Production of a Sustainable Primary Airway Cell Model for Respiratory Research

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Abstract

The airway epithelium is one of the body's front-line defences against environmental factors. It is known that in diseases such as asthma and COPD the epithelial layer is susceptible to damage which propagates inflammation and airway remodelling. Whilst primary airway epithelial cells have been key to furthering our knowledge of respiratory diseases, their practicality in research is limited by their short lifespan when cultured *in vitro*. Work completed by our research group previously highlighted the potential of BMI-1 in extending the lifespan, whilst maintaining plasticity, of bronchial epithelial cells for research purposes. The aim of this project was to build further on this work and determine whether HBECs engineered to express BMI-1 are comparable to normal human bronchial epithelial cells (NHBECs).

Using lentivirus transduction, eight primary human bronchial epithelial cell (HBEC) donors were infected to stably overexpress BMI-1. Cells overexpressing BMI-1 had an evident proliferative advantage in comparison to cells engineered to express an empty vector, as these cells underwent senescence within 3 passages, following lentivirus infection. By performing an MTT assay, cells modified to overexpress BMI-1 were shown to have greater metabolic activity than NHBECs. The impact of BMI-1 on cell properties were assessed using electric cell substrate impedance sensing (ECIS) and a two-step cell cycle assay. BMI-1 upregulation does not significantly alter cell adherence and cell spreading of HBECs. In addition, overexpression of BMI-1 did not impact the ability of HBECs to form a resistive barrier. It was also shown that the frequency of cells in the G1/G0 phase of the cell cycle was significantly reduced in cells engineered to overexpress BMI-1 in comparison to NHBECs. Preliminary RNA-sequencing analyses suggest alterations in gene expression exist as anticipated but these are modest and identifiable, representing just 2.3% of the genome (5% FDR).

Together these results show that the use of lentivirus to deliver and promote BMI-1 expression in HBECs is an effective genetic manipulation system. BMI-1 expression provides cells with a proliferative advantage and enhanced metabolic activity within cells. Furthermore, it has been shown that HBECs engineered to overexpress BMI-1 behave almost identically to NHBECs when cultured *in vitro*.

Acknowledgments

I would like to thank my supervisors Professor Ian Sayers and Dr Cynthia Bosquillon for giving me the opportunity to work on this project. Ian thank you for your patience, support and continued guidance throughout the year.

I would also like to thank all within the respiratory medicine department for making this an enjoyable year. In particular I'd like to thank Mike Portelli and Marlies Ketelaar for taking the time out to train and advise me during the project. I would also like to thank Amanda Henry for the preliminary RNA sequencing data.

Covering Letter

The following report has been revised and corrected based on the examiners comments. The comments have been addressed with the exception of a couple of points which I will now clarify. Unfortunately, the RNA sequencing data was analysed just over a week before the deadline and as a result I did not have the available time to write a complete chapter dedicated to the RNA sequencing. To overcome this, we added the available preliminary data to the final discussion chapter to highlight some of the initial findings. However, a more extensive RNA sequencing analysis will feature as a component of a paper later this year.

An additional point mentioned was the use of cell viability on the x-axis of ***Figure 2.10*** – ***Figure 2.12***. This was following the work performed previously in our research group and in alignment with the figures used in Torr et al., 2018. What an MTT assay truly measures has been subject to debate. Research groups have used MTT assays as a measure of proliferation, but mitochondrial activity is potentially more related to cell viability rather than proliferation.

