirrer at 500 rpm (Table 2.12 and Table 2.13). For histological analysis, scaffolds were fixed in 4% PFA.

Table 2.12. Rat trachea decellularisation protocols 1-5. The table shows the treatments, duration of treatments, number of treatment cycles, cannula width and pump speed of the different protocols attempted for rat trachea decellularisation.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overnight H₂O</td>
<td>Overnight H₂O</td>
<td>Overnight H₂O</td>
<td>Overnight H₂O</td>
<td>Overnight H₂O</td>
</tr>
<tr>
<td></td>
<td>4h 4% SDC</td>
<td>4h 4% SDC</td>
<td>2h 4% SDC</td>
<td>4h 4% SDC</td>
<td>4h 4% SDC</td>
</tr>
<tr>
<td></td>
<td>30 min PBS</td>
<td>30 min PBS</td>
<td>30 min PBS</td>
<td>30 min PBS</td>
<td>30 min PBS</td>
</tr>
<tr>
<td></td>
<td>3h DNase (NaCl)</td>
<td>3h DNase (NaCl)</td>
<td>3h DNase (NaCl)</td>
<td>3h DNase (NaCl)</td>
<td>3h DNase (NaCl)</td>
</tr>
<tr>
<td>No of cycles</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cannula width</td>
<td>0.7 mm</td>
<td>0.7 mm</td>
<td>0.7 mm</td>
<td><strong>1.5 mm</strong></td>
<td>1.5 mm</td>
</tr>
<tr>
<td>Pump speed</td>
<td>1 mL/min</td>
<td>1 mL/min</td>
<td>1 mL/min</td>
<td>1 mL/min</td>
<td>1 mL/min</td>
</tr>
</tbody>
</table>
Table 2.13. Rat trachea decellularisation protocols 6-8. The table shows the treatments, duration of treatments, number of treatment cycles, cannula width and pump speed of the different protocols attempted for rat trachea decellularisation.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 1</td>
<td>Overnight H2O</td>
<td>Overnight H2O</td>
<td>4h 1% SDS</td>
</tr>
<tr>
<td>4h 4% SDC</td>
<td>4h 1% SDS</td>
<td>4h 1% SDS</td>
<td></td>
</tr>
<tr>
<td>30 min H2O</td>
<td>Overnight H2O</td>
<td>Overnight H2O</td>
<td></td>
</tr>
<tr>
<td>4h 4% SDC</td>
<td>15 min DNase (MgCl2)</td>
<td>4h 1% SDS</td>
<td></td>
</tr>
<tr>
<td>Overnight H2O</td>
<td>Overnight H2O</td>
<td>1h H2O</td>
<td></td>
</tr>
<tr>
<td>4h 4% SDC</td>
<td></td>
<td>15 min DNase (MgCl2)</td>
<td></td>
</tr>
<tr>
<td>1h PBS</td>
<td></td>
<td>Overnight H2O</td>
<td></td>
</tr>
<tr>
<td>1h DNAse (NaCl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3h H2O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No of cycles</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cannula width</td>
<td>Stirrer: 500 rpm</td>
<td>1.5 mm</td>
<td>1.5 mm</td>
</tr>
<tr>
<td>Pump speed</td>
<td>2 mL/min</td>
<td>2 mL/min</td>
<td></td>
</tr>
</tbody>
</table>

Rat tracheas were provided after surgery by Asllan Gjinovci (GOS Institute of Child Health, UCL, London).

2.29. Airway epithelial cell seeding on decellularised rat trachea scaffolds

Rat trachea scaffolds decellularised with Protocol 8 and γ-irradiated at 1782 Gy were used for the seeding of airway epithelial cells. The proximal (attached to cannula) and distal (bronchi) parts of the trachea were removed and the trachea was sliced open to create a flat scaffold. The flat scaffold was divided into 2 smaller square pieces of approximately 5 mm x 5 mm. The scaffolds were pinned on 0.4 μm pore PET cell culture inserts to keep flat with the luminal side facing upwards (Figure 2.9). 10⁵ cells were seeded on the scaffolds resuspended in 30 μl of cFAD medium supplemented with Y-2763. After 1 hour, top and bottom of insert were fully supplemented with medium. The medium was maintained for several days or alternatively changed to ALI differentiation medium after 48h.
2.30. \textit{In vivo} experiments in small animal models

B6.Cg-Gt(ROSA)26Sor<tm14(CAG-tdTomato)Hze>/J, NSG or NOD.Cg-Foxn1em1DvPrkdscidl2rgtm1Wgl/J hairy mice were anaesthetised with an intraperitoneal injection of Midazolam (5 \( \mu \)g/g) and Ketamine (75 \( \mu \)g/g) or Ketamine alone (100-120 \( \mu \)g/g). Where Midazolam was injected, 0.5 \( \mu \)g/g Flumazenil was intraperitoneally injected for reversal. After the anesthesia took effect, mice were suspended from the top incisors to allow access to the trachea and were intratracheally intubated with an I.V. 22 Gauge cannula (Sentrawin). 50 \( \mu \)l of 2\% w/v polidocanol - nonaethylene glycol monododecyl ether (Sigma) in PBS or 10\(^{6}\) mCherry+DF508 nasal epithelial cells in 100 \( \mu \)l cFAD medium were administered via the cannula using a Hamilton\textsuperscript{®} Microsyringe (Sigma). Where the volume of administered solution was under 100 \( \mu \)l, additional air was administered with the microsyringe, up to a total volume of 100 \( \mu \)l.

Mice were culled with an intraperitoneal injection of 100\(\mu\)l of equal volumes of Lignol\textsuperscript{®} (2\% w/v) and Euthatal\textsuperscript{®} Pentobarbital Sodium (200 mg/mL). After the paw pinch reflex was lost, the
abdominal aorta was severed for permanent cessation of circulation. Lungs were subsequently harvested and retained in 4% PFA until processing.

*In vivo* experiments were performed under supervision from Robin McAnulty (Respiratory Centre for Inflammation and Tissue Repair, UCL, London) or Ruhina Maeshima (GOS Institute of Child Health, UCL, London).
CHAPTER 3. CULTURE AND CHARACTERISATION OF PRIMARY HUMAN AIRWAY EPITHELIAL CELLS
3.1. Introduction

To study a disease of the airways such as asthma, chronic obstructive pulmonary disease (COPD) and Cystic Fibrosis (CF), it is crucial to be able to work with the appropriate cell models and assays. In the context of CF, immortalised cell lines of airway epithelial cells have in the past contributed greatly to research; however, their generation is inefficient and does not represent patient to patient variability. Only rare clones are able to expand and be maintained under the immortalisation procedure and these clones have undergone significant genetic changes, no longer retaining the parental cells’ features (Awatade et al., 2018). In many occasions, these cells are also unable to polarise and undergo mucociliary differentiation, especially after a few passages (Randell et al., 2011).

The need for models that are more patient specific and that accurately represent *in vivo* processes has introduced cell models derived from human embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells derived from the airways. Ethical concerns concerning ESCs limit their use while iPSCs demonstrate karyotype-instability, tumorigenicity and altered antigenicity (Suprynowicz et al., 2012).

Primary airway epithelial cells, directly derived from the airways of patients, maintain the gene expression and phenotype observed *in vivo* and are not prone to mutations when dividing (Bhowmick and Gappa-Fahlenkamp, 2016). They can represent the disease manifestation in individual patients and therefore constitute excellent material for disease modelling and drug testing. Primary cells however, are very sensitive to culture conditions and can be expanded for a limited number of passages, before becoming terminally differentiated and reaching senescence.

Conventionally, human airway epithelial cells are cultured as monolayers on plastic (2D). This expansion phase is crucial to generate enough cell numbers for differentiation of cells under air-liquid interphase conditions (ALI). Airway epithelial cells differentiated on ALI cultures demonstrate a phenotype resembling the lower airway epithelium; a pseudostratified epithelium with mucus secretion and beating cilia (mucociliary differentiation). They also form tight junctions and develop trans-epithelial resistance (TEER) (Bérubé et al., 2010). The differentiated cultures are crucial for measurement of CFTR function *in vitro*, as the protein is expressed only in differentiated cells (Awatade et al., 2018). For decades it was believed that
CFTR could be found in the apical surface of ciliated cells, however recent findings locate the majority of CFTR transcripts in a specific type of differentiated cells, called ionocytes (Montoro et al.; 2018; Plasschaert et al., 2018).

In order to extend the lifespan of primary airway epithelial cells in vitro and delay senescence, culture conditions that include the presence of an irradiated feeder layer of mouse embryonic fibroblasts and the Rho-associated protein kinase inhibitor (Y-27632) are increasingly being used. Upon removal of the feeder layer and Y-27632, cells can be successfully differentiated in ALI cultures (Reynolds et al., 2016; Butler et al., 2016; Martinovich et al., 2017).

Other culture conditions to extend the lifespan of primary airway epithelial cells include a feeder-free protocol which disrupts the SMAD signaling pathway by dual inhibition of TGFβ/BMP, but have so far not been adopted widely (Mou et al., 2016).

Primary airway epithelial cells of the lower airways can be sourced from biopsy samples, lung explants and cadavers. All these sources provide high cell yield, however only a biopsy sample can be acquired from a living donor and this procedure is highly invasive (Awatade et al., 2018). As a result, epithelial cells from the upper airways (nasal cells) are often used as an alternative (Courcey et al., 2012; Brewington et al, 2018). Nasal cells can be acquired through minimally invasive nasal brushings and maintain the characteristics of lower airway cells’ such as the ability to differentiate to a polarised mucociliary epithelium and the expression of CFTR (Awatade et al., 2018).

For a combined cell and gene therapy for CF, not only do we need a cell model which accurately represents in vivo functions, we imperatively need non-immortalised primary cells which can be edited in vitro and then returned to the patient airways as a transplant. Maintaining all the characteristics and processes of these cells while achieving a high number of cells during expansion is crucial both for in vitro manipulation and in vivo transplantation.

An important limitation of airway epithelial cells is their poor transfection efficiency. It is widely accepted that airway epithelial cells are resistant to transfection, even more so when already differentiated in ALI cultures (Randell et al., 2011). Liposome – DNA complexes only transflect cells at the edges of epithelial cell colonies and this accounts for poor efficiencies (Matsui et al., 1997). For successful and efficient gene editing, this limitation needs to be overcome and an efficient transfection method should be optimised for airway epithelial cells. An alternative
technique for transfection, nucleofection, has been used effectively for immortalised airway epithelial cells and primary alveolar epithelial cells and resulted in high numbers of transfected cells (Maurice et al., 2010; Grzesik et al., 2013).

3.2. Aims

In this chapter I aim to:

1. Establish cell culture conditions for the long-term expansion of different types of primary airway epithelial cells.
2. Differentiate primary airway epithelial cells in air-liquid interphase (ALI) cultures.
3. Characterise primary airway epithelial cells through immunofluorescence during expansion and differentiation conditions.
4. Demonstrate CFTR expression and function in differentiated primary airway epithelial cells through immunofluorescence, Western Blots and Ussing chamber measurements.
5. Optimise transfection conditions for primary airway epithelial cells.
3.3. Isolation, culture and characterisation of Human Fetal Tracheal Cells

Human fetal material was provided by the Joint MRC/Wellcome Trust Human Developmental Biology Resource (HDBR) and tracheal tissue was collected from 3 fetuses (Table 3.1). The epithelium was detached and dissociated to single cells in order to isolate Human Fetal Tracheal Cells (HuFeTRC) (Figure 3.1 and Figure 3.2). Paraffin sections of the fetal tracheal tissue were stained with Haematoxylin & Eosin (H&E) staining before and after the removal of the epithelium.

Table 3.1. Details of human fetal tracheas processed. The table shows the number of samples, their respective developmental ages and the name of the successfully isolated cells.

<table>
<thead>
<tr>
<th>Sample no</th>
<th>Weeks post conception (wpc)</th>
<th>Name of Cultivated cells</th>
<th>Whole trachea sample - fixed and embedded in paraffin</th>
<th>Sample with removed epithelium - fixed and embedded in paraffin</th>
</tr>
</thead>
<tbody>
<tr>
<td>13426</td>
<td>20</td>
<td>HuFeTRC01</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>13510</td>
<td>13</td>
<td>HuFeTRC02</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>13536</td>
<td>20</td>
<td>HuFeTRC03</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
Figure 3.1. Human trachea isolated from a 20 weeks post conception (wpc) fetus. (A) Gross microscopy of 20 wpc human trachea. Scale bar = 2 mm; (B) Fresh tissue after removal of the epithelial layer. Scale bar = 0.5 mm; (C) Haematoxylin & Eosin (H&E) of 20 wpc human trachea consisting of the respiratory epithelium (★), the sub-mucosa, the cartilage and smooth muscle layer; (D) H&E stain of human trachea after removal of the epithelial layer, used for dissociation to derive cells in culture. Scale bars = 100 μm.

Figure 3.2. Human trachea isolated from a 13 weeks post conception (wpc) fetus. (A) Gross microscopy of 13 wpc human trachea. (B) Appearance of the trachea after removal of the epithelial layer. Scale bars = 2 mm.
Isolated HuFeTRC were cultured over a lethally irradiated feeder layer of 3T3-J2 mouse embryonic fibroblasts without the use of Y-27632 and were passaged every 7 days. The HuFeTRC expand and displace the surrounding fibroblasts. They give rise to round colonies with small, compact cells at early passages and larger, more elongated cells towards the later passages. At passages 10-12 large cells can be observed in the center of the colonies (Figure 3.3).

Figure 3.3. Human Fetal Tracheal Epithelial cells expanded in co-culture with irradiated feeder layer (3T3). Phase contrast images of HuFeTRC01, passages 1-12. Epithelial cells grow in round colonies and displace the surrounding fibroblasts, as indicated in panel 1. Scale bar = 50 μm
HuFeTRC01 were maintained in culture for at least 13 passages (84 days in culture) and had a cumulative cell number of $10^{15}$ (30 population doublings) (Figure 3.4). HuFeTRC01 were stained for immunofluorescence and were positive for epithelial cell marker E-cadherin, proliferation marker ki67 and basal cell markers CK5/14 and p63 (Figure 3.5).

**HuFeTRC01 growth curve**

![Graph showing growth curve of HuFeTRC01](image)

Figure 3.4. Growth curve of HuFeTRC01. The cumulative cell number of HuFeTRC01 across 13 passages (84 days in culture) is $10^{15}$ which equals to 30 population doublings. Curve represents results from one experiment.
Figure 3.5. Immunofluorescence of HuFeTRC01 passage 3, expanded *in vitro*. HuFeTRC01 are (A) positive for epithelial cell marker E-cadherin (green) and proliferation marker ki67 (red); (B) positive for basal cell marker CK5/14 (red) and transcription factor p63 (green). (C) No primary antibody negative control. Scale bars = 50 μm.

Both HuFeTRC02 and HuFeTRC03 cultures displayed mesenchymal cell morphology after 4 and 2 passages respectively (Figure 3.6). As a result, no further experiments were conducted using
these cells. Further investigation on the causes of mesenchymal transition and optimisation of the fetal culture was not conducted as it was not within the scope of this thesis.

Figure 3.6. Human Fetal Tracheal Epithelial cells are expanded in co-culture with irradiated feeder layer (3T3). Phase contrast images of: (A) HuFeTRC02 passage 1, day 7, epithelial cell colony; (B) HuFeTRC02 passage 4, day 7, mesenchymal transition; (C) HuFeTRC03 passage 1, day 7, epithelial colony; (D) HuFeTRC03 passage 2, day 7, mesenchymal transition. Scale bar = 50μm.

3.4. Liposome transfection of Human Fetal Tracheal Cells

HuFeTRC01 were used to test the efficiency of liposome based transfections in airway epithelial cells. HuFeTRC01 were transfected with 2 μg of pEGFP-N1 using 1.5 μl, 2 μl or 3 μl of Lipofectamine 3000. FACS analysis 24 hours post transfection demonstrated that 10.5% of the cells were GFP+ when 3 μl of Lipofectamine 3000 was used. Transfections with lower amounts of Lipofectamine resulted in less GFP+ cells (Figure 3.7). Feeder layer cells were not separated.
from the epithelial cells with cell markers or via physical parameters during sorting, however transfection of 3T3 cells alone, resulted in 0.8% GFP+ cells.
Figure 3.7. Transfection of HuFeTRC with pEGFP-N1 using Lipofectamine 3000. FACS plots show gating for (A) total cells; (B) live (DAPI negative) cells; (C) single cells and (D) GFP+ cells in non-transfected control. Transfection efficiency is (E) 4.10% for cells transfected with 1.5 μl of lipofectamine per well; (F) 8.63% for cells transfected with 2 μl of lipofectamine per well, (G) 10.50% for cells transfected with 3 μl of lipofectamine per well; (H) 0.80% for 3T3-J2 cells. The number of cells per reaction was approx. 10000. Plots represent results of one experiment per condition.

3.5. Culture and characterisation of Adult Airway Epithelial Cells

One vial of Normal Human Bronchial Epithelial cells (NHBEs) was acquired from the McGill University Primary Airway Cell Biobank (PACB) and cells were expanded in four different conditions: in co-culture with irradiated 3T3 feeder layer with and without the use of Y-27632 (cFAD medium), and on plastic with and without the use of Y-27632 in BEGM medium.

In presence of irradiated 3T3 feeder layer, the cells formed distinct round colonies, while on plastic the cells spread uniformly across the flask (Figure 3.8). Importantly, cells plated on plastic could not be expanded longer than passage 4, whereas cells on irradiated 3T3 feeder layer could be expanded for more than 7 passages. The use of Y-27632 helped to achieve higher cell numbers at each passage (Figure 3.9).
Figure 3.8. Expansion of Normal Human Bronchial Epithelial Cells (NHBEs). Phase contrast images of NHBE colonies expanded in co-culture with irradiated feeder layer (3T3) after 7 passages (42 days in culture): cells can be expanded (A) with Y-27632 and (B) without Y-27632 and form round colonies. Phase contrast images of NHBE expanded on plastic after 2 passages (14 days in culture): cells can be expanded (C) with Y-27632 and (D) without Y-27632. Scale bar = 100 μm.
Figure 3.9. Growth curve of NHBE. The cumulative cell number of NHBEs across 8 passages (49 days in culture) is $10^{21}$ (47 population doublings) for cells grown with 3T3 + Y-27632, $10^{19}$ (42 population doublings) for cells grown with 3T3 and no Y-27632, $10^{10}$ (16 population doublings) for cells grown on plastic with Y-27632 and $10^9$ (14 population doublings) for cells grown on plastic without Y-27632. Curves represent results of one experiment per condition.