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Abbreviations

ALI – Air Liquid Interface

AUC – Area Under the Curve

BEGM – Bronchial Epithelial Cell Growth Medium

BLF – British Lung Foundation

BMI-1 – B Cell-Specific Moloney Murine Leukemia Virus Integration Site 1

COPD – Chronic Obstructive Pulmonary Disease

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

DAMPs – Damage-Associated Molecular Patterns

DNA – Deoxyribose Nucleic Acid

ECIS – Electric Cell Substrate Impedance Sensing

EGF – Epidermal Growth Factor

EV – Empty Vector

FDR – False Discovery rate

FKPM – Fragment Per Kilobase Million

GWAS – Genome Wide Association Study

HBEC – Human Bronchial Epithelial Cell

hES – Human Embryonic Stem Cell

hTERT – Human Telomerase Reverse Transcriptase

hOEG – Human Olfactory Ensheathing Glia

ILC – Innate Lymphoid Cell

NHBEC – Normal Human Bronchial Epithelial Cell

NHOK – Normal Human Oral Keratinocytes

NHS – National Health Service

PAMPs – Pathogen-Associated Molecular Pattern

PBS – Phosphate Buffered Saline

PcG – Polycomb Group Protein

PDL – Populations Doublings

PLAUR – Urokinase Plasminogen Activated Receptor

PRR – Pattern Recognition Receptor

qPCR – Quantitative Polymerase Chain Reaction

RNA – Ribose Nucleic Acid

ROCK – Rho-associated Protein Kinase

RPM – Revolutions Per Minute

SNP – Single Nucleotide Polymorphism

TEER – Trans-epithelial Electric Resistance

1 Chapter 1: Introduction to the airway epithelium in health and disease.

1.1 Airway disease

A comprehensive British Lung Foundation (BLF) survey has estimated that lung disease contributes to 20% of deaths in the UK (Snell et al., 2016). Asthma and chronic obstructive pulmonary disease (COPD) are the two most common airway diseases in the UK and place a significant burden on the National Health Service (NHS). Asthma and COPD are both chronic obstructive respiratory diseases which can be characterised by reoccurring episodes of breathlessness, coughing and wheezing. It is estimated that asthma affects up to 300 million people worldwide (Lambrecht and Hammad, 2015). The prevalence of asthma is particularly high within developed countries including the UK were the clinical asthma incidence is thought to be as high as 18% (To et al., 2012). Currently there is no cure for asthma but only measures to control the clinical symptoms which can differ drastically in severity. For mild intermittent asthma short-acting β2 agonist inhalers which induce bronchial dilation are usually sufficient. However, in cases of moderate to severe asthma corticosteroids such as prednisolone or hydrocortisone are required in combination with long-acting β2 agonist inhalers.

1.2 Allergic and non-allergic asthma

To simplify, the hallmark of allergy-induced asthma is inflammation of the airways stimulated by mast cells, eosinophils and T cells. In reality asthma is a much more complex disease as I will discuss in greater detail later in the chapter (Anderson and Coyle, 1994). The majority of children and approximately 50% of adults suffer from allergic asthma, whilst non-atopic asthma generally develops later in life. The factors responsible for inducing non-atopic asthma are poorly defined but are not thought to involve IgE sensitivity and T helper 2 (Th2) driven adaptive immunity, which are common features of allergic-asthma. There is growing evidence to suggest that

separating asthma into two clinical subgroups (allergic and non-allergic) is an oversimplification and that instead clinically different asthma should be separated into asthma endotypes, that take into consideration key variables including; genetic susceptibility, environmental risk, age of onset, clinical presentation and prognosis (Anderson, 2008).

1.3 The airway epithelium

In this study I will focus on the airway epithelium, a critical barrier which separates the body from the environment. As a result, epithelial cells are the host's first line of defence against bacteria, viruses, toxic chemicals and allergens. Epithelial cells express an extensive repertoire of pattern recognition receptors (PRRs) which allows them to respond rapidly to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Therefore, epithelial cells play a key role in eliciting immune responses to a wide range of factors via the activation of dendritic cells (Lambrecht and Hammad, 2012).

The airway epithelium is made up of predominantly three specialised cell types; ciliated columnar cells, mucin-secreting goblet cells and basal cells as displayed in ***Figure 1.1***.

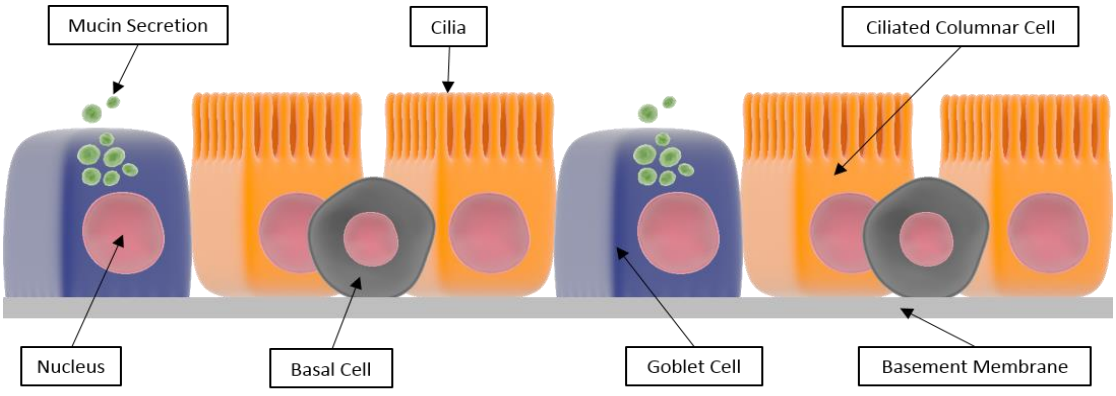


Figure 1.1. The Bronchial Epithelial Layer. The bronchial epithelium consisting of 3 main cell types: Mucin secreting goblet cells, ciliated columnar cells and undifferentiated basal cells.