One vial of nasal Epithelial Cells with the ΔF508 CFTR mutation (ΔF508 NECs) were acquired from the GOS Institute of Child Health UCL Living Airway Biobank and the cells were expanded in two different conditions: in the presence of irradiated 3T3 feeder layer with and without the use of Y-27632. Since cells were originally expanded with the use of Y-27632 at the biobank facility, it was only possible to remove Y-27632 starting at passage 4. The cells on the irradiated 3T3 feeder layer formed round, distinct colonies and could be expanded for more than 11 passages (Figure 3.10).
ΔF508 Nasal Epithelial Cells (ΔF508 NECs) expanded in co-culture with irradiated feeder layer (3T3). Phase contrast images of ΔF508 NEC colonies after 9 passages (45 days in culture): cells can be expanded (A) with Y-27632 and (B) without Y-27632 and form round colonies. (C) The cumulative cell number of ΔF508 NECs across 11 passages (60 days in culture) is $10^{18}$ (44 population doublings) for cells grown with 3T3 + Y-27632 and (D) the cumulative cell number of NECs across 9 passages (69 days in culture) is $10^{14}$ (27 population doublings) for cells grown with 3T3 and no Y-27632 (Y-27632 was removed after passage 4). Curves represent results of one experiment per condition. Scale bars = 100 μm.

NHBEs and ΔF508 NECs were expanded repeatedly for different experiments. The cumulative cell numbers and population doublings were calculated and found to be comparable in two independent experiments. Figure 3.11 represents a comparison between two growth curves of ΔF508 NECs expanded with 3T3 and Y-27632. The cells were expanded for 11-12 passages and the cumulative cell number was $10^{18}$-$10^{19}$ (42-44 population doublings).
Figure 3.11. Comparison of two independent ΔF508 NEC growth curves. Curve 1 represents cells expanded for 11 passages (60 days in culture) with a cumulative cell number of $10^{19}$ (44 population doublings). Curve 2 represents cells expanded for 12 passages (67 days in culture) with a cumulative cell number of $10^{18}$ (42 population doublings). Curves represent n=2 of independent experiments.

NHBEs and ΔF508 NECS were stained for immunofluorescence and were positive for E-cadherin, proliferation marker ki67, basal cell markers CK5/14 and p63, epithelial cell marker CK8, epithelial cell adhesion molecule EpCAM and transcription factor SOX9 (Figure 3.12 and Figure 3.13).
Figure 3.12. Immunofluorescence of NHBEs passage 4, expanded in vitro on irradiated feeder layer without Y-27632. NHBEs are (A) positive for epithelial cell marker E-cadherin (green) and proliferation marker ki67 (red); (B) positive for basal cell marker CK5/14 (red) and transcription factor p63 (green); (C) positive for airway epithelial cell marker CK8 (red) and epithelial adhesion molecule EpCAM (grey) and (D) positive for transcription factor SOX9 (red). (E) No primary antibody negative control. Very bright fluorescent formations next to the main colony in panel C are single epithelial cells with strong CK8 expression. Strong fluorescence in panel D on the epithelial colony indicates terminal differentiation of epithelial cells and cell pile-up. Scale bars = 50μm.
Figure 3.13. Immunofluorescence of ΔF508 NEC passage 10, expanded in vitro on irradiated feeder layer with Y-27632. ΔF508 NEC are (A) positive for epithelial cell marker E-cadherin (green) and proliferation marker ki67 (red); (B) positive for basal cell marker CK5/14 (red) and transcription factor p63 (green); (C) positive for airway epithelial cell marker CK8 (red) and epithelial adhesion molecule EpCAM (grey) and (D) positive for transcription factor SOX9 (red). (E) No primary antibody negative control. Very bright fluorescent formations next to the main colony in panel C are single epithelial cells with strong CK8 expression. Strong fluorescence in panel D on the epithelial colony indicates terminal differentiation of epithelial cells and cell pile-up. Scale bars = 50μm.

In order to test in vitro differentiation potency of airway epithelial cells, NHBEs and ΔF508 NECs were plated in Air Liquid Interface (ALI) cultures. Cells formed a monolayer on cell culture inserts within 2 days post seeding and beating motile cilia were observed under the microscope after 3 or 4 weeks, depending on the culture. Short movies of the beating cilia were acquired and subsequently the cultures were fixed and stained for immunofluorescence analysis. Confocal images were acquired and processed for 3D reconstruction with Volocity software. Differentiated ΔF508 NECs were positive for airway epithelial cell marker CK8, differentiated airway epithelium mucosecretory marker MUC5AC, tight junctions marker ZO-1 and cilia marker acetylated tubulin (Figure 3.14). When using Pneumacult ALI differentiation media, NHBEs and ΔF508 NECs were also observed to be positive for FOXI1 ionocyte marker and importantly CFTR (Figure 3.15).
Figure 3.14. 3D reconstruction of confocal images of NHBEs and ΔF508 NECs differentiated in vitro in air liquid interface (ALI) cultures with BEBM/DMEM + Retinoic Acid+ BulletKit. NHBEs are (A) positive for airway epithelial cell marker CK8 (red) and differentiated airway epithelium mucosecretory marker MUC5AC (green), (B, C) positive for tight junctions marker ZO-1 (red) and cilia marker acetylated tubulin (green). ΔF508 NECs are (D) positive for CK8 (red) and MUC5AC (green), (E, F) positive for ZO-1 (red) and acetylated tubulin (green). (G) No primary antibody negative control. Scale bars = 13.5 μm.
Figure 3.15. 3D reconstruction of confocal images of NHBEs and ΔF508 NECs differentiated in vitro in air liquid interface (ALI) cultures with Pneumacult media. ΔF508 NECs are (A) positive for FOXI1 ionocyte marker (green) and (B) positive for CFTR (green). NHBEs are (C) positive for FOXI1 ionocyte marker (green) and (D) positive for CFTR (green). (E) No primary antibody negative control.

NHBEs and ΔF508 NECs were also cultured in ALI cultures with the purpose of measuring the resistance and short circuit current of in vitro differentiated cells by using Ussing chambers. Cells that were expanded on irradiated 3T3 feeder layer and successfully differentiated in ALI cultures
with BEBM/DMEM + Retinoic Acid + BulletKit were highly resistant and showed large currents (Table 3.2 and Figure 3.16). Cells that were expanded on irradiated 3T3 feeder layer and were fed in ALI cultures with cFAD + Retinoic Acid did not fully differentiate, as they showed very low resistance and minimal currents. Both NHBEs and ΔF508 NECs showed Epithelial Sodium Channel (ENaC) inhibition when treated with amiloride, whereas only NHBEs showed CFTR activation and inhibition when treated with forskolin & IBMX (3-isobutyl-1-methylxanthine) and CFTR inhibitor 172 respectively (Figure 3.16). These chloride channel responses were expected and confirmed the phenotype of healthy (responsive) or CF (not responsive) cells.

Table 3.2. Transepithelial resistance of cell monolayers on Ussing chambers, calculated using Ohm’s law from stimulated short circuit (I_sc) measurements, before treatment. Cells differentiated with BEBM/DMEM + Retinoic Acid + BulletKit show high resistance while cells fed with cFAD + Retinoic Acid show minimal resistance.

<table>
<thead>
<tr>
<th>CULTURE</th>
<th>RESISTANCE (Ω)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHBE P7 BEBM/DMEM + Retinoic Acid + BulletKit</td>
<td>690</td>
</tr>
<tr>
<td>ΔF508 NEC P10 BEBM/DMEM + Retinoic Acid + BulletKit</td>
<td>1285</td>
</tr>
<tr>
<td>NHBE P7 cFAD + Retinoic Acid</td>
<td>25</td>
</tr>
<tr>
<td>ΔF508 NEC P10 cFAD + Retinoic Acid</td>
<td>19</td>
</tr>
</tbody>
</table>

Figure 3.16. Effects of epithelial sodium channel (ENaC) inhibitor (amiloride), CFTR activator (forskolin + IBMX) and CFTR inhibitor 172 in stimulated short circuit current (I_sc) of NHBE and ΔF508 NEC cultures. (A) NHBEs and ΔF508 NECs fed with BEBM/DMEM + Retinoic Acid+ BulletKit in ALI cultures both show ENaC
inhibition. NHBEs additionally show CFTR activation and inhibition while ΔF508 cells lack CFTR responses; (B) NHBEs and ΔF508 NECs fed with cFAD + Retinoic Acid in ALI cultures show minimal ENaC and CFTR responses (Graphs by Max Woodall). Measurements represent results of one experiment per cell type and differentiation state.

3.6. Detection of CFTR protein in NHBEs and ΔF508 NECs by Western Blot

Protein lysate from cells differentiated on ALI cultures in Pneumacult media was used to detect CFTR protein. Protein was detected in both NHBEs and ΔF508 NECs with the NHBEs presenting both the B and C bands and ΔF508 NECs presenting only the B band. CFTR is expressed in very low levels while the overexpressed (NHBEs + CFTR mRNA) controls contain very high amounts of protein without being very concentrated. As a result, the Na+/K+-ATPase in the loading control is not detectable (Figure 3.17).

![Western Blot Image](image)

**Figure 3.17.** Western blots of NHBEs and ΔF508 NECs for CFTR protein. (A) CFTR appears in the NHBEs as bands B (150 kDa) and C (170-180 kDa). Na+/K+-ATPase was used as a loading control. The overexpressed (NHBEs + CFTR mRNA) positive control contains very high amounts of protein without sample being concentrated. As a result, Na+/K+-ATPase in the positive control is not detectable. (B) CFTR appears in the ΔF508 NECs as band B (150 kDa). Total protein content was measured with Coomassie stain. Blots represent one experiment for each of the two cell types.
3.7. Nucleofection of Adult Airway Epithelial Cells

In order to establish optimal conditions for the transfection of airway epithelial cells, NHBEs were nucleofected with 5 μg pmaxGFP using different pulse settings. Pulse width was kept at 20 ms and pulse number at 2, and the range of pulse voltage ranged from 1100 mV to 1500 mV. 48 hours post nucleofection, images were captured that show GFP+ (green) cells under every condition (Figure 3.18).

On the same day, cells were harvested, fixed (FACS facility of institute required fixation of human material) and stained for EpCAM (Alexa Fluor 660) before FACS analysis. Staining for EpCAM allowed to distinguish epithelial cells from the 3T3 feeder cells. After gating on EpCAM+ cells, the GFP+ cells within the epithelial cell population were 45.2% at 1100 mV, 63.7% at 1200 mV, 65% at 1300 mV, 63.8% at 1400 mV and 42% at 1500 mV (Figure 3.19). Based on these results, the best settings for nucleofection of NHBE is 2 pulses of 20 ms pulse width and 1200 mV pulse voltage, since among voltages with similar transfection efficiency, the lowest one will also result in lower cell death.
Figure 3.19. Nucleofection of NHBEs with pmaxGFP. FACS plots show gating for (A) total cells, (B) single cells, (C) EpCAM+ cells in GFP+ (single) control (D) EpCAM positive cells in EpCAM positive (single) control, (E) GFP+ cells in EpCAM positive (single) control. Nucleofection efficiency is (F) 45.2% for cells nucleofected at 1100 mV, (G) 63.7% for cells nucleofected at 1200 mV (H) 65% for cells nucleofected at 1300 mV, (I) 63.8% for cells nucleofected at 1400 mV and (J) 42% for cells nucleofected at 1500 mV. The pulse width remained constant at 20 ms and the pulse number was 2. The number of cells per reaction was approx. 90000. Plots represent results of one experiment per condition.

Next, the best conditions and settings for nucleofection of NHBEs were tested in the ΔF508 NECs. ΔF508 NECs were nucleofected with 5 μg GFP-N1 at 2 pulses of 20 ms pulse width and 1200 mV pulse voltage. 48 hours post nucleofection, the cells were harvested and stained for anti-feeder antibody (PE) which allowed separation of the epithelial cells from the 3T3 cells. After gating on the PE- cells, the GFP+ cells within the epithelial cell population were 65.7% (Figure 3.20). Nucleofection settings at 2 pulses of 20 ms pulse width and 1200 mV pulse voltage were used for all following experiments.
Figure 3.20. Nucleofection of ΔF508 NECs with pEGFP-N1. FACS plots show gating for (A) total cells, (B) live cells, (C) single cells, (D) Feeder cells (PE+) in GFP+ (single) control (E) Feeder cells (PE+) in PE positive (single) control, (F) GFP+ cells in PE+ (single) control. Nucleofection efficiency is (G) 65.7% for cells nucleofected at 1200 mV, with a pulse width of 20 ms and a pulse number was 2. The number of cells was approx. 400000. Plots represent results of one experiment per condition.
3.8. Discussion

The type and quality of cells used for cell therapy are very important factors for its success. Crucially, we need to maintain the progenitor pool to ensure lifelong capability of airway epithelial cells to self-renew and expand. We believe that patients’ airway epithelial cells represent the most appropriate cell type for CF cell therapy. These cells can be expanded in the presence of irradiated 3T3 feeder layer, to increase their proliferation capacity, a key feature for in vitro gene editing and cell transplantation (Butler et al., 2016, Gentzsch et al., 2017)

We have obtained CF patient cells through nasal brushings, a minimally invasive procedure, which can be applied routinely in the clinic. Alternative methods for airway epithelial cell harvesting, like bronchoscopies or bronchial biopsies could potentially provide a larger number of cells, but are significantly more invasive. To our knowledge, there are no studies demonstrating basal cell enrichment or better suitability for cell therapy of cells originating from these sources.

The nasal brushing procedure provided us with enough cells to start an epithelial cell culture and achieve, after passaging, cumulative cell numbers which exceed $10^{19}$ (with 3T3 and Y-27632). These numbers already exceed by several orders of magnitude the cell number required for in vivo studies in small animal models and even human cell therapy. The number of epithelial cells used for repopulation of murine lungs is usually around the range of $10^6$ (Milman Krentsis et al., 2018, Ghosh et al., 2017), whereas estimates of number of cells needed for repopulating human lung epithelium are in the range of $4.5 \times 10^{10}$ (Calle et al., 2014, LaRanger et al., 2017). The numbers we achieve are, in addition, higher than those reported in the literature. Gentzsch et al. (Gentzsch et al., 2017) can achieve less than 30 population doublings over 40 days for CF patient cells with the ΔF508 mutation when using irradiated feeder layer and Y-27632, while we can achieve at least 42 population doublings over 67 days under the same conditions, in n=2 independent experiments.

We have demonstrated that the ΔF508 NEC cells are positive for basal cell markers (CK5/14 and p63) while in expansion conditions and can be differentiated in ALI cultures. Following differentiation, the cells are positive for ciliation (acetylated-tubulin) and mucus (MUC5AC) markers. Importantly they differentiate towards ionocytes (FOXI1 positive cells), express CFTR and are functionally responsive in stimulated short circuit current after at least nine passages.
These results show that we can cultivate and characterise nasal epithelial cells from CF patients with the ΔF508 mutation (ΔF508 NECs). With our culture conditions, we can both retain long term expansion and basal cell state and subsequently differentiate these cells into a functional epithelium *in vitro*.

Normal fetal cells are a promising alternative for cell therapy applications. Fetal cells are capable of faster and more frequent proliferation than adult cells and fetal tissue is less promptly rejected by the recipient during transplantation due to lower levels of histocompatibility antigens. Fetal cells are also known to survive grafting and low oxygen conditions during transplantation better (Bhattacharya et al., 2004).

We have isolated and expanded HuFeTRC over an irradiated feeder layer, demonstrating their potential for long term expansion and shown that HuFeTRC are positive for basal cell markers throughout their submerged culture conditions.

We were unable to maintain the culture of two out of the three isolated HuFeTRC, due to the appearance of mesenchymal cells. We did not perform any assays to study why our culture behaved in this way however, we hypothesise that the mesenchymal cells could have either a. originated from the tracheal tissue and overgrown in the culture through passages or b. been the product of epithelial-mesenchymal transition (EMT). Normally, the irradiated 3T3 feeder layer inhibits the growth of mesenchymal cells, therefore mesenchymal cells originating from the tracheal tissue should not survive under our culture conditions. Stressful culture conditions however, can cause EMT, especially in fetal cells from early stages that are less committed. As an indication, fetal hepatocytes have been observed to undergo EMT in low-density cultures (Chinnici et al., 2017).

Due to further problems with growing and expanding HuFeTRC we were not able to culture HuFeTRC on ALI to demonstrate whether they are capable of differentiation, similarly to cells harvested postnatally. In the future, further work on fetal cells could help to provide suitable material for *in vivo* transplantation (Rosen et. al, 2015).
Adult airway epithelial cells with a healthy phenotype can provide excellent cell models for experiments and be used as controls.

We have successfully cultured NHBEs over an irradiated feeder layer and demonstrated their potential for long term expansion with and without Y-27632. We report 47 population doublings over 49 days for cells grown with irradiated feeder layer and Y-27632, a number higher than previous literature reports, which is however based on one growth curve experiment. For normal (healthy) airway epithelial cells grown under the same conditions, Gentzsch et al. (Gentzsch et al., 2017) achieve less than 35 population doublings over 40 days and Butler et al. (Butler et al., 2016) achieve 23 population doublings over 50 days. NHBEs, similarly to the ΔF508 NECs, can also be differentiated on ALI cultures, express CFTR and show activation and inhibition currents in Ussing chambers.

NHBEs cannot be used for the development of CF ex vivo gene therapy, however they can be used for the generation of CFTR knockouts. This will allow us to create cell models of the disease and use in parallel the same WT (healthy) cells for comparison. Such comparisons could give insights on differences between healthy and CF cells under expansion and differentiation conditions, in Ussing chambers and very importantly, at seeding and engraftment experiments.

ALI cultures are a very informative assay to study the differentiation capacity of airway cells. Throughout our experiments, we used two different media commonly used for ALI cultures: the standard 1:1 BEGM/DMEM + BulletKit and the newer Pneumacult. While using the BEGM/DMEM + BulletKit we often observed “islands” of ciliated cells, instead of an even distribution across the cell culture insert. Cells took longer than 3 weeks to differentiate and ciliation was observed at the edges of the cell culture inserts more than in the middle. Changing ALI medium to Pneumacult, cells ciliated in 2 weeks approximately and a uniform distribution of ciliated cells was observed. Importantly, the presence of ionocytes and a positive CFTR stain and Western Blot were only observed under differentiation in Pneumacult medium. Rayner et al. similarly observe superior differentiation of NHBEs under Pneumacult medium. Interestingly, with their expansion culture method, which does not include a feeder layer, they can only differentiate cells up to passage 4, while we have observed successful differentiation up to the 10th passage (Rayner et al., 2019).
In terms of protein expression, we were able to visualise CFTR via immunofluorescence and record the positive response in Ussing chambers in NHBEs, while in ΔF508 NECs we acquired a positive stain but no response in Ussing chambers, as anticipated. In Western blots, CFTR appeared as both band C (complex glycosylated form) and B (immature form) in NHBEs and only as band B in ΔF508 NECs. This result is consistent with findings of other studies, where it is reported that the ΔF508 mutation causes protein mislocalisation and improper glycosylation. As a consequence, only band B is present in cells with this mutation (Cebotaru et al., 2008). Western blots will be useful to confirm correction of CFTR in ΔF508 NECs as well as successful knockout of the protein in NHBEs.

Liposome based GFP transfections of airway epithelial cells, as demonstrated with the HuFeTRC, are inefficient, however, for the aims of this project, where gene therapy is attempted ex vivo, it is not necessary to use liposome based transfections as is in in vivo gene therapy. Higher transfection efficiencies can be achieved with the help of electric pulses (nucleofection). We have shown that NHBEs and ΔF508 NECs can be nucleofected efficiently with GFP. For our nucleofections we used the Neon system as opposed to the most cited Amaxa nucleofection system (Lonza) (Maurisse et al., 2010; Rapiteanu et al., 2019). The advantage of the Neon system is that settings can be optimised for different cell types, by adjusting three different parameters: pulse width, pulse number and pulse voltage. This gives the user freedom, as opposed to working with the Amaxa where programs are cryptic. However, full optimisation with the Neon system, constitutes a very laborious and expensive procedure where all the different combinations of settings need to be tested.

The optimised conditions of the GFP nucleofections can be used as a guideline for the CRISPR/Cas9 experiments even though our CRISPR/Cas9 strategy involves transfection of three separate components (Cas9 protein, sgRNA and repair plasmid). With nucleofection, we transfect and edit basal cells that can be subsequently differentiated into mature cell types expressing CFTR. Differentiation is possible either in vitro in ALI cultures or in vivo after transplantation, as a means to correct cystic fibrosis by cell therapy.
CHAPTER 4. CRISPR/CAS9 CORRECTION OF CFTR IN ΔF508 NASAL EPITHELIAL CELLS
4.1. Introduction

*Ex vivo* gene therapy for cystic fibrosis requires the restoration of a functional CFTR gene in the diseased patient cells. An obvious way of achieving this is through a virus: indeed, delivery of CFTR via retrovirus, lentivirus, adenovirus and adeno-associated virus has been attempted in various studies, primarily as a means to target the airway epithelium for *in vivo* gene therapy (Johnson, 2001; Castellani and Conese, 2010). For *ex vivo* gene therapy, lentiviral delivery of CFTR to the cells *in vitro* should be a straightforward task. However, CFTR integration in random parts of the genome, which could include regulatory elements and proto-oncogenes, makes such an approach problematic for therapeutic purposes.

An alternative approach for restoring CFTR is offered through CRISPR/Cas9, which allows precise manipulation of genes via gene editing. CRISPR/Cas9 can precisely target genes to achieve knockouts with high efficiency or correct genes utilising the HDR pathway. Correction of genes by HDR is limited by the fact that it is an inefficient process, where one out of hundreds or thousands of cells will be corrected. For primary cells, where single cell cloning is very difficult, if not impossible, identifying a corrected cell and expanding it to numbers necessary for downstream experiments is an insurmountable hurdle. For cell therapy applications, single cell cloning creates an additional issue, as every cell carries the same, potentially detrimental, genetic changes.

Standard HDR requires a repair template which includes the corrected sequence of the gene and long homologous arms for precise targeting. This template usually comes in the form of a single-stranded oligo DNA nucleotide (ssODN). To increase the efficiency of HDR, various methods have been suggested: longer homology arms, synchronisation of cells in the G2/S phase of the cell cycle and compounds that can act as HDR enhancers have been used in different studies (Liu et al., 2019).

A way to increase the number of corrected cells in culture rather than increase HDR efficiency itself, is to include a selection marker in the repair template, in the form of an antibiotic or a fluorescent protein. Here, cells that have been corrected with HDR can be selected through their resistance to antibiotics or with FACS, while non-corrected cells are eliminated, bypassing the need for single cell cloning. Two issues are associated with the use of a selection marker in HDR experiments. The first is the size of the repair template: selection markers are a few kb of DNA.
long and when combined with long homology arms, they cannot be delivered as ssODNs but rather have to be delivered in the form of a plasmid. The second issue has to do with the position of the selection marker in the corrected cells. For scarless correction and restoration of the gene function the selection marker has to be removed after the HDR correction. Such removal can prove cumbersome as it relies on molecular tools other than CRISPR/Cas9, such as Cre/loxP or piggyBac. Alternatively, a marker can be designed to incorporate into an intronic region and therefore removal is not required for gene functionality.

An important step for successful HDR that can be optimised to a greater degree, is the efficiency of double strand breaks (DSBs) created by CRISPR/Cas9 close to the mutation location. HDR can only repair sequences that have first been broken; increasing the percentage of broken sequences will, as a consequence, increase HDR. In the early days of CRISPR/Cas9, most studies used plasmids which expressed both the Cas9 and guide RNAs, for the creation of these DSBs. More recently, the option to use Cas9 in the form of a protein and guide RNAs as synthetic guide RNAs is becoming more popular. The advantages of not using plasmids are clear: random plasmid integrations in the genome are avoided and as Cas9 protein does not require transcription, it is immediately available to be used for editing. Additionally, protein will be turned over by the cell faster than plasmid, thus avoiding further cutting at off-target sites (Kim et al., 2014; Liang et al., 2015).

4.2. Aims

In this chapter I aim to:

1. Construct a repair plasmid for CRISPR/Cas9 HDR correction of primary nasal epithelial cells with the ΔF508 CFTR mutation (ΔF508 NECs).
2. Optimise CRISPR/Cas9 mediated double strand breaks (DSBs) with a selected guide RNA located close to the ΔF508 mutation in ΔF508 NECs.
3. Select optimal puromycin concentration for puromycin selection of ΔF508 NECs.
4. Create puromycin-resistant 3T3-J2 cells for support of the epithelial culture during puromycin selection.
5. Correct the ΔF508 CFTR mutation in ΔF508 NECs via the CRISPR/Cas9 HDR pathway.
4.3. Construction of two repair templates for CRISPR/Cas9 correction of the ΔF508 mutation via the HDR pathway

Sanger sequencing was conducted in ΔF508 NECs in order to confirm the ΔF508 CFTR mutation. According to Ensembl, in the human population, there are two polymorphisms resulting in the deletion of the Phenylalanine amino acid, named TMP_ESP_7_117199645_117199647 and rs113993960. Polymorphism TMP_ESP_7_117199645_117199647 was found to be present in the ΔF508 NECs, meaning that a nucleotide triplet of TCT is missing from CFTR compared to the healthy (WT) sequence. Figure 4.1 shows the ΔF508 NEC sequencing result of CFTR exon 10, with indications at the ΔF508 mutation site and the targeting site of the sgRNA. The full sequence of the cells 1000 bp upstream and 1000 bp downstream of the mutation was not printed and included in this report, but it is available as digital file.
Figure 4.1. ΔF508 NECs Sanger sequencing result of CFTR exon 10. The figure demonstrates the nucleotide sequence and the amino acid translation. The target site of the sgRNA is shown and the missing Phenylalanine amino acid point is indicated by a small arrow. The healthy sequence would read ATC TTT instead of ATT, at the arrow point.

A repair plasmid was designed, to serve as a template for HDR correction of the ΔF508 NECs. This construct includes a puromycin/Thymidine kinase (TK) selection cassette, flanked by piggyBac excision transposase recognition sites and arms homologous to the CFTR sequence which include the correcting ΔF508 mutation. The cells where HDR results in the insertion of the cassette can be selected through their puromycin resistance; furthermore, the entire cassette can be removed scarlessly by the piggyBac transposase. Cells where the cassette is not removed are sensitive to ganciclovir, as a result of Thymidine Kinase expression (Firth et al., 2015).

Releasing the repair template from the plasmid backbone with the same sgRNA which will create the Double Strand Break (DSB) has been reported to increase HDR efficiency (Zhang et al., 2017). For this reason, two almost identical plasmids were designed, one with and one without flanking sgRNA recognition sites which will and will not, respectively, allow the release of the repair template from the plasmid backbone (Figure 4.2).

Figure 4.2. Schematic of CRISPR/Cas9 approach for the correction of the ΔF508 mutation in ΔF508 NECs. ΔF508 NECs will be transfected with a. Cas9 protein, b. sgRNA (gRNA) and c. a plasmid containing a
puromycin/Thimidine Kinase cassette flanked by piggyBac recognition sites along with homology arms with the CFTR sequence (which includes a correction mutation). Releasing the repair template from the plasmid using the same sgRNA that creates the Double Strand Break has been shown to increase HDR efficiency. Selection of corrected cells is possible with puromycin and the cassette can be excised with the piggyBac transposase. Unexcised cells can be selected with ganciclovir (sensitivity of cells expressing TK).

PCR reactions were performed to create overlapping fragments, so the homology arms and the puromycin/TK selection cassette could be assembled within the pUC19 plasmid backbone using the Gibson Assembly cloning method for the creation of the two repair plasmids (Figure 4.3).

Figure 4.3. Agarose electrophoresis of fragments used for Gibson Assembly. PCR bands of: (A) LHA (left) and RHA (right) for the construct without the sgRNA recognition sites, (B) RHA for the construct with the sgRNA recognition sites, (C) selection cassette, used for both constructs. 1 Kb ladder, band sizes starting from the bottom: 500 bp, 1000 bp, 1500 bp, 2000 bp, 3000 bp (stronger), 4000 bp, 5000 bp, 6000 bp, 8000 bp, 10000 bp.

After the fragments were assembled, 10 minipreps of each of the two constructs (with and without sgRNA recognition sites) were digested with EcoRI restriction enzyme to assess desired assembly. The digestion products were separated on an agarose gel by electrophoresis. Plasmids with the expected fragment sizes after digestion indicated desired assembly of the constructs (Figure 4.4). Finally, plasmids with the expected fragment sizes were sequenced with Sanger sequencing: one construct without sgRNA recognition sites was identified as exact match to the
desired sequence, however it was not possible to identify an exact match for the construct with
the sgRNA sequences.

Figure 4.4. Agarose electrophoresis of repair plasmids digested with EcoRI. Bands show digestions of
plasmids: (A) with the sgRNA recognition sites (expected band sizes: 1165, 2881, 3333 and 7379), (B)
without the sgRNA recognition sites (expected band sizes: 3333, 4000 and 7333). 1 Kb ladder, band sizes
starting from the bottom: 500 bp, 1000 bp, 1500 bp, 2000 bp, 3000 bp (stronger), 4000 bp, 5000 bp, 6000
bp, 8000 bp, 10000 bp.

The repair template which included the sgRNA recognition sites was instead assembled with
pGEM-T easy plasmid as backbone. Twelve minipreps of the construct were digested with EcoRI
restriction enzyme to assess desired assembly. The digestion products were separated on an
agarose gel (Figure 4.5). One plasmid with the expected fragment sizes after digestion was
sequenced with Sanger sequencing, which confirmed the desired sequence. The sequences of
the two constructs are available as digital files.

Figure 4.5. Agarose electrophoresis of repair plasmid digested with EcoRI. Bands show digestions of
plasmids with the sgRNA recognition sites (expected band sizes: 199, 1175, 2997, 3333 and 7704. 1 Kb
ladder, band sizes starting from the bottom: 500 bp, 1000 bp, 1500 bp, 2000 bp, 3000 bp (stronger), 4000 bp, 5000 bp, 6000 bp, 8000 bp, 10000 bp.

4.4. Optimisation of CRISPR/Cas9 mediated Double Strand Breaks in ΔF508 NECs with guide 7

In order to establish optimal conditions and reagents for creating a high number of CRISPR/Cas9 mediated Double Strand Breaks (DSB) at the targeting site of guide 7, we initially nucleofected ΔF508 NECs with 2.5 μg plasmid px330 (expressing hSpCas9 and guide 7) and 2.5 μg pEGFP-N1. 48 hours after nucleofection, cells were sorted by FACS and the GFP+ cells were expanded in culture (Figure 4.6). Genomic DNA from GFP+ cells was amplified by PCR around the guide 7 cut site and PCR products were sequenced by Sanger sequencing. The sanger sequencing chromatograms were analysed with TIDE software.
Figure 4.6. Nucleofection of ΔF508 NECs with px330 and pEGFP-N1. FACS plots show gating for (A) total cells, (B) live cells, (C) single cells, (D) Feeder cells (PE+) in GFP+ (single) control (E) Feeder cells (PE+) in PE+ (single) control, (F) GFP+ cells in PE+ (single) control. (G) 47.6% of epithelial cells are GFP+. The number of cells was approx. 375000. Plots represent results of one experiment per condition.

TIDE analysis in Figure 4.7, shows that nucleofection of ΔF508 NECs with px330 plasmid encoding guide 7 and Cas9 created indels in 2.8 % of the CFTR alleles (as an average of the results from primers T1 and T2).
Figure 4.7. Indels created by nucleofection of ΔF508 NECs with px330 and pEGFP-N1. (A) TIDE plot shows that the total percentage of sequences presenting with indels is 3.3% (T1 primer) with 1% of these being
a +1 insertion. (B) Average (T1 and T2 primers) of sequences presenting with indels is 2.8%. TIDE analysis represents results from one experiment.

To observe the growth rate of GFP+ ΔF508 NECs nucleofected with px330 and pEGFP-N1, the cells were maintained in culture after FACS and a cumulative cell curve was created, versus the cumulative cell curve of non-nucleofected cells in culture. After 20 days in culture, the nucleofected cell population was not able to achieve as high cumulative cell numbers as the non-nucleofected culture (Figure 4.8).

![Cumulative growth curve](image)

**Figure 4.8.** Growth curve of GFP+ ΔF508 NECs nucleofected with px330 and pEGFP-N1 versus standard non-nucleofected culture. 20 days after nucleofection, the nucleofected cells achieve a cumulative cell number of $10^{14}$ cells while the non-nucleofected culture achieves $10^{16}$ cells. Curves represent results from one experiment per condition.

In order to increase the percentages of DSBs in ΔF508 NECs, we subsequently used guide 7 as sgRNA (Sigma) and Cas9 as protein (NEB). We also compared RNP sgRNA/Cas9 molar ratios ranging from 1:1 to 3:1. Figure 4.9 shows that CRISPR/Cas9 mediated DSB with guide 7, when
guide 7 is nucleofected to the cells as sgRNA (Sigma) and Cas9 as protein (NEB), created indels in 10.4% of the sequences (as an average of the results from primers T1 and T2) when the sgRNA/Cas9 ratio is 3:1. Lower percentages of indels are observed with lower sgRNA/Cas9 ratios (Figure 4.9).
A

**Indel Spectrum**

<table>
<thead>
<tr>
<th>% of sequences</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>2.1</td>
</tr>
<tr>
<td>20</td>
<td>7.1</td>
</tr>
<tr>
<td>30</td>
<td>86.7</td>
</tr>
<tr>
<td>40</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

**Quality control - Aberrant sequence signal**

B

**T1 & T2 average**

<table>
<thead>
<tr>
<th>Molar ratio (sgRNA/Cas9)</th>
<th>Indels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>3.5 %</td>
</tr>
<tr>
<td>1.25:1</td>
<td>3 %</td>
</tr>
<tr>
<td>1.5:1</td>
<td>7.3 %</td>
</tr>
<tr>
<td>2:1</td>
<td>7.8 %</td>
</tr>
<tr>
<td>3:1</td>
<td>10.4 %</td>
</tr>
</tbody>
</table>
Figure 4.9. Indels created by nucleofection of ΔF508 NECs with sgRNA (Sigma) and Cas9 protein (NEB). (A) TIDE plot shows that the total percentage of sequences presenting with indels is 10.7% (T1 primer) with 7.1% of these being a +1 insertion, when the sgRNA/Cas9 ratio is 3:1. (B) Average (T1 and T2 primers) of sequences presenting with indels is 3.5% for sgRNA/Cas9 ratio of 1:1, 3% for ratio 1.25:1, 7.3% for ratio 1.5:1, 7.8% for ratio 2:1 and 10.4% for ratio 3:1. TIDE analysis represents results from one experiment per condition.

ΔF508 NECs nucleofected with sgRNA (Sigma) and Cas9 protein (NEB) were maintained in culture and a cumulative cell curve was created, versus the cumulative cell curve of non-nucleofected cells in culture. After 14 days in culture, the nucleofected cell population recovered and achieved the same cumulative cell number as the non-nucleofected culture.

Figure 4.10. Growth curve of ΔF508 NECs nucleofected with sgRNA (Sigma) and Cas9 protein (NEB), versus standard non-nucleofected culture. 14 days after nucleofection, both nucleofected cells and non-nucleofected culture achieve a cumulative cell number of $10^{11}$ cells. Curves represent results from one experiment per condition.

In order to further improve the percentage of DSBs in ΔF508 NECs, we repeated nucleofections with sgRNA and Cas9 protein, however this time we used sgRNAs from Synthego and Cas9 from
ThermoFisher Scientific. RNP sgRNA/Cas9 molar ratios from 3:1 to 9:1 were also compared. Figure 4.11 shows that CRISPR/Cas9 mediated DSB with guide 7, when guide 7 was nucleofected to the cells as sgRNA (Synthego) and Cas9 as protein (Thermo), now created indels in 44.9% of the sequences (as an average of the results from primers T1 and T2) when the sgRNA/Cas9 ratio was 9:1. Lower percentages of indels were observed with lower sgRNA/Cas9 ratios. At a sgRNA/Cas9 ratio of 3:1, indels were present at 35.7% of sequences with Synthego sgRNA and Thermo Cas9, versus only 10.4% with Sigma sgRNA and NEB Cas9.
### A

**Indel Spectrum**

- total eff = 44.2%
- Sample

![Graph showing indel spectrum with bars indicating percentages and p-values](image)

- Red bar: p < 0.001
- Black bar: p ≥ 0.001

**Quality control - Aberrant sequence signal**

- Region for decomposition
- Expected cut at 203bp

![Graph showing aberrant sequence signal with control and test samples](image)

### B

<table>
<thead>
<tr>
<th>Molar ratio (sgRNA/Cas9)</th>
<th>Indels</th>
</tr>
</thead>
<tbody>
<tr>
<td>3:1</td>
<td>35.7%</td>
</tr>
<tr>
<td>6:1</td>
<td>43.1%</td>
</tr>
<tr>
<td>9:1</td>
<td>44.9%</td>
</tr>
</tbody>
</table>
Figure 4.11. Indels created by nucleofection of ΔF508 NECs with sgRNA (Synthego) and Cas9 protein (Thermo). (A) TIDE plot shows that the total percentage of sequences presenting with indels is 44.2% (T1 primer) with 28.3% of these being a +1 insertion, when the sgRNA/Cas9 ratio is 9:1. (B) Average (T1 and T2 primers) of sequences presenting with indels is 35.7% for sgRNA/Cas9 ratio of 3:1, 43.1% for ratio 6:1 and 44.9% for ratio 9:1. TIDE analysis represents results from one experiment per condition.

ΔF508 NECs nucleofected with sgRNA (Synthego) and Cas9 protein (Thermo) were maintained in culture and a cumulative cell curve was created, versus the cumulative cell curve of non-nucleofected cells in culture. After 15 days in culture, the nucleofected cell population was able to recover and achieved the same cumulative cell number as the non-nucleofected culture (Figure 4.12).

Cumulative growth curve
ΔF508 NEC DSB sgRNA (Synthego) + Cas9 (Thermo)

Figure 4.12. Growth curve of ΔF508 NECs nucleofected with sgRNA (Synthego) and Cas9 protein (Thermo), versus standard non-nucleofected culture. 14 days after nucleofection, both nucleofected cells and non-nucleofected culture achieve a cumulative cell number of $10^{17}$ cells. Curves represent results from one experiment per condition.

4.5. Puromycin selection optimisation in ΔF508 NECs
The appropriate concentration of puromycin needed for the selection of epithelial cells was optimised in ΔF508 NECs. Cells were expanded and treated with concentrations of puromycin ranging from 0 μg/mL to 1 μg/mL. The concentration of puromycin that killed all cells (no cells visible under the microscope) in one week was 1 μg/mL and therefore was chosen for all subsequent experiments (Figure 4.13).
Figure 4.13. Puromycin selection of ΔF508 NECs. ΔF508 NECs are treated with 0 μg/mL, 0.5 μg/mL and 1 μg/mL puromycin. After 8 days there are no visible cells under the microscope in the culture treated with 1 μg/mL puromycin (only dead cells and debris present) while cells treated with 0 μg/mL puromycin have not been affected. The orange circles show epithelial cell colonies. Scale bar: 200 μm.

4.6. Creation of puromycin-resistant 3T3-J2 cells

Selection of ΔF508_NEC with puromycin kills cells that have not acquired the puromycin/TK cassette via HDR. At the same time, it also kills the entire irradiated feeder layer of 3T3 cells. In order to support the epithelial cell culture of ΔF508_NEC during puromycin selection, puromycin resistant 3T3-J2 cells were created with the use of a lentivirus. The pSicoR-EF1a-mCh-Puro plasmid used to prepare the lentivirus, contains both mCherry and a puromycin resistance gene and was initially titrated in HEK293 cells. Transduction of 8.5x10^4 HEK293 cells with 1 μl of undiluted virus resulted in 20% mCherry+ cells (Figure 4.14). The titre of the virus was calculated as 78.7 x10^6 TU/mL.
A

B

Gated on Total Cells

C

Gated on Live Cells

D

mCherry-control
Gated on Single Cells

E

1 μl virus

F

0.2 μl virus

G

0.04 μl virus

H

0.005 μl virus

20%

5.7%

1.6%

0.2%
Figure 4.14. mCherry/puromycin lentivirus titration in HEK293. FACS plots show gating for (A) total cells, (B) live cells, (C) single cells, (D) mCherry+ cells in DAPI (single) control. (E) 20% of HEK293 cells are mCherry+ when transduced with 1 μl of virus, (F) 5.7% of HEK293 cells are mCherry+ when transduced with 0.2 μl of virus, (G) 1.6% of HEK293 cells are mCherry+ when transduced with 0.04 μl of virus and (H) 0.2% of HEK293 cells are mCherry+ when transduced with 0.2 μl of virus. The number of cells was 393333 per reaction.

Titration in HEK293 cells is not indicative of the efficiency with which 3T3-J2 cells will be transduced by the lentivirus, therefore the transduction was additionally optimised for the feeder cells. 3T3-J2 were transduced with different dilutions of the virus. Transduction of 8.5x10^4 3T3-J2 cells with 1 μl of undiluted virus resulted in 16% mCherry+ cells (Figure 4.15). Here, the titre of the virus was calculated as 13.6 x10^6 TU/mL. Following, 5x10^6 3T3-J2 were transduced with an MOI of 0.3 and maintained in culture. After 48 hours, the cells were selected with 1 μg/mL puromycin for the duration of a week to eliminate mCherry/puromycin negative cells.
Figure 4.15. mCherry/puromycin lentivirus optimisation in 3T3-J2 cells. FACS plots show gating for (A) total cells, (B) live cells, (C) single cells, (D) mCherry+ cells in DAPI (single) control. (E) 16% of 3T3-J2 cells are mCherry+ when transduced with 1 μl of virus and (F) 0.2% of 3T3-J2 cells are mCherry+ when transduced with 0.2 μl of virus. The number of cells was 85000 per reaction.

4.7. CRISPR/Cas9 correction of ΔF508 NECs via the HDR pathway with sgRNA, Cas9 and repair plasmid

For the correction of ΔF508 NECs via the HDR pathway, cells were nucleofected with the optimal 9:1 ratio of sgRNA (Synthego) to Cas9 (Thermo) protein and 15 μg of repair construct without the sgRNA recognition sites. A control reaction was performed where cells were nucleofected with Cas9 and repair construct but no sgRNA, in order to observe random plasmid integrations that can give puromycin resistance to cells. 48 hours after nucleofection, cells were selected with 1 μg/mL puromycin for the duration of a week. Cells in the mock transfection were fully
eliminated, while in the control reaction very few puromycin-resistant colonies could be observed. In the HDR reaction a number of puromycin-resistant colonies were visible among the feeder layer. After replating, no cells survived in the control, while cells expanded in the HDR reaction (Figure 4.16).
Figure 4.16. Puromycin selection of ΔF508 NECs nucleofected with sgRNA (Synthego), Cas9 protein (Thermo) and repair plasmid without sgRNA recognition sites. ΔF508 NECs are treated with 1 μg/mL puromycin. After one week, epithelial cells in the mock nucleofection are eliminated, while colonies can be observed in the control and HDR (Cas9/sgRNA/plasmid) reaction. At replating, no cells survive in the control while cells expand in the HDR reaction. Orange circles show puromycin resistant epithelial colonies. Scale bar: 200 μm.

Genotyping of the puromycin-resistant ΔF508 NECs confirmed the insertion of the selection puromycin/TK cassette and the correction of the ΔF508 mutation. In-out PCRs at the 3’ and 5’ ends of the cassette give products of the expected sizes which perfectly match the template sequence when sequenced with Sanger sequencing (Figure 4.17).
At this point, puromycin-resistant ΔF508 NECs were single cell cloned, however no colonies grew out of 144 individually plated cells (one experiment).

Puromycin-resistant ΔF508 NECs were expanded in culture and subsequently nucleofected with the piggyBac excision transposase, in order to excise the selection puromycin/TK cassette. 48 hours after transfection, cells were selected with 3 μg/mL ganciclovir to eliminate cells which still retained the cassette. Following ganciclovir selection, cells could not be maintained in culture (with less than 2x10^4 cells at splitting), however the cell pellet was collected for genotyping. Genotyping was not indicative of the expected cassette excision in either of two independent piggyBac excision nucleofections. The cell population still retained the integrated cassette in the cell sequence, as shown by 3’ and 5’ in-out PCRs (data not shown). ICE analysis around the sgRNA cut site indicated a wide range of indels (Figure 4.18).
Figure 4.18. ICE analysis of ΔF508 NECs after piggyBac excision. (A) and (B) Two independent excision reactions and subsequent ganciclovir selections show indels around the sgRNA cut site.
In order to increase the number and clonogenicity of puromycin-resistant cells after HDR correction, the previous experiment was repeated with a few alterations. Here, the repair plasmid without the sgRNA recognition sites was substituted for the one with sgRNA recognition sites and an HDR enhancer was included in the culture after nucleofection. Puromycin selection was started 24 hours after nucleofection. To avoid colony overgrowth and terminal differentiation, the cells under selection were replated 72 hours post nucleofection, while the 7 day puromycin selection continued to the following passage. Genotyping of the puromycin-resistant cells confirmed the presence of the cassette and the correction of the ΔF508. Following piggyBac excision transposase nucleofection and ganciclovir selection, a larger number of cells was acquired compared to the previous experiment (18.6x10^4 cells at splitting) which, however, still could not be expanded further. The cells were genotyped, however the puromycin/TK cassette was still found to be incorporated in the cells. ICE analysis around the sgRNA cut site demonstrated that 93.5% of sequences carried the same 19 bp deletion (first two lines show the same deletion) (Figure 4.19).
Figure 4.19. ICE analysis of ΔF508 NECs after improved HDR and piggyBac excision. After the excision reaction and subsequent ganciclovir selection ICE analysis around the sgRNA cut site shows 93.5% of sequences carrying an identical 19 bp deletion (first two lines show the same deletion). ICE results are based on one experiment.

As an alternative to the correction approach where the selection puromycin/TK cassette needs to be removed with the piggyBac excision transposase, a different repair plasmid (Donor-G) was used for HDR correction. This plasmid contains a repair template with homology arms and a puromycin selection cassette which is designed to incorporate in the intron between exons 10 and 11. As a result, it does not require to be excised for the CFTR correction.

ΔF508 NECs were nucleofected with the optimal 9:1 ratio of sgRNA (Synthego) to Cas9 (Thermo) protein and 15 μg of Donor-G. As previously, a control reaction was performed as well as a mock nucleofection. Cells were expanded and passaged once before they were selected with 1 μg/mL puromycin for the duration of a week. Cells in the mock transfection were fully eliminated, while in the control reaction very few puromycin-resistant colonies could be observed. In the HDR reaction a number of puromycin-resistant colonies were visible among the feeder layer (Figure 4.20).
Figure 4.20. Puromycin selection of ΔF508 NECs nucleofected with sgRNA (Synthego), Cas9 protein (Thermo) and Donor-G repair plasmid. ΔF508 NECs are treated with 1 μg/mL puromycin. After one week, epithelial cells in the mock nucleofection are eliminated, while colonies can be observed in the control and HDR (Cas9/sgRNA/plasmid) reaction. Orange circles show puromycin resistant epithelial colonies. Scale bar: 200 μm.

The puromycin-resistant ΔF508 NECs were genotyped with 3’ end and 5’ end in-out PCRs which confirmed the incorporation of the cassette in the cells (Figure 4.21) and expanded in culture. While enough cells were generated to plate in an ALI culture (approximately 140000 cells), cells were not able to be expanded further. Protein lysate from the ALI culture was used for Western blotting, which showed correction of CFTR and expression of the gene, with both bands C and B present (Figure 4.22).
Figure 4.21. Genotyping of puromycin-resistant ΔF508 NECs nucleofected with sgRNA (Synthego), Cas9 protein (Thermo) and Donor-G repair plasmid. Sanger sequencing of (A) 3’ end in-out PCR and (B) 5’ end in-out PCR.
Figure 4.22. Western blot of ΔF508 NECs corrected with Donor-G. CFTR appears in the corrected ΔF508 NECs as bands B (150 kDa) and C (170-180 kDa). CFTR band B is not detectable in the non-corrected ΔF508 NECs, possibly due to sample degradation (sample same as in Figure 3.17). GAPDH was used as a loading control and shows slightly increased loading at the non-corrected ΔF508 NECs versus the corrected. Results represent one western blot assay.
4.8. Discussion

The aim of the work described in this chapter, was to correct the ΔF508 mutation in primary nasal epithelial cells from CF patients (ΔF508 NECs) utilising the HDR CRISPR/Cas9 pathway. The correction of primary cells is the first step to a successful cell therapy.

As mentioned in the introduction of this chapter, the efficiency of an HDR CRISPR/Cas9 experiment is dependent on many factors. Here, we designed and constructed two repair plasmids, as templates for HDR, which included a puromycin/TK selection marker. This approach is similar to the strategy used by Firth et al., however with a few significant differences. In their study, Firth et al., corrected iPSCs while we used primary airway epithelial cells. Secondly, we have created a new construct where the selection cassette was designed to disrupt the guide RNA sequence in the repair template. Therefore, unlike the construct of Firth et al., our template was not broken by the CRISPR/Cas9 machinery targeting the template itself (Firth et al., 2015). Lastly, in one of the two constructs, the repair template was designed so that it could be released from the plasmid backbone with the same guide RNAs that create the double strand breaks in the genome. This is believed to improve HDR efficiency in cases where the repair template cannot be delivered as a linear ssODN due to its size (Zhang et al., 2017).

Cloning the repair templates in the pUC19 plasmid backbone was straightforward for the template without the guide RNA recognition sites, while it proved difficult and was unsuccessful for the construct with the guide RNA recognition sequences. We hypothesise, that the addition of the guide RNA recognition sequences in the template sequence, altered the reading frame of the downstream genes and sequences and as a result possibly expressed a product that was toxic to the bacterial culture. The repair template with the guide RNA recognition sequences was cloned without further issues, in a different plasmid backbone, the pGEM-T easy. We hypothesise that this was possible because the cloning reading frame in this plasmid was different.

As explained in the material and methods section, this particular guide RNA target site was selected for DSB creation, because of its good efficiency and specificity score. Its proximity to the ΔF508 mutation is not ideal as it is 65 bp away. However, this site is the best and essentially only available guide target for the ΔF508 mutation when using SpCas9. SpCas9 was chosen for these experiments as it is the most widely used and well characterised Cas9, with proven good
on-target activity. DSB efficiency was assessed by TIDE software analysis of Sanger sequencing chromatograms, rather than with the T7 endonuclease 1, as T7E1 does not reflect the real sgRNA activity and in many cases underreports the percentage of DSBs (Sentmanat et al., 2018).

We initially attempted to induce CRISPR/Cas9 mediated DSBs in primary epithelial cells by nucleofecting the cells with the px330 plasmid, which expressed both Cas9 and our selected guide RNA. In this experiment, we co-transfected cell with a GFP plasmid to be certain we are expanding cells which have been positively transfected. Although GFP+ cells do not necessarily contain px330, it is likely that both plasmids have entered the cells. After DNA sequence analysis, we discovered that the efficiency of DSBs in the cell population was very low, while cell culture growth curves showed that the cell clonogenicity was decreased. Here, we hypothesise that primary cells were greatly affected by the toxicity of DNA delivery and potentially from Cas9 and guide RNAs without chemical modifications (Hendel et al., 2015).

Our hypothesis is strengthened by our following experiments where nucleofections with Cas9 protein and sgRNAs lead to greater efficiency of DSBs. We can also observe that not all Cas9 protein and sgRNAs are equivalent: sgRNAs from Synthego are one-part fusion guide RNAs of the crRNA and tracrRNA and are reported to carry chemical modifications which greatly improve DSB efficiency (Holden et al. no date). These modifications allow Synthego sgRNAs to provide higher stability and to decrease the innate cellular immune response (Schubert et al., 2018; Kim et al., 2018). As a result, they outperform the two-part cr:tracrRNA from Sigma.

The superiority of one-part sgRNA versus the 2 part crRNA:tracrRNA can also be attributed to inefficiencies in annealing the two-piece system, as 1:1 annealing is unlikely to be 100%, and to the tendency of the tracrRNA to form tetramers with itself (from Synthego).

The case for Cas9 protein seems to be less clear as different labs have been using different brands of Cas9 with good results. Our decision to change the Cas9 brand from NEB to Thermo Scientific was more for consistency with results of our collaborators. Newer versions of Cas9 protein generally also carry modifications which enhance specificity and decrease off-target effects (Slaymaker et al., 2016; Kleistiver et al., 2016), however the exact specifications of the reagents are proprietary.

We optimised the ratio of sgRNA/Cas9, by trying different molar ratios of the reagents. As advised by the manuals of the reagents, we always kept the sgRNA in excess of the Cas9 protein.
We found that increasing the ratio of sgRNA versus Cas9 leads to higher DSB efficiency until the ratio of 9:1 where the improvement seems to slowly plateau. Previous studies have used ratios from as low as 1:1 (Liang et al., 2015), however recently, Min et al., showed that a 10:1 ratio improved efficiency in their experiments. Here, our results are limited by the lack of further independent experiments that would allow the comparison of different sgRNA/Cas9 ratios with statistical significance, making this a descriptive but informative observation which can constitute the basis for subsequent investigation.

We are aware that efficiency of DSBs is also dependent on the specific guide target site and its sequence. The impact of the genome and epigenome at the target locus can affect the Cas9 binding and cleavage. GC content of the guide RNA sequence can also affect efficiency, as guides with a very low or very high GC content have shown to be less effective against their targets (Gisler et al., 2019).

As demonstrated in Chapter 3, the presence of the irradiated feeder layer of 3T3 mouse embryonic fibroblasts, is paramount to the long term survival and expansion of the epithelial cells. For our HDR CRISPR/Cas9 correction, puromycin selection would eliminate the feeder layer, leaving the epithelial cells without support. As a consequence, it was necessary to make puromycin-resistant 3T3-J2 cells to provide support for the epithelial culture. We achieved this through the transduction of the cells with an mCherry/puromycin lentivirus. The mCherry gene was included for ease of titration and to allow the separation of the 3T3 from the epithelial cells via FACS, if needed in the future.

Combining our optimised nucleofection approach with optimised sgRNA/Cas9 protein reagents and ratios and repair template, resulted in a successful HDR correction experiment. This was confirmed by genotyping, which demonstrated the incorporation of the puromycin/TK selection marker and the correction of the ΔF508 mutation in ΔF508 NECs. However, a very small percentage of cells appeared to be corrected by HDR and as a result of the puromycin depletion of all the non-corrected cells, the culture could be maintained with difficulty and single cell cloning was impossible. Using the repair template that could be linearised with the sgRNAs and
an HDR enhancer, only marginally improved HDR levels, as demonstrated by the low survival of the cells.

PiggyBac excision of the puromycin/TK selection cassette posed an additional problem to the cell survival, as the cell population was further depleted by the ganciclovir selection. Even more concerning, was that the remaining cell population presented with the selection cassette still in the genome (demonstrated by in-out PCRs). Genomic PCRs around the guide cleavage site showed a wide range of indels, which can most probably be attributed not to imperfect piggyBac excision but to sequences where NHEJ caused indel formation, instead of HDR inserting the selection cassette. The cells carrying these sequences may have survived the puromycin selection due to random puromycin cassette integrations.

The piggyBac system has been used effectively in a number of studies (Eggenschwiler et al., 2016; Nishizawa-yokoi et al., 2016; Wang et al., 2017), and is unclear why it did not demonstrate the expected results in our experiments. A possible explanation may implicate the primary epithelial cells, as the piggyBac system has not been previously shown to be effective in these cells. Importantly, recent studies, have demonstrated that breaks in the genome are very toxic to primary cells in a p53 dependent manner. Single DSBs might be tolerated, however additional ones, in this case created by the piggyBac excision transposase, can increase the overall burden and trigger apoptosis (Ihry et al. 2018; Schiroli et al. 2019). As mentioned earlier, primary cells are also sensitive to DNA delivery and the piggyBac excision transposase is expressed by a DNA vector.

A way to test the above hypothesis, would be to repeat experiments with the transient inhibition of p53. Short-term p53 inhibition has been shown not to damage the DNA damage response (DDR) of the cells and to maintain the DNA repair. Additionally, no chromosomal aberrations or mutational burden have been recorded (Schiroli et al. 2019). Of course, for any cell therapy applications of the CRISPR/Cas9 system, the effects of p53 inhibition should be closely monitored.

Our correction of the ΔF508 NECs using a repair template with a puromycin selection marker that incorporates in the intron (Donor-G), demonstrated that such an approach is possible for primary epithelial cells, as this template had previously been used successfully to correct iPSCs by ZNF technology (Crane et. al. 2015). However, the issue with very few surviving cells still
remained and is prohibitive for any downstream transplantation experiments. Theoretically, a system where the repair template carries a fluorescent marker which incorporates in the intron, would be superior than the puromycin one, as the selection of cells would be faster, could happen on a much larger scale and would not further exhaust the cells through additional culture expansion. However, the limiting factor would probably still be the seemingly very low levels of HDR in primary epithelial airway cells. In addition, the stable integration of any marker would make this approach not clinically translatable.

Western blotting, is the gold standard for demonstrating protein expression after gene correction. Here, corrected ΔF508 NECs with Donor-G, show expression of CFTR protein with both bands C and B visible in the western blot.

Correcting CF mutations with gene editing can potentially be an easier task, which does not use HDR editing strategies, depending on the characteristics of the specific mutation. Recently, strategies to correct exon skipping mutations have been published, but these approaches utilise the more efficient NHEJ pathway to remove a part of the gene that includes the mutation (Sanz et al. 2017, Maule et al., 2019). With the mutation removed, the cell can splice exons physiologically and the CF phenotype of the cells is reversed. Other approaches take advantage of the new CRISPR/Cas9 base editors, which however can be used only for point changes and can still not make all possible nucleotide substitutions (Mention et al., 2019). As a result, they can correct very few CF mutations. Both the above strategies, ultimately correct mutations that affect a very small percentage of CF patients.

Our correction approach is designed for the ΔF508 mutation, the most common CF mutation, which involves three missing nucleotides and cannot be corrected with base editors. This approach should theoretically be possible to adapt for any CFTR mutation, irrespective of its location or the number of base pairs it affects. For this reason, it is particularly important for mutations that result in no protein production, as it is this class of mutations that cannot be rescued by any of the corrector and potentiator drugs.

Previous studies succeeding to correct CFTR have done so in cell lines, iPSCs (Firth et al., 2015) or intestinal organoids (Schwank et al., 2013). Such cell types are easier to work with, primarily because of their greater proliferation capacity and ability to single cell clone, if needed. However,
any attempt to generate corrected CF cells for cell therapy should aim to correct a cell type suitable for *in vivo* airway transplantation, like primary epithelial airway cells.
CHAPTER 5. CRISPR/CAS9 KNOCKOUT OF CFTR IN NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS
5.1. Introduction

An important question for any *in vivo* or *ex vivo* gene therapy, is how many cells would need to be corrected in order to see an improvement in the lung function of patients. For *in vivo* gene therapy, this translates to the number of cells that will have to be successfully transduced for example with a viral vector, while for *ex vivo* approaches it demonstrates the number of corrected cells that would have to successfully engraft in the airways.

The lack of robust *in vivo* models of cystic fibrosis has made these questions very hard to answer. *Cftr*\(^{-/-}\) mice do not exhibit a CF lung disease phenotype probably due to the existence of alternative chloride channels, which help restore the ion transport defect. As a result, cell replacement studies in mice cannot give insights on functional improvement. CFTR deficient ferrets and pigs develop CF lung disease, which includes bacterial infections, mucus plugging and inflammation (Mall and Hartl, 2014a). However, these models are difficult and expensive to maintain (CF pigs require gastrointestinal surgery to correct the obstruction caused by meconium ileus at birth) and are available in limited numbers in few centers around the world (Ballard et al., 2016).

For the above reasons, research has to rely on *in vitro* cell models in an attempt to give an estimate of the percentage of normal/corrected cells that can significantly improve overall CFTR activity when transplanted among CF cells. As primary cells can be extremely heterogeneous between donors, (Bhowmick and Gappa-Fahlenkamp, 2016) working with an *in vitro* model where healthy and CF cells are seeded together on ALI cultures in different percentages, to measure functional CFTR, could lead to serious misinterpretations. Additionally, the lack of pre-existing CF lung pathophysiology (such as inflammation, infection, mucus, or altered bicarbonate levels) in such models makes them less relevant to patient systems.

A way to develop a better model for estimating the number of engrafted cells needed for cell therapy would be the creation of isogenic CFTR knockouts from primary airway cells of healthy donors. In this way, the heterogeneity is minimised since healthy and CFTR deficient cells have the same genetic background. In order to mimic the CF lung environment during CFTR function measurements, sputum collected from CF patients can be added to the ALI cultures seeded with different percentages of healthy/CFTR knockout cells.
CFTR isogenic knockouts could be further used in an array of other experiments, for example for better understanding of CFTR function and interactions or for screening drugs. They could also be used for in vivo cell therapy studies in order to compare cell engraftment between healthy and CF cells.

5.2. Aims

In this chapter I aim to:

1. Optimise CRISPR/Cas9 mediated double strand breaks (DSBs) with a selected guide RNA located close to the beginning of the CFTR gene in NHBEs.
2. Assess the DSB percentage stability across cell culture passages in a mixed NHBE population.
3. Increase the percentage of DSBs in NHBEs by repeated nucleofection of Cas9 and guide RNA.
4. Perform single cell cloning of a mixed cell population of NHBEs with DSBs in order to identify NHBE clones with knockout and wild type genotypes.
5. Attempt to expand single cell clones differentiate expanded cells in air-liquid interphase (ALI) cultures.
6. Confirm that mRNA and protein expression in single cell clones is consistent with their genotype.
7. Characterise single cell clones through immunofluorescence during expansion and differentiation conditions.
5.3. Optimisation of CRISPR/Cas9 mediated Double Strand Breaks in NHBE cells with guide 1

In order to establish optimal conditions and reagents for creating a high number of CRISPR/Cas9 mediated Double Strand Breaks (DSB) at the targeting site of guide 1, NHBEs were nucleofected with sgRNA (Synthego) and Cas9 protein (Thermo). sgRNA/Cas9 molar ratios from 6:1 to 9:1 were also compared. Figure 5.1 shows that CRISPR/Cas9 mediated DSB with guide 1, when guide 1 is nucleofected to the cells as sgRNA (Synthego) and Cas9 as protein (Thermo), create indels in 47.9% of the sequences when the sgRNA/Cas9 ratio is 9:1 while they create indels in 37.2% of sequences when the ratio is 6:1.

![Figure 5.1. Indels created by nucleofection of NHBE cells with sgRNA (Synthego) and Cas9 protein (Thermo). (A) TIDE plot shows that the total percentage of sequences presenting with indels is 47.9% (T3 primer) with 24.3% of these being a -1 deletion, when the sgRNA/Cas9 ratio is 9:1. (B) Sequences presenting with indels is 37.2% for sgRNA/Cas9 ratio of 6:1 and 47.9% for ratio 9:1. TIDE analysis represents results from one experiment per condition.](image)

NHBEs nucleofected with a 9:1 ratio of sgRNA/Cas9 were maintained in culture for two further passages and DNA was collected for genotyping and assessing the percentage of DSBs at the end of each passage. At the end of passage 2, the percentage of DSBs in the sequences had fallen to 40.3%, while at the end of passage 3 the percentage of DSBs was at 41.5% (Figure 5.2). This change in percentages indicated that cells with indels could be proliferating at different rates than cells without indels.
Figure 5.2. Percentage of sequences presenting with indels in NHBE cells nucleofected with 9:1 sgRNA/Cas9 ratio, after the first, second and third passage. After the 1st passage 47.9% of sequences present with indels, after the 2nd passage 40.3% of sequences present with indels and after the 3rd passage 41.5% of sequences present with indels. Graph represents results of one experiment.

In order to further increase the percentage of sequences with DSBs in the NHBE cells, NHBEs were nucleofected with a 9:1 sgRNA/Cas9 ratio three consequent times, spaced two days apart. Cells were collected for genotyping after each nucleofection to assess the percentage of DSBs. After the first nucleofection, the percentage of sequences with DSBs was 18.5%, while following the third nucleofection the percentage of sequences with DSBs had risen to 77.2%. More than 73% of sequences appear to carry indels that are not multipliers of 3 and therefore could disrupt the reading frame and create stop codons (Figure 5.3). However, this corresponds to a much lower percentage of cells carrying indels in both CFTR alleles which would result in homozygous or compound heterozygous knockouts.
Figure 5.3. Indels created by 3 repeated nucleofections of NHBE cells with a 9:1 ratio of sgRNA (Synthego) and Cas9 protein (Thermo). TIDE plots show that the total percentage of sequences presenting with indels after (A) the first nucleofection is 18.5% with 10.4% of these being a -1 deletion; (B) the second nucleofection is 51.4% with 25% of these being a -1 deletion; (C) the third nucleofection is 77.2% with 31.1% of these being a -1 deletion. (D) Plot shows the increase of the percentage of sequences with indels after 3 repeated rounds of nucleofections. TIDE analyses represent results from one experiment of 3 consequent (repeated) nucleofections.

Single cell cloning of NHBE cells with 77.2% of sequences presenting with indels

As further work with a mixed cell population with an unknown percentage of knockouts (Figure 5.3) which could also fluctuate between passages (Figure 5.2) would not be optimal, NHBEs with 77.2% indels were single cell cloned. 17 out of 144 plated cells and 40 out of 144 plated cells gave rise to single colonies in two independent cloning experiments. Figure 5.4 shows clones of the first cloning experiment during the first passage. Clones 1.4, 1.6, 1.10 and 1.13 show proliferating potential, while clone 1.7 shows a non-proliferating colony. Clones were only selected for further expansion when a single colony, which was showing proliferating potential, was visible per cell culture well.
Figure 5.4. Phase contrast images of selected NHBE single cell clones during passage 1. Clone 1.7 shows a non-proliferating colony while all other images show colonies with good proliferating capacity. Images on the left show that the colonies are larger than the microscope field of view. Images on the right show the borders of each colony. Scale bar: 200μm.
Single cell clones which could be expanded enough to extract DNA, were genotyped and the sequence was analysed by TIDE. TIDE showed the presence and type of indels in the cell sequences and additionally confirmed that clones were not contaminated with foreign cells, by demonstrating existence of either one or maximum two different types of sequences in every case (representing the two alleles). The percentages of the two sequences were proportionate, with small differences when one of the alleles was shorter that the other and therefore would potentially have been favoured in the PCR amplification step which preceded sequencing and TIDE analysis. TIDE analysis of single cells clones from cloning experiment 1, showed a variety of genotypes, including Wild Types (no indels in both alleles), Heterozygotes (indels in one of the two alleles) and Compound Heterozygotes (indels in both alleles) (Figure 5.5 and Table 5.1). The full spectrum of genotypes which were identified after analysis of single cell clones from cloning experiments 1 and 2 are shown on Table 5.1. In some instances, even though DNA had been isolated, TIDE analysis was not possible because the PCR generated two bands or no band at all. These were likely caused by larger deletions in one or both alleles, however since no cells could be frozen for stock in all cases, further characterisation was not possible.
Figure 5.5. TIDE plots of selected NHBE single cell clones of cloning experiment 1. (A) Single cell clone 1.2 is a heterozygote with a genotype of 0/+1; (B) Single cell clone 1.4 is a heterozygote with a genotype of 0/-1; (C) Single cell clone 1.6 is a Wild type with a genotype of 0/0; (D) Single cell clone 1.10 is a Wild type with a genotype of 0/0; (E) Single cell clone 1.13 is a heterozygote with a genotype of 1/-4.

Table 5.1. NHBE single cell clones isolated by two cloning experiments. The table shows the name of the clone, the number of cells frozen for stock, the genotype of the clone as indicated by TIDE analysis and the reasons for not acquiring a genotype through TIDE when this was not possible.

<table>
<thead>
<tr>
<th>Clone name</th>
<th>No of cells frozen</th>
<th>Genotype</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 1.2</td>
<td>413000</td>
<td>0/+1</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>Clone 1.4</td>
<td>493000</td>
<td>0/-1</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>Clone 1.5</td>
<td>-</td>
<td>-</td>
<td>No PCR band</td>
</tr>
<tr>
<td>Clone 1.6</td>
<td>100000</td>
<td>0/0</td>
<td>Wild type</td>
</tr>
<tr>
<td>Clone 1.7</td>
<td>-</td>
<td>-</td>
<td>Two PCR bands</td>
</tr>
<tr>
<td>Clone 1.10</td>
<td>1280000</td>
<td>0/0</td>
<td>Wild type</td>
</tr>
<tr>
<td>Clone 1.13</td>
<td>-</td>
<td>1/-4</td>
<td>Compound heterozygous</td>
</tr>
<tr>
<td>Clone 2.2</td>
<td>1114000</td>
<td>22/-2</td>
<td>Compound heterozygous</td>
</tr>
<tr>
<td>Clone 2.5</td>
<td>140000</td>
<td>0/-23</td>
<td>Heterozygous</td>
</tr>
</tbody>
</table>
Out of the 10 single cell clones that yielded enough cells for frozen stocks, single cell clones 2.2 (Compound heterozygote) and 2.20 (Wild type) were expanded further for rt-qPCR, western blotting and further characterisation. Single cell clone 2.2 was selected as a possible knockout as both its alleles carry indels that are not multipliers of 3 and create stop codons (see Appendix). In expansion conditions, the morphology of cells of both clones was indistinguishable from the original NHBE culture when expanded in the presence of the irradiated feeder layer with Y-27632 (Figure 5.6).
5.4. Characterisation of NHBE single cell clone 2.2 and single cell clone 2.20

NHBE single cell clones 2.2 and 2.20 were stained for immunofluorescence during expansion and were positive for epithelial cell marker E-cadherin, proliferation marker ki67 and basal cell markers CK5/14 and p63 (Figure 5.7 and Figure 5.8).
Figure 5.7. Immunofluorescence of NHBE single cell clone 2.2, expanded in vitro on irradiated feeder layer with Y-27632. Clone 2.2 is (A) positive for epithelial cell marker E-cadherin (green) and proliferation marker ki67 (red); (B) positive for basal cell marker CK5/14 (red) and transcription factor p63 (green). Scale bars = 50μm. No primary antibody negative control in Figure 5.9.

Figure 5.8. Immunofluorescence of NHBE single cell clone 2.20, expanded in vitro on irradiated feeder layer with Y-27632. Clone 2.20 is (A) positive for epithelial cell marker E-cadherin (green) and proliferation marker ki67 (red); (B) positive for basal cell marker CK5/14 (red) and transcription factor p63 (green). Scale bars = 50μm. No primary antibody negative control in Figure 5.9.
Single cell clones 2.2 and 2.20 were also plated for differentiation in ALI cultures, in Pneumacult medium. After 15 days in culture, the differentiated cells were stained for immunofluorescent markers and were positive for airway epithelial cell marker CK8, differentiated airway epithelium mucosecretory marker MUC5AC, tight junctions marker ZO-1 and cilia marker acetylated tubulin. Single cell clone 2.20 (Wild type) was also found to express CFTR, while in single cell clone 2.2 (Compound heterozygote) no expression of CFTR could be detected (Figure 5.9).
Figure 5.9. 3D reconstruction of confocal images of NHBE single cell clones 2.2 and 2.20 differentiated in vitro in air liquid interface (ALI) cultures. Clone 2.20 is (A) positive for airway epithelial cell marker CK8 (red) (B) positive for differentiated airway epithelium mucosecretory marker MUC5AC (green), (C) positive for tight junctions marker ZO-1 (red) and cilia marker acetylated tubulin (green), (D) positive for CFTR (green). Clone 2.2 is (E) positive for CK8 (red), (F) MUC5AC (green), (G) positive for ZO-1 (red) and acetylated tubulin (green) and (H) negative for CFTR. (I) No primary antibody negative control.

Differentiated cells from ALI cultures of clones 2.2 and 2.20 were used for RNA and protein extraction. Rt-qPCR analysis, showed a 1.2-fold reduction of CFTR expression ($2^{\Delta \Delta C_{T}} = 0.85$) in clone 2.2 (Compound heterozygote) compared to clone 2.20 (Wild type) (Figure 5.10), while western blotting showed no CFTR protein present in clone 2.2 (Figure 5.11).

**CFTR expression fold change**

![CFTR expression fold change](image)

Figure 5.10. Rt-qpcr of NHBE single cell clones 2.20 and 2.2. Clone 2.2 CFTR expression is reduced 1.2-fold ($2^{\Delta \Delta C_{T}} = 0.85$) compared to Clone 2.20. Results represent an n=3 of technical replicates.
Figure 5.11. Western blot of NHBE single cell clone 2.2. CFTR protein is absent from clone 2.20. Na+/K+-ATPase was used as a loading control. The overexpressed (NHBEs + CFTR mRNA) positive control contains very high amounts of protein without sample being concentrated. As a result, Na+/K+-ATPase in the positive control is not detectable. Blot represents one experiment.
5.5. Discussion

The optimisation of ratios of sgRNA and Cas9 protein for the creation of double strand breaks (DSBs) in NHBE cells in this chapter, confirms the findings of chapter 4. The use of TrueCut Cas9 protein and sgRNA from Synthego can efficiently create DSBs, which increase as the molar ratio of sgRNA/Cas9 increases from 6:1 to 9:1. Similar to chapter 4, our results are limited by the lack of further independent experiments that would allow the comparison of different sgRNA/Cas9 ratios with statistical significance. As a consequence, the observation regarding the DSB increase as a result of the sgRNA/Cas9 molar ratio increase is descriptive but nevertheless informative and can constitute the basis for subsequent investigation. As the guide RNA used for the experiments in this chapter is different that the guide RNA used in chapter 4, we the differences in DSB percentages between NHBEs and ΔF508 NECs could be attributed to genomic and epigenomic effects at each target locus.

By monitoring the NHBE population with DSBs across cell culture passages, we observed that the percentage of DSBs does not remain constant, with the percentage of cells without DSB rising through passages. This is possibly a result of cells without DSBs growing at faster rates than cells with DSBs or alternatively cells with DSBs becoming terminally differentiated at higher rates.

Higher DSB percentages were made possible through repeated nucleofections, without impact on the growth of cells. However, even with 77.2% DSBs, a mixed NHBE population will not retain this percentage stable and additionally, it is unknown how many of the cells in this population will be true knockouts. Single cell cloning can remove this uncertainty by isolating individual cells which can be genotyped accurately via TIDE. TIDE not only does provide the cells’ genotype as a specific insertion or deletion at the DSB site, it additionally provides evidence that only two alleles were identified at maximum, confirming the populations’ clonal origin.

The full spectrum of genotypes identified in single cell clones contains wild type, heterozygous and compound heterozygous populations. Between the compound heterozygous clones, where both alleles of a clone carry indels that are not multipliers of 3, the clone could be a CFTR knockout. The identified genotypes show an enrichment for -1 and +1 indels, which can be a characteristic of this specific guide RNA (Chakrabarti et al., 2019).

Single cell cloning of primary NHBEs was successful with an efficiency of 12-28% (emerging clones/plated single cells). This technique has only very recently been demonstrated for primary
epithelial airway cells, in two publications from the Shay lab: by La Ranger et al. (La Ranger et al., 2018) and by Peter-Halls et al. (Peters-Hall et al., 2018). Both publications achieve single cell cloning with cells grown in the presence of an irradiated feeder layer and Y-27632. In their paper of 2017, La Ranger et al. claim to be the first to achieve differentiation of clones in ALI cultures. In 2018, the Peter-Halls et al. study isolates CRISPR/Cas9 treated clones with knockout and Wild type genotypes, which can be differentiated in ALI, and again claim to be the first to achieve this. However, this publication does not give good evidence of the knockout genotypes and cannot support the clonal origin of their cells as robustly as we can with the help of the TIDE assay.

In our experiments, plating the single cells on an irradiated feeder layer of 3T3 fibroblasts in the presence of Y-27632, as done routinely for the normal culture, was sufficient to sustain the emergence of clonal colonies. As expected, the different colonies demonstrated variable clonogenic potential as not all clones could be expanded beyond the 48 well plate. However, 10 clones were able to expand to hundreds of thousands of cells and beyond, allowing further analysis and work with these cells.

A pending question is why single cell cloning was successful for NHBEs but unsuccessful for the puromycin-resistant ΔF508 NECs of chapter 4. As mentioned earlier, we believe that the puromycin selection vastly depletes the population of cells and with it, also eliminates the clonogenic cells. Assessing the clonogenic potential of early passage NHBEs and ΔF508 NECs before any manipulation can give insight on differences between the original clonogenic potential of the cells which would be due to donor variability or be attributed to the different origin of cells (bronchial versus nasal).

Here, successful single cell cloning is a remarkable achievement as it has the potential to revolutionise gene editing in primary airway cells, including for gene correction strategies. It can also open avenues for other types of experiments which can provide knowledge for gene and cell therapy strategies, such as assessing the clonogenic potential between cell types and different donors or discovering markers specific to a highly clonogenic, multipotent lung stem cell population (basal cell subpopulation).

The single cell clones examined further, had a physiological airway epithelial marker expression during expansion conditions, confirming their origin and non-differentiated basal cell state. Very importantly, they were able to differentiate in ALI cultures to a variety of cell types, including
ciliated and mucous cells. This is supportive of the concept that single cell clones of basal cells can still maintain the multipotent differentiation capacity of the parental cell population. These basal cells represent the progenitor/stem cells of the human airways and have the ability to self-renew and differentiate into all mature cell types.

Comparison of CFTR mRNA expression levels via rt-qPCR demonstrated a 1.2-fold reduction of expression in the Compound heterozygous clone compared to the Wild type clone. This reduction is insignificant to seem consistent with a gene knockout. However, mRNA levels in gene knockouts can still be high, when transcripts are not recognised by Nonsense Mediated Decay (NMD), the cell’s control mechanism that degrades mRNAs with premature stop codons. If mRNAs contain a stop codon in the last exon or within < 55 nt from the last exon-exon junction in the penultimate exon, they are not efficiently recognised by NMD (Popp and Maquat, 2016).

Modelling of the indels of both alleles of the Compound heterozygous clone, demonstrates that they contain many stop codons, including in exon 2 and in the last exon (see Appendix). Stop codons in exon 2 will be responsible for a truncated, non-functional protein, however the stop codons in the last exon could be allowing the transcripts to evade NMD. In addition to this, many transcripts containing stop codons are reported to evade NMD, in ways that are not fully understood. Indeed, the 55 nt rule only explains around 50% of the variance of NMD efficiency (Dyle et al., 2019). The mechanism behind this is not well understood, however it is believed that a combination of NMD-promoting and NMD-antagonising features contribute to determine the susceptibility of any transcript to NMD (Hug et al., 2016).

Lack of CFTR protein in the Compound heterozygote clone is demonstrated by the negative western blot and immunofluorescent stain, thereby confirming that the genotype leads to a total protein knockout. Further analysis of these cells could include electrophysiological measurements to assess channel activity and measurements of the air-surface liquid in ALI cultures. Confirmed and robust isogenic knockout cells will be valuable for downstream experiments, as outlined in the introduction of this chapter.
CHAPTER 6. SEEDING OF ΔF508 NASAL EPITHELIAL CELLS ON DECELLULARISED RAT TRACHEA SCAFFOLDS
6.1. Introduction

Ex vivo gene therapy for CF requires, apart from cells with functional CFTR, cells that can adhere to the basal lamina of the airways. Before testing such engraftment of epithelial cells in vivo, an intermediate model can provide early results and reduce the use of animals for experiments, as well as the associated costs and labour. This model can present in the form of decellularised scaffolds of natural organs. These organs can originate from humans, but more often, they can be harvested from various animal sources. After decellularisation, the remaining scaffolds of the organs can provide an excellent matrix for testing the seeding and engraftment of airway epithelial cells. Contrary to in vivo cell delivery, decellularised scaffolds provide an environment where cell engraftment can be tested in the absence of native epithelium competition.

Decellularising airway organs and tissue can follow methods of decellularisation of other organs, and can involve freezing and thawing, agitation and sonication (physical methods) or enzymatic and chemical methods. A combination of such methods is sometimes required for effective removal of the cells from the organ (Evangelatov and Pankov, 2013).

A successful decellularisation protocol should remove the cells from the organ but leave the extra-cellular matrix (ECM) intact. The ECM is crucial for the engraftment of cells as it provides the 3D environment, sets boundaries between the different types of tissue in the organ and also provides elasticity and integrity. The ECM is additionally important for mechanical signaling and for the regulation of growth factors (Evangelatov and Pankov, 2013).

For the purposes of using a decellularised airway organ as matrix for testing the adherence of epithelial cells, the full lung can generate unnecessary challenges in its decellularisation and cell seeding, such as the need for vascular perfusion of solutions and cells (Doi et al., 2017; Skolasinski et al., 2018; LaRanger et al., 2018). Meanwhile, CF is mainly a disease of the upper airways and therefore it is possible and somewhat more appropriate to test airway epithelial cell engraftment on tracheas. As human cadaveric tracheas are rare and in this case not essential, the tracheas of animal models can provide an excellent alternative. Specifically, the tracheas of rats, being larger than these of mice, are easier to harvest and convenient in size for downstream experiments. They are also significantly cheaper and easier to decellularise than the tracheas of larger models such as pigs.
Detergent-enzymatic treatment (DET) protocols for decellularisation are preferred over physical methods, such as freeze/thaw cycles which may inadequately lyse the cells of the organ. Even in the case where lysis has been achieved throughout the scaffold, the cell debris and the DNA are not removed with the procedure (Xing et al., 2015). Additionally, they tend to have a more detrimental effect on the ECM of the organ (Gilbert et al., 2006).

6.2. Aims

In this chapter I aim to:

1. Optimise a detergent-enzymatic treatment (DET) protocol for full decellularisation of rat tracheas.
2. Assess the effect of decellularisation on the Extra Cellular Matrix (ECM) of rat trachea scaffolds by comparison to fresh rat tracheas through histological staining.
3. Test engraftment of primary airway epithelial cells on decellularised rat trachea scaffolds.
4. Attempt to differentiate primary airway epithelial cells seeded on decellularised rat trachea scaffolds in an ALI culture type model.
6.3. Optimisation of a protocol for full decellularisation of Rat Tracheas

The first protocols tested for decellularisation of rat tracheas consisted of a DET with 4% Sodium deoxycholate (SDC) where 1 or 2 cycles with the detergent were performed and pump perfusion rate was set at 1 mL/min (protocols 1-3). These protocols failed to decellularise the tracheas, as many nuclei were visible in the scaffold after histological analysis (Figure 6.1, Panels 1-3).

With the hypothesis that unsuccessful decellularisation was due to the cannula connecting the pump to the tracheas not being wide enough to fill up the trachea lumen with solutions during perfusion, the 0.7 mm cannulas were substituted with 1.5 mm cannulas. Protocols of 1 and 2 cycles of 4% SDC were used (protocols 4 and 5), but again failed to sufficiently decellularise the tracheas (Figure 6.1, Panels 4 and 5).

For the next experiment, a decellularisation protocol using a glass bottle on a magnetic stirrer (protocol 6) was attempted, which again did not sufficiently remove cells from the tracheas and additionally destroyed parts of the scaffold (Figure 6.1, panel 6).
Figure 6.1. Hematoxylin and Eosin images of decellularised rat tracheas. Protocols 1-6 (see Materials and Methods section) are not sufficient to remove cells/nuclei (arrows) from fresh rat tracheas. Panels 1-6, Scale bars = 100μm.

Finally, the detergent was changed from 4% SDC to 1% SDS, the perfusion rate was increased from 1 mL/min to 2 mL/min and 1 and 2 cycles of the detergent solution were performed (protocols 7 and 8). Here, the protocol with 1 cycle did not decellularise the scaffold adequately; however the protocol with 2 cycles provided good decellularisation without visible nuclei in the cartilage or epithelial layer (Figure 6.2).
Figure 6.2. Protocols 7 and 8 for the decellularisation of rat tracheas. (A), (B) Hematoxylin and Eosin stain shows that 1 cycle of DET with 1% SDS (protocol 7) cannot sufficiently remove nuclei from the scaffolds, (C), (D) Hematoxylin and Eosin stain shows that 2 cycles of DET with 1% SDS (protocol 8) remove the nuclei from the scaffolds. (E) DAPI stain of scaffolds decellularised with protocol 8 shows that all nuclei are removed from the epithelium and the majority of nuclei are removed from the cartilage. Panels A, C and E, Scale bars = 250 μm. Panels B and D, Scale bars = 100 μm.

In order to assess decellularisation protocol 8 for its effect on the extracellular matrix (ECM) we compared decellularised scaffolds with fresh rat tracheas. Mason’s Trichrome stain showed lack of brown nuclei in the decellularised scaffold compared to the fresh trachea, while collagen (stained blue) was slightly fainter in the decellularised scaffold but comparable to fresh. Picro-Sirius Red stain showed slightly weaker collagen composition (fainter red stain) in the decellularised scaffold, however the two scaffolds presented similar. Alcian Blue stain showed reduction of mucus substances on the decellularised scaffold (weaker blue stain). H&E comparison of the tracheas demonstrated the lack of nuclei and a weaker stain in the decellularised scaffold (Figure 6.3).
Figure 6.3. Histological comparison of decellularised rat trachea scaffolds (protocol 8) with fresh rat tracheas. (A) Mason’s Trichrome stain of decellularised scaffold, with lack of brown nuclei and faint collagen (blue stain) (B) Mason’s Trichrome stain of fresh trachea, with brown nuclei present and collagen (C) Picro-Sirius red stain of decellularised scaffold, with weak collagen expression (faint red stain) (D) Picro-Sirius red stain of fresh trachea, showing more intense collagen staining (E) Alcian Blue stain of decellularised scaffold, with reduction of proteoglycans (weak blue stain). (F) Alcian Blue stain of fresh trachea, staining proteoglycans (G) H&E stain of decellularised scaffold, with lack of nuclei and a weaker stain (H) H&E stain of fresh trachea with visible blue nuclei. Scale bars = 100 μm.

6.4. Seeding of ΔF508 Nasal Epithelial Cells (NECs) on decellularised rat trachea scaffolds

Following the optimisation of the decellularisation of the rat tracheas, ΔF508 NECs were seeded on decellularised scaffolds and maintained in culture under submerged conditions (cFAD medium), for 3 to 6 days. Histological analysis of the seeded scaffolds demonstrated that cells successfully adhere on the scaffold and form a layer of cells that tends to become increasingly stratified the longer it is retained in culture (Figure 6.4).
Figure 6.4. Hematoxylin and Eosin stain of decellularised rat trachea scaffolds seeded with ΔF508 NECs in submerged conditions. (A), (B) Scaffold maintained in culture for 3 days, (C) Scaffold maintained in culture for 6 days. (D) Decellurised rat trachea scaffold with no seeded cells. Scale bars = 50 μm.

Immunofluorescent staining of the seeded scaffolds confirmed that the adherent ΔF508 NECs retain their identity as airway epithelial cells after 3 and 6 days in submerged conditions and are CK5/14 positive. The basal layer of cells is also p63 positive and CK8 positive (Figure 6.5 and Figure 6.6).
Figure 6.5. Immunofluorescence of decellularised trachea scaffold seeded with ΔF508 NECs for 3 days in submerged conditions. ΔF508 NECs on scaffold are (A) positive for basal cell marker CK5/14 (red) and transcription factor p63 (green); (B) positive for airway epithelial cell marker CK8 (red). (C) Negative control: no primary antibody. Scale bars: 50 μm.
Figure 6.6. Immunofluorescence of decellularised trachea scaffold seeded with ΔF508 NECs for 6 days in submerged conditions. ΔF508 NECs on scaffold are (A) positive for basal cell marker CK5/14 (red) and transcription factor p63 (green); (B) positive for airway epithelial cell marker CK8 (red). No primary antibody negative control in Figure 6.5. Scale bars: 50 μm.

After the successful engraftment of cells on the decellularised scaffolds the next step was to attempt to differentiate the cells on the scaffold in an ALI culture type model. As the scaffold cartilage is not penetrable by the cell culture medium, exposing the top layer of cells to air, as in ALI cultures, is not possible. For this reason, scaffolds with seeded cells were changed from cFAD to Pneumacult differentiation media 48 hours after seeding. Pneumacult media was added to the bottom of the cell culture inserts and also on top of the insert as a thin film layer on top and around the scaffold. Histological analysis demonstrated that cells were adherent on the scaffold after 5 and 12 exposure days. The morphology of the cells was irregular and their distribution was stratified. The cell layer was also prone to separate from the scaffold, however this could be due to the microtome sectioning (Figure 6.7).

Figure 6.7. Hematoxylin and Eosin stain of decellularised rat trachea scaffolds seeded with ΔF508 NECs in exposure conditions. (A) Scaffold exposed in culture for 5 days, (B) Scaffold exposed in culture for 12 days. Seeded cells (arrows) are detected on the scaffold on both time points and have irregular morphology and stratified distribution. Scale bar = 100 μm.
6.5. Discussion

In this chapter, we tested different decellularisation protocols for rat tracheas, based on established protocols for the decellularisation of other rat organs, like the liver, the small bowel and more recently the oesophagus (Totonelli et al., 2012; Maghsoudlou et al., 2016; Urbani et al., 2017). Similar protocols have also been used for the decellularisation of pig and human tracheas (Conconi et al., 2005; Baiguera et al., 2010). These protocols use 4% SDC as the main surfactant to wash away cells and DNase-I in 1 M NaCl for DNA removal. The solutions are delivered via a pump and depending on the organ, multiple cycles of the DET are used.

Our attempts to decellularise a rat trachea with variations of these protocols was unsuccessful, and for this reason we decided to substitute the detergent from 4% SDC with the stronger 1% SDS and increase the pump speed (perfusion rate) from 1 mL/min to 2 mL/min. We also substituted the 1 M NaCl with 0.025 mM MgCl₂, as DNase-I requires Mg²⁺ for hydrolyzing double-stranded DNA (Guérout et al., 2010). Using two cycles of the amended protocol, we were able to achieve full decellularisation of the rat tracheas.

We observed that full decellularisation does not exert major detrimental effects on the ECM proteins, after various histological stains. Collagen levels are comparable to fresh rat tracheas while the different trachea layers and structures seem well preserved. Further assessment of the scaffold could include DNA quantification, as well as collagen, elastin and glycosaminoglycan quantification (Totonelli et al., 2012). Additionally, mechanical testing for measuring tensile strength and elastance is sometimes performed (Totonelli et al., 2012; Urbano et al., 2017). However, as our scaffolds are not prepared for the purposes of bioengineering organs in vitro, such an extensive characterisation was not deemed necessary.

The successful adherence of primary airway epithelial cells on the decellularised scaffolds confirmed that the decellularisation protocol created a supportive matrix and as ΔF508 NECs are capable of engrafting they are promising for in vivo delivery experiments. Importantly, the cells retained their airway identity on the scaffold, where they formed a non-differentiated layer under submerged conditions. Similarly to our experiments, successful engraftment of human bronchial epithelial cells (HBECs) on decellularised scaffolds has been reported by Butler et al., 2016 and LaRanger et al., 2018.
Making cells differentiate on the decellularised scaffolds can be challenging. The cartilage of the trachea seems not to be penetrable by media in the same way as ALI culture inserts are, therefore prohibiting the full exposure of the top layer to air as in ALI cultures. The apical side of the cells needs to be covered with media as it is not possible to feed them from the basal side. Because of this covering with media, there seem to be no strong forces to direct the epithelial cells towards differentiation. Even though airway cells could potentially still be able to differentiate when covered with differentiation media, this is a process that might take much longer than the standard ALI culture differentiation and therefore longer time points should be tested. Alternatively, seeded scaffolds could be assembled on a bioreactor, which would maintain a continuous flow of media. In this case, the flow can be the decisive factor that directs the cells towards differentiation, similarly to the La Ranger et al., 2018 HBEC differentiation. Lastly, Butler et al., 2016 manage to achieve differentiation of HBECs through subcutaneous transplantation of the seeded scaffold in NSG mice.

Apart from using the decellularised scaffold system for testing the engraftment and differentiation of cultivated airway epithelial cells ex vivo, before proceeding to in vivo transplantation experiments in animal models, in fact, a scaffold with successfully differentiated cells could also be used for optimising cell transfection and delivery of gene-editing components for potential in vivo CF gene therapy.
CHAPTER 7. IN VIVO DELIVERY OF ΔF508 NASAL EPITHELIAL CELLS IN MOUSE AIRWAYS
7.1. Introduction

The most challenging step in an ex vivo gene therapy for cystic fibrosis is the delivery and subsequent engraftment of cells in the airways. As the epithelium is by definition a continuous layer of cells tightly interconnected without gaps, any delivered cells will not be able to dislodge the native cells and adhere. Previous research has indicated that this is possible following airway injury, which will interrupt the epithelial layer, and in this way allow foreign cells to engraft (Berical et al., 2019).

Various compounds have been used for lung injury, with some, including radiation, hyperoxic agents and bleomycin, targeting the alveolar regions of the airways while sulfur dioxide, naphthalene and polidocanol being better suited to target the proximal airways (Liu et al., 2006). 2% w/v polidocanol injury has been used for upper airway injury effectively in a number of studies (Suzuki et al., 2000; Borthwick et al. 2001; Gui et al., 2015; Farrow et al., 2018), with intratracheal instillation being the preferred method for its administration. Contrary to oropharyngeal delivery, which directs compounds to the periphery of the lung, intratracheal administration deposits them directly to the proximal airways.

Injury in the lungs should be followed by cell delivery at the appropriate time point, when the damaged epithelium can be replaced by the administered cells before it regenerates endogenously, via its own stem cell populations and mechanisms. As the mouse airway epithelium repairs very rapidly, the instillation needs to take place quickly after injury, though the exact time point might also be dependent on the presence of inflammation, which can potentially affect engraftment in a negative way. Depending on the type of injury, cell delivery can take place from as early as 4 hours for irradiation injury, to as late as 9 days after infection for influenza injury (Rosen et al., 2015; Zuo et al., 2015). For polidocanol injury of the upper airways, a time point of 24 hours has been reported (Gui et al., 2015).

Detection of delivered human cells in the mouse airways is possible with anti-human antibodies, however these can be unreliable and difficult to optimise. Another approach includes fluorescent labelling of the delivered cells, which can then be analysed by flow cytometry (FACS) or visualised with histological methods (Ma et al., 2018). Detection of labelled cells can be further enhanced with the help of an antibody targeting the fluorescent marker (Zuo et al., 2015). For
non-invasive cell detection, Farrow et al., use luciferase as a reporter to label cells and subsequently detect them via bioluminescence imaging.

7.2. Aims

In this chapter I aim to:

1. Create lung injury in mice with intratracheal administration of 2% w/v polidocanol.
2. Monitor the progression of proximal airway epithelial injury and repair after the administration of 2% w/v polidocanol at different time points.
3. Label ΔF508 Nasal Epithelial Cells (ΔF508 NECs) with a fluorescent marker for easier detection in the airways after transplantation.
4. Characterise labelled ΔF508 NECs through immunofluorescence during expansion and differentiation conditions.
5. Transplant ΔF508 NECs in mouse airways following polidocanol injury and detect engrafted cells at different time points.
7.3. Optimisation of upper airway injury in mice

In order to monitor the progression of airway injury in mice after the delivery of 2% w/v polidocanol, mice were sacrificed on day 1, 2 and 4 after the intratracheal administration. 24 hours after delivery, epithelial injury with shedding was observed, as well as indication of widespread inflammatory response with neutrophil recruitment. At 48 hours after delivery, the inflammatory response had declined and the airway injury was more widespread, with complete shedding of the epithelium at multiple areas. 96 hours after injury, the epithelium seemed to already have been repaired at most areas, however short stretches of epithelial injury could still be observed (Figure 7.1). Based on these observations, it was decided that the optimal time point for cell delivery to the airways is 48 hours after injury.
Figure 7.1. Airway injury with 2% w/v polidocanol in mice. (A), (B) 24 hours after delivery, the epithelium presents shedding and widespread inflammatory response. (C), (D) 48 hours after delivery, the epithelial injury is widespread and there is no evidence of inflammation. (E), (F) 96 hours after delivery, the epithelium is repairing with only small areas of injury present. Panels A and E, Scale bars = 50 μm. Panels B, D and F, Scale bars = 20 μm. Panel C, Scale bar = 200 μm.

7.4. Labelling of ΔF508 NECs with mCherry by lentiviral vector transduction

For easier detection of the epithelial cells in the airways after delivery, ΔF508 NECs were labelled with the pSicoR-EF1a-mCh-Puro lentivirus described in Chapter 4. This lentivirus carries apart
from the puromycin resistance gene, an mCherry gene which can be used for detection of cells via fluorescence. The transduction efficiency of the virus was tested in ΔF508 NECs under different conditions and amounts of viral vector. The cells were transduced with the highest efficiency when using 1 μl virus, 8 μg/mL polybrene and OptiMEM and the efficiency was 67.1% (Figure 7.2). From here, the viral vector titer was calculated to be $6.7 \times 10^6$ TU/mL. Following, 1.5 x10^6 ΔF508 NECs were tranduced with an MOI of 0.3 and maintained in culture. After 72 hours, the cells were FACs sorted to select for mCherry+ cells. Feeder cells were not stained as there was no need to separate them from the epithelial culture and the epithelial cells were separated in 3 different tubes (Figure 7.3).
Figure 7.2. mCherry/puromycin lentivirus optimisation in ΔF508 NECs. FACS plots show gating for (A) total cells, (B) live cells, (C) single cells, (D) mCherry+ cells in DAPI (single) control. (E) Feeder cells (PE+) in PE+ (single) control, (F) GFP+ cells in PE+ (single) control. (G) 18.9% of ΔF508 NECs are mCherry+ when transduced with 1 μl of virus, (H) 43% of ΔF508 NECs are mCherry+ when transduced with 2 μl of virus, (I) 55.2% of ΔF508 NECs are mCherry+ when transduced with 1 μl of virus and 8 μg/mL polybrene, (J) 56.1% of ΔF508 NECs are mCherry+ when transduced with 2 μl of virus and 8 μg/mL polybrene, (K) 67.1% of ΔF508 NECs are mCherry+ when transduced with 1 μl of virus and 8 μg/mL polybrene and OptiMEM. The number of cells was 10000 per reaction.
Figure 7.3. mCherry/puromycin lentivirus transduction of ΔF508 NECs. FACS plots show gating for (A) total cells, (B) live cells, (C) single cells, (D) mCherry+ cells in DAPI (single) control. (E) (F) (G) Approximately 30% of ΔF508 NECs are mCherry+ when transduced with 1 μl of virus and 8 μg/mL polybrene and OptiMEM.

7.5. Characterisation of mCherry+ ΔF508 NECs

After transduction, the mCherry+ ΔF508 NECs were expanded in culture and frozen for stock. They were also stained for immunofluorescence during expansion and were positive for epithelial cell marker E-cadherin, proliferation marker ki67 and basal cell markers CK5/14 and p63. The cells were not endogenously fluorescent under the red channel, however they were positive when stained with an anti-mCherry primary antibody and secondary fluorescent antibody (Figure 7.4).
Figure 7.4. Immunofluorescence of mCherry+ ΔF508 NECs, expanded in vitro on irradiated feeder layer with Y-27632. mCherry+ ΔF508 NECs are (A) positive for epithelial cell marker E-cadherin (green) and proliferation marker ki67 (grey); (B) positive for basal cell marker CK5/14 (grey) and transcription factor p63 (green); (C) not endogenously fluorescent under the red channel (D) positive for anti-mCherry. (E) No primary antibody negative control. Scale bars = 50μm.

mCherry+ ΔF508 NECs were also plated on ALI cultures for differentiation. After 15 days in culture, the differentiated cells were stained for immunofluorescent markers and were positive
for airway epithelial cell marker CK8, differentiated airway epithelium mucosecretory marker MUC5AC and cilia marker acetylated tubulin. The cells also expressed CFTR (Figure 7.5).

Figure 7.5. 3D reconstruction of confocal images of mCherry+ ΔF508 NECs, differentiated in vitro in air liquid interface (ALI) cultures. mCherry+ ΔF508 NECs are (A) positive for cilia marker acetylated tubulin (green); (B) positive for airway epithelial cell marker CK8 (red) and differentiated airway epithelium mucosecretory marker MUC5AC (green), (C) positive for CFTR (green). (D) Negative control: no primary antibody. Scale bars = 50 μm.

7.6. In vivo transplantation of mCherry+ ΔF508 NECs in mouse upper airways

For the transplantation of human airway epithelial cells in the airways of mice, mouse airways were first injured with 2% w/v polidocanol, as described previously. Two days after the injury, $10^6$ mCherry+ ΔF508 NECs were delivered in each mouse airway via intratracheal administration. Mice were sacrificed one, two, seven and fourteen days after cell delivery and cell engraftment was assessed via immunostaining and histological analysis of the harvested lungs. For this purpose, mouse lungs were initially stained for mCherry together with an anti-rabbit-Cy3 (red)
secondary antibody, however it was observed that the red autofluorescence of the mouse lung was prohibitory for the detection of true mCherry+ cells. An anti-rabbit-647 (far red) secondary antibody demonstrated that there was less far red than red autofluorescence present in the mouse lung, however the image was still not ideal for the detection of transplanted cells. As a result, immunohistochemistry was attempted, as this method is not hindered by autofluorescence of the tissue. Two antibodies targeting human cells (anti-hnuclei and STEM121) were tested on human lung tissue (positive control) but were unable to stain cells positive. Finally, the mCherry antibody was tested for immunohistochemistry on mCherry+ tissue from a transgenic mouse (positive control) and the detection of cells was successful (Figure 7.6).
Figure 7.6. Optimisation of antibodies and staining protocols for the detection of mCherry+ ΔF508 NECs in mouse lungs. (A) Immunofluorescent staining with mCherry primary antibody and Cy3 secondary antibody on mouse lung. Scale bar = 100 μm. (B) Immunofluorescent staining with mCherry primary antibody and
647 secondary antibody on mouse lung. Scale bar = 100 μm. (C) Immunofluorescent staining with mCherry primary antibody and 596 secondary antibody on mCherry+ tissue (probably kidney from transgenic mouse). Scale bar = 50 μm. (D) Immunohistochemistry stain with anti-human nuclei primary antibody on human lung. Scale bar = 100 μm. (E) Immunohistochemistry stain with STEM121 primary antibody on human lung. Scale bar = 100 μm. (F) Immunohistochemistry stain with mCherry primary antibody on mCherry+ tissue. Scale bar = 100 μm.

The immunohistochemistry protocol with the mCherry antibody was used to stain sections across the whole mouse lung from all time points of the experiment (7 mice in total). After whole slide imaging, the lung images were thoroughly examined for engrafted cells. Injury was observed at 24 hours and 48 hours after cell delivery (72 and 96 hours after polidocanol delivery respectively), however, no mCherry+ cells were detected at any time point (Figure 7.7).

Figure 7.7. In vivo transplantation of mCherry+ ΔF508 NECs in mouse airways. Immunohistochemistry staining shows injury in the airways but no mCherry+ engrafted cells: (A) 24 hours after cell delivery. (B) 48 hours after cell delivery. Scale bars = 100 μm.
7.7. Discussion

In this chapter, we attempted to establish a protocol for the transplantation of primary human nasal epithelial cells into mouse lungs, as a paradigm for ex vivo gene therapy. Initially, intratracheal instillation of 2% w/v polidocanol, was able to create injury in the upper airways of mice, which was evident 24 hours later but more established at 48 hours. To our knowledge, no previous study has focused on monitoring the progression of injury by polidocanol. For example, Borthwick et al., only provide images of epithelial injury at 24 hours after polidocanol instillation. At this time point, their findings are similar to ours, as they also observe shedding of the epithelium at different areas. Notably, we conducted all our experiments delivering 50 μl of polidocanol, a much higher dose than previous studies, which delivered 10 or 20 μl, however we have no indication of how this may have affected the airway injury (Borthwick et al., 2001; Gui et al., 2015).

The observed injury progression led to the decision that the epithelial cell delivery should take place 48 hours after polidocanol administration. Previously, other studies have used shorter intervals, with Borthwick et al. and Gui et al. transplanting at 24 hours, while Farrow et al, who is however injuring the mouse nose with polidocanol, transplant 2 hours later. All three studies, successfully observe engrafted cells, either by in vivo imaging or via histological methods.

In order not to be solely reliant on anti-human antibodies for the detection of engrafted cells, we decided to label ΔF508 NECs with an mCherry fluorescent marker. The mCherry/puromycin lentivirus had originally been prepared for a different experiment (see Chapter 4) and its efficiency and transduction conditions needed to be optimised in epithelial cells. We observed that the addition of polybrene and the change of the cell culture media from cFAD to OptiMEM at transduction, yielded higher transduction efficiency. However, as our earliest ΔF508 NECs stock was P3, the lentiviral transduction protocol together with the pre- and post-expansion cultures resulted in mCherry+ ΔF508 NECs of P7.

mCherry+ ΔF508 NECs were characterised by immunofluorescence during expansion and differentiation conditions to confirm physiological expression of markers and of CFTR after the transduction. We also tested whether the endogenous fluorescence of the mCherry+ cells was adequate for detection, however we realised that the addition of an anti-mCherry antibody was necessary.
Following, we tried to optimise antibodies and methods for detection of engrafted cells in mouse airways. The presence of autofluorescence in the mouse lung, both red and far red, made the detection of fluorescent cells via immunofluorescence stain impossible. We are aware of protocols that aim to reduce tissue autofluorescence for immunofluorescent stains, however on this occasion, we preferred an immunohistochemistry protocol to eliminate the autofluorescence issue. Using the immunohistochemistry protocol, we were unable to optimise antibodies that target human cells, however the detection of mCherry+ cells in a positive control was possible, thus providing us a way to detect engrafted mCherry+ ΔF508 NECs.

We selected NSG mice for the delivery of human cells to avoid rejection. However, as these mice do not develop CF lung disease, there is always the limitation of not being able to detect any functional restoration or symptom improvement.

After thorough examination of the lung sections, we were unable to detect any mCherry+ cells at any sacrifice time point. Reasons for the unsuccessful engraftment could include 1. selection of a wrong time point for cell delivery, 2. late passage of transplanted cells, 3. clumped/dying cells, as the length of the delivery procedure was approximately 1.5 hours for all 10 mice of the experiment and the cells had to remain in a tube throughout, and 4. delivery of insufficient number of cells. In order to examine these issues and better optimise a protocol for in vivo cell transplantation in mouse lungs, more experiments are needed, however, this was not possible during the timeline of this project.
CHAPTER 8. DISCUSSION
8.1. Main findings

In this study, I have attempted to address a number of issues concerning *ex vivo* gene therapy for cystic fibrosis. Specifically, I have demonstrated that I can culture and expand tissue-specific lung stem cells (epithelial basal cells) as a starting material for such therapies. These cells retain their physiological marker profile in culture and can be differentiated in ALI cultures. After optimisation of the gene editing reagents, conditions and strategy, I showed that CF mutations can be corrected in primary airway epithelial cells with CRISPR/Cas9 technology and result in physiological CFTR expression. CRISPR/Cas9 was also used to create isogenic CFTR knockouts in primary airway epithelial cells. Importantly, I demonstrated that single-cell cloning is possible for primary airway epithelial cells and this opens new avenues for gene editing and experiments which will aim to determine highly clonogenic subpopulations of basal cells. Airway epithelial cells additionally engraft in decellularised scaffolds, as a first step to successful *in vivo* transplantation. Finally, I have initiated experiments for the engraftment of primary airway epithelial cells in the airways of mice, following airway injury.

8.2. Achievements, limitations, challenges and future work

Cystic fibrosis is a life-limiting condition affecting an estimated 70,000 people worldwide. Early diagnosis and symptom management have contributed to a rising life expectancy in recent years. However, the burden of disease, which includes morbidity, mortality and costs, remains high and most patients will die from terminal lung disease before they turn 50 years of age. Recently, the licensing of small molecule compounds has offered more targeted therapies to many CF patients. Still, these drugs are not suitable for every CF mutation and have variable effectiveness on different patients. Their long-term positive effect to the disease progression or their side effects have not yet been monitored and their action is reliant on continuous administration.

Gene therapies have the potential to offer a universal therapy for all patients, including those with mutations of Class I, where premature stop codons result in no protein production. Such therapies could provide a molecular cure of the disease and therefore require a few, or even one administration.

Here, *in vivo* gene therapy needs to overcome challenges, which involve mainly the efficient transfection of the airways *in vivo*, something that can be especially challenging in the CF lung,
which is already ridden by accumulation of mucus and inflammation. In order to be effective and permanent, *in vivo* gene therapy needs additionally to be able to target the basal cells/progenitors of the lung in the stem cell niches, as any CFTR correction on differentiated cells will last for as long as that particular cell survives. As the most promising strategy for *in vivo* gene therapy consists of gene delivery via a lentiviral vector, gene insertions in random genome sites can be an added complication.

*Ex vivo* gene therapy on the other hand, requires the harvest of an appropriate cell type followed by its maintenance and expansion in culture. The method for its correction needs to be both efficient and accurate, without unwanted off-target effects or insertions. The greatest challenge is however, the transplantation of the cells back to the airways, where they should be able to engraft and replace the native epithelium despite the hostile pathophysiology of the CF lung.

### 8.2.1. Cell type, expansion and differentiation

A key factor for a successful cell therapy is the selection of a suitable cell type and its subsequent maintenance and expansion *in vitro* without loss of differentiation potency. Based on the paradigms of skin and cornea transplantation, I believe that primary airway epithelial cells are the appropriate cell type for cell therapy in the lung (Green et al., 1979; Rama et al., 2017). Achieving numbers and quality of these cells adequate to support *in vivo* transplantation can be especially challenging, since primary cells reach senescence in culture after a few passages. Here, I have shown that a co-culture system which includes an irradiated feeder layer of 3T3 mouse fibroblasts and Y-27632 is sufficient to expand primary airway epithelial cells to numbers suitable for repopulating the human lung epithelium.

Current knowledge identifies basal cells as the long-lived progenitors of the airways and suggests that the human upper airway epithelium is maintained by an equipotent basal progenitor cell population (Texeira et al., 2013). I have demonstrated that, through the co-culture method, the long-term maintenance of the basal cell population is possible. However, as the basal cell population could be heterogeneous and contain subpopulations with different proliferation and differentiation capacity, CF cell therapy might in the future need to focus in identifying, selecting and maintaining these cells. Cells exhibiting an extensive growth potential and differentiation capacity would be advantageous for an efficacious CF cell therapy.
Future CF cell therapy studies should also focus on whether nasal or bronchial cells have comparable stem-cell and growth potential and which would be the ideal way to harvest this starting material. Nasal cells can be acquired less invasively than bronchial cells, however it would be important to demonstrate that by opting for these, there is no compromise on the quality of the therapeutic product.

In my experiments I have shown that cells expanded in the co-culture system, apart from retaining a physiological basal cell profile, are able to differentiate in ALI and can generate different types of cells, including ciliated cells, mucus cells and ionocytes. The cells also demonstrated transepithelial resistance and could maintain the expression of CFTR throughout passages. It is important that these features are successfully maintained in vitro in order to guarantee the cells’ functionality after in vivo transplantation.

8.2.2. Correction

In this study, I have shown that the correction of primary airway epithelial CF cells is possible via the CRISPR/Cas9 HDR pathway. The repair of the ΔF508 mutation was achieved with a repair template which included a selection marker and resulted in cells which expressed the physiological CFTR protein. Our approach is similar to those of Firth et al. and Crane et al. who corrected the ΔF508 mutation with the help of a selection marker in iPSCs (Firth et al., 2015; Crane et al., 2015). Even earlier than that, Schwank et al. were the first to correct the ΔF508 mutation, however this work was on intestinal organoids generated from CF patients cells (Schwandt et al., 2013). This team did not use a selection marker for the correction and only reported functional restoration and no actual HDR efficiency. At the beginning of our project, primary airway epithelial cell correction via HDR had not been achieved, though in February 2019, a paper by Vaidyanathan et al. reported correction of the ΔF508 mutation in these cells (see below) (Vaidyanathan et al., 2019).

Primary airway epithelial cells, might have an extensive proliferation potential in the co-culture system, however it is not unlimited and neither is comparable to this of cell lines. A low HDR efficiency, results in a small subsection of the cell population being corrected and this reduces greatly the clonogenic potential and the subsequent proliferation capacity of the cells. Even with improved transfection and DSB efficiency (guide targeting) the limiting factor appear to be the levels of HDR and future work should focus on improving these rates. Additionally, the insertion
of a selection cassette in the genome, which helps select the corrected cells, is not ideal for human therapeutic applications.

The optimal way to restore CFTR expression in CF cells is still a topic of discussion. Depending on the specific mutation of the patient there can be approaches that do not utilise the HDR pathway. Splicing mutations can be corrected via the NHEJ pathway and specific point mutations can be corrected with base editors (Sanz et al., 2017; Maule et al., 2019; Mention et al., 2019). Nevertheless, this includes a small number of CF patients who carry these mutations. A system based on HDR has the advantage that it can correct any type of mutation, from point mutations to large deletions. However, the HDR template needs to be specifically designed for each one of them, making this strategy inefficient in terms of time and costs. A suggestion for a universal template to correct any mutation, comes in the form of a super-exon (cDNA containing all but the first exon of the gene) that can be inserted in the first intron of the gene and subsequently correct any mutation from the second exon onwards (Bednarski et al., 2016). The super-exon can be inserted via homology-independent targeted integration (HITI), an NHEJ based pathway, however such attempts have reported low efficiency rates (Suzuki et al., 2016).

Interestingly, an efficient way to correct any CF mutation in patients’ cells would be with a lentiviral vector. Admittedly, lentiviral vectors can integrate in random genetic positions and interact with the genome in unpredictable ways. However, no insertional mutagenesis has been reported in now a dozen of trials using lentiviral vectors (Biasco et al., 2017). Moreover, the recent creation of a transgenic skin transplant via a γ-retrovirus, showed no enrichment for integrations in cancer-associated regions or clonal selection of such cells, suggesting that such fears may have been exaggerated (Hirsch et al., 2017).

As mentioned earlier, Vaidyanathan et al. recently introduced a novel CRISPR/Cas9 system which includes the delivery of an ssODN repair template without a selection marker via the adeno-associated virus 6 (AAV6). The efficiency of HDR correction in their work is the highest ever reported (at minimum 28%) however, the mechanism of action of this system is still unknown (Vaidyanathan et al., 2019). Their method is currently the most promising approach for primary airway epithelial cell correction and can be modified accordingly to correct any CF mutation.

Any cells that are prepared for cell therapy and especially those that have undergone gene editing should be monitored for karyotypic abnormalities, off-target mutations and p53 stability.
Here, an advantage of ex vivo gene therapy is that corrected cells can be tested before they are delivered as a therapy. Especially for p53 mutations, it is reported that a no-break correction system, as are for example CRISPR/Cas9 base editors, would be advantageous. DSBs are known to trigger apoptosis and therefore gene edited cells where breaks were induced, tend to preferentially survive if the apoptosis pathway is somehow inhibited (Ihry et al., 2018; Schirol et al., 2019).

8.2.3. Knockouts and single cell cloning

Differences between CF and healthy donor cells have been observed in multiple studies, but in many occasions, it is difficult to distinguish true phenotypes from donor variability (Mayer et al., 2013; Balloy et al., 2015; Perra et al., 2018). For many areas of CF research, including for those that deal with drug responses and inflammation, it is important to understand the inherent differences of CF cells in primary patient material and not in immortalised cell lines. Therefore, the creation of CFTR gene knockouts in normal primary airway epithelial cells can help answer many questions, some of which are directly relevant to CF cell and gene therapy.

As part of this work, I have showed that by optimising the transfection efficiency and rate of DSBs in epithelial cells, which are notoriously difficult to transfect, I can achieve a mixed population of cells with a high percentage of desired (knockout) genotypes. Being primary and not immortalised, individually these cells have a very variable growth and survival potential and therefore this high percentage is necessary for the isolation of the desired genotypes.

Following transfection, I was able to achieve single cell cloning of the primary cells, something that had not yet been reported when this study begun, but was published in 2018 by La Ranger et al. (LaRanger et al., 2018) and by Peter-Halls et al. (Peters-Hall et al., 2018) respectively. Nevertheless, in my work, I have shown more accurate genotyping and a much more extensive characterisation of the clones. Importantly, I have demonstrated the ability of a single cell to expand and subsequently differentiate in all daughter cell types of basal cells.

For cell and gene therapy, an important question is how many cells need to be corrected in the CF lung (either through in vivo correction or transplantation of corrected cells) to restore the functionality of the epithelium. An indication can be given by in vitro experiments that measure chloride currents in mixed populations of cells that express and not express CFTR. When these cells are isogenic, heterogeneity between donors is minimised.
Future studies, should utilise the single cell cloning technique to discover highly clonogenic subpopulations of basal cells and whether nasal and bronchial cells are similarly clonogenic. Combined with the knockout strategy, single cell cloning can help the creation of isogenic knockouts of other CF relevant genes, for example those of the inflammasome or those related to bicarbonate transport.

8.2.4. Engraftment

Cell therapy in the lung needs to guarantee the successful engraftment of the delivered cells. During this study, I have created a fully decellularised rat trachea scaffold, which maintains the properties of the ECM of the organ. Decellularised organ scaffolds that preserve the properties of the native organ, are used as matrices for the development of organs for transplantation. My aim here however, was not to create a bio-engineered trachea for transplantation purposes but rather to use the scaffold for seeding and engraftment experiments.

Seeded cells successfully engrafted in the decellularised scaffold, demonstrating that this is possible when there is no competition with the native epithelium. Contrary, our efforts to differentiate the cells seeded on the scaffold were unsuccessful. However, the timeline of this project did not allow adequate optimisation, which could have included longer differentiation periods and dynamic movement of media or scaffold (Maughan et al., 2017).

In the future, decellularised airway scaffolds with successfully differentiated airway epithelial cells, can be used to optimise transfection and gene correction in an airway model system, as a precursor for the development of in vivo CF gene therapy.

In order to test the ability of cells to engraft in vivo where they face competition with the native epithelium, many studies have shown that airway injury is necessary (Berical et al., 2019). Here, I have successfully induced airway epithelial shedding in mice via the intratracheal delivery of polidocanol. Subsequent delivery of CF primary airway epithelial cells did not result in engraftment, however the protocol and procedure were not adequately optimised due to time constraints. Future work should focus on achieving pre clearing of host lung progenitor cells and establishing the appropriate time point for cell delivery, something that could be aided by in vivo tracking of delivered cells.

A limitation of such engraftment experiments, is that CF mouse models do not develop lung disease. As a consequence, transplantation experiments are performed in lungs without chronic
inflammation or infection thus making it difficult to predict how engraftment would be affected by preexisting respiratory disease in CF patients.

8.2.5. General limitations

As mentioned in earlier chapters, a general limitation of some of the experiments of this study regards the lack of replicates. The reasons behind this were directly associated with time constrictions. Where the importance of testing the feasibility of a certain approach in primary airway epithelial cells e.g. correction via a CRISPR/Cas9 selection system or creation of isogenic knock-outs from single cells, was prioritised, full optimisation of nucleofection conditions was not pursued. As a result, nucleofection conditions cannot be compared with statistical significance. Similarly, growth curves of cells have in all but one instance, not been repeated and the results represent what is possible rather than what would be achieved as an average. In some instances, time did not allow replication of long experiments with negative or not promising results, and neither their full investigation and troubleshooting. In the case of Western blots, the very limited material with very low CFTR concentration, even when the lysates from multiple ALI cultures were pooled together, resulted in single replications of the assay.

Nevertheless, single observations have been in all occasions informative for directing subsequent research and will form the basis for subsequent investigation.

8.3. From bench to bedtime

In order to translate results from the research lab to a human ex vivo cell therapy for CF, the first challenge is the correction of primary airway epithelial cells in an efficient and safe way. Secondly, is the maintenance of a stem cell population in the corrected cells that is able to expand, differentiate and repopulate the host lung. Thirdly, is the development of clinically appropriate compounds for human lung injury and of an efficient way to deliver a large number of cells to the human airways. Importantly, technological advances will be needed to support an injured and regenerating human lung in the clinic. Lastly, all experiments should abide by GMP standards and this includes the cell harvesting procedure, cell culture reagents and gene editing compounds.

8.4. Conclusion
In this study, I sought to work towards a proof of principle ex vivo gene therapy for CF. Progress was achieved on multiple fronts, notably expansion and maintenance of a suitable cell type in vitro as well as gene editing, single cell cloning and in vitro engraftment of these cells. Challenges still remain for the delivery of such therapies to the clinic and hopefully future studies will endeavor to address them.
REFERENCES


Hong KU, Reynolds SD, Giangreco A, Hurley CM and Stripp BR. Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion (2001). Am J Respir Cell Mol Biol. 24:671-81.


Mention K, Santos L and Harrison PT. Gene and Base Editing as a Therapeutic Option for Cystic Fibrosis-Learning from Other Diseases (2019). *Genes (Basel).* 10(5).


Posters
Barr J Sr. Optimizing CRISPR ribonucleoprotein components for precision genome editing. Integrated DNA Technologies.

From Biorxiv
Geisinger JM and StearnsT. CRISPR/Cas9 Treatment Causes Extended TP53-Dependent Cell Cycle Arrest In Human Cells (2019).

Online resources:


http://blog.addgene.org/genome-engineering-using-cas9/grna-ribonucleoproteins-rnps

http://www.histology.leeds.ac.uk/respiratory/index.php

https://cftr2.org/mutations_history

https://commons.wikimedia.org/w/index.php?curid=30131299

http://cnx.org/content/col11496/1.6/

https://pocketdentistry.com/5-the-respiratory-system/

https://www.synthego.com

https://www.addgene.org

https://www.idtdna.com
APPENDIX

Modelling of indels in the two alleles of clone 2.2. Both the -2 and +22 mutations create stop codons.
# Mycoplasma testing

<table>
<thead>
<tr>
<th>Date</th>
<th>Samples</th>
<th>Reading A</th>
<th>Reading B</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.04.2018</td>
<td>DF508 P5</td>
<td>274</td>
<td>141</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>HuFeTRC01 P5</td>
<td>267</td>
<td>207</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>Negative control</td>
<td>416</td>
<td>50</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Positive control</td>
<td>238</td>
<td>12942</td>
<td>54.38</td>
</tr>
<tr>
<td>10.05.2018</td>
<td>HuFeTRC01 P6</td>
<td>385</td>
<td>238</td>
<td>0.618</td>
</tr>
<tr>
<td></td>
<td>DF508 P6 Crispr 27.04.18</td>
<td>223</td>
<td>183</td>
<td>0.821</td>
</tr>
<tr>
<td></td>
<td>DF508 P6 27.04.18</td>
<td>216</td>
<td>216</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>DF508 P6 20.04.18</td>
<td>432</td>
<td>323</td>
<td>0.748</td>
</tr>
<tr>
<td></td>
<td>DF508 Crispr 30.04.18</td>
<td>419</td>
<td>295</td>
<td>0.704</td>
</tr>
<tr>
<td></td>
<td>Negative control</td>
<td>585</td>
<td>146</td>
<td>0.250</td>
</tr>
<tr>
<td></td>
<td>Positive control</td>
<td>178</td>
<td>9998</td>
<td>56.169</td>
</tr>
<tr>
<td>06.12.2018</td>
<td>Clone 20 - WT</td>
<td>3189</td>
<td>2427</td>
<td>0.761054</td>
</tr>
<tr>
<td></td>
<td>Clone 2 - MT</td>
<td>4496</td>
<td>3299</td>
<td>0.733763</td>
</tr>
<tr>
<td></td>
<td>PI</td>
<td>4054</td>
<td>2960</td>
<td>0.730143</td>
</tr>
<tr>
<td></td>
<td>DF508 - CR7</td>
<td>8608</td>
<td>2917</td>
<td>0.338871</td>
</tr>
<tr>
<td></td>
<td>Negative control</td>
<td>916</td>
<td>143</td>
<td>0.156114</td>
</tr>
<tr>
<td></td>
<td>Positive control</td>
<td>657</td>
<td>529382</td>
<td>805.7565</td>
</tr>
<tr>
<td>25.06.2019</td>
<td>CR10-Donor-G</td>
<td>621</td>
<td>355</td>
<td>0.571659</td>
</tr>
<tr>
<td></td>
<td>Negative control</td>
<td>963</td>
<td>132</td>
<td>0.137072</td>
</tr>
<tr>
<td></td>
<td>Positive control</td>
<td>442</td>
<td>36112</td>
<td>81.70136</td>
</tr>
</tbody>
</table>