It is important to highlight that the composition of the three main cell types varies along the respiratory tract, from the trachea down to the terminal bronchiole. There is an abundance of pseudostratified ciliated columnar cells and mucin-secreting goblet cells in the epithelium of the upper respiratory tract, including the bronchial epithelium, which provide mechanical strength and immune protection against inhaled pathogens. In the lower respiratory tract there is an absence of goblet cells and a greater presence of secretory cells which release surfactant and facilitate gas exchange as shown in ***Figure 1.2*** (Iwasaki et al., 2017).

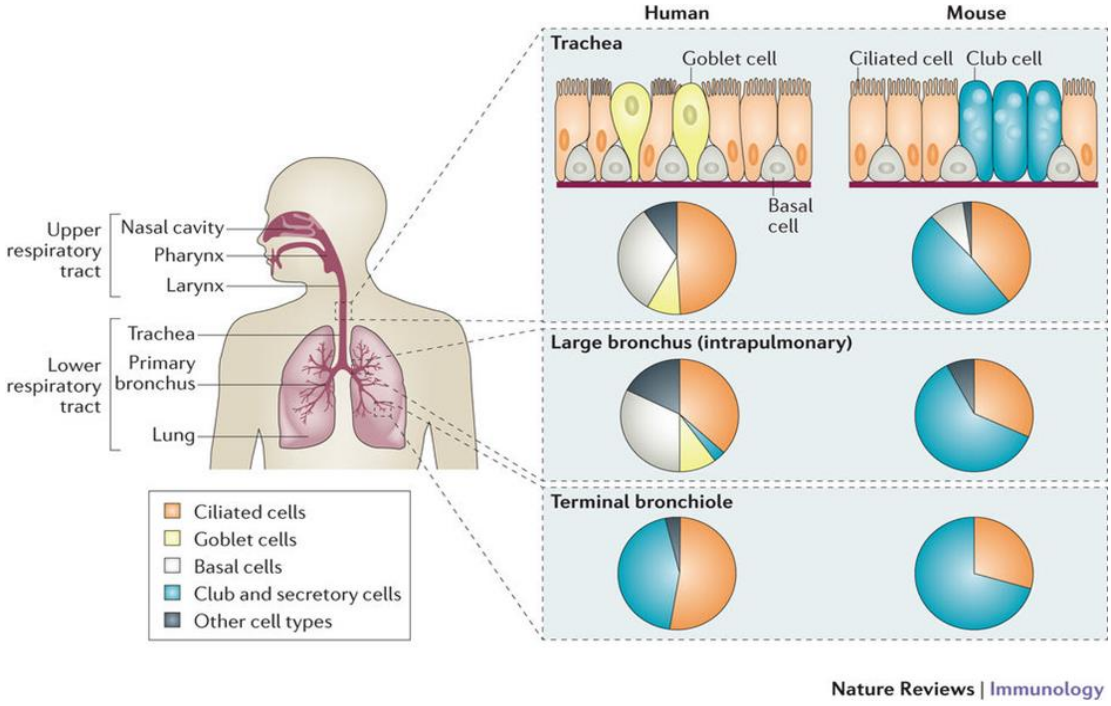


Figure 1.2. Cell Composition of the Airway Epithelium in Humans and Mice. This figure highlights how the proportion of specific cell types differs along the airways. Goblet cells and basal cells are highly abundant in the upper respiratory tract but not in the lower respiratory tract where secretory cells are present more readily. This figure also outlines how the mouse airway epithelium differs from humans (Iwasaki et al., 2017).

Due to the significant differences between the function of each cell type, it is expected that the balance of epithelial cell types is key to maintaining airway homeostasis and that profound changes to cell composition would lead to respiratory complications.

Figure 1.3 highlights how changes in airway cell composition can trigger asthma symptoms such as breathlessness and wheezing. It shows the disruptive effect of airway remodelling on the airways which includes; goblet cell hyperplasia, increased mucin secretion, thickened basement membrane, hypertrophy of the airway smooth muscle and airway narrowing. I will discuss in greater detail later in the chapter, how disruptive airway remodelling is and how it contributes significantly to chronic airway diseases such as asthma and COPD.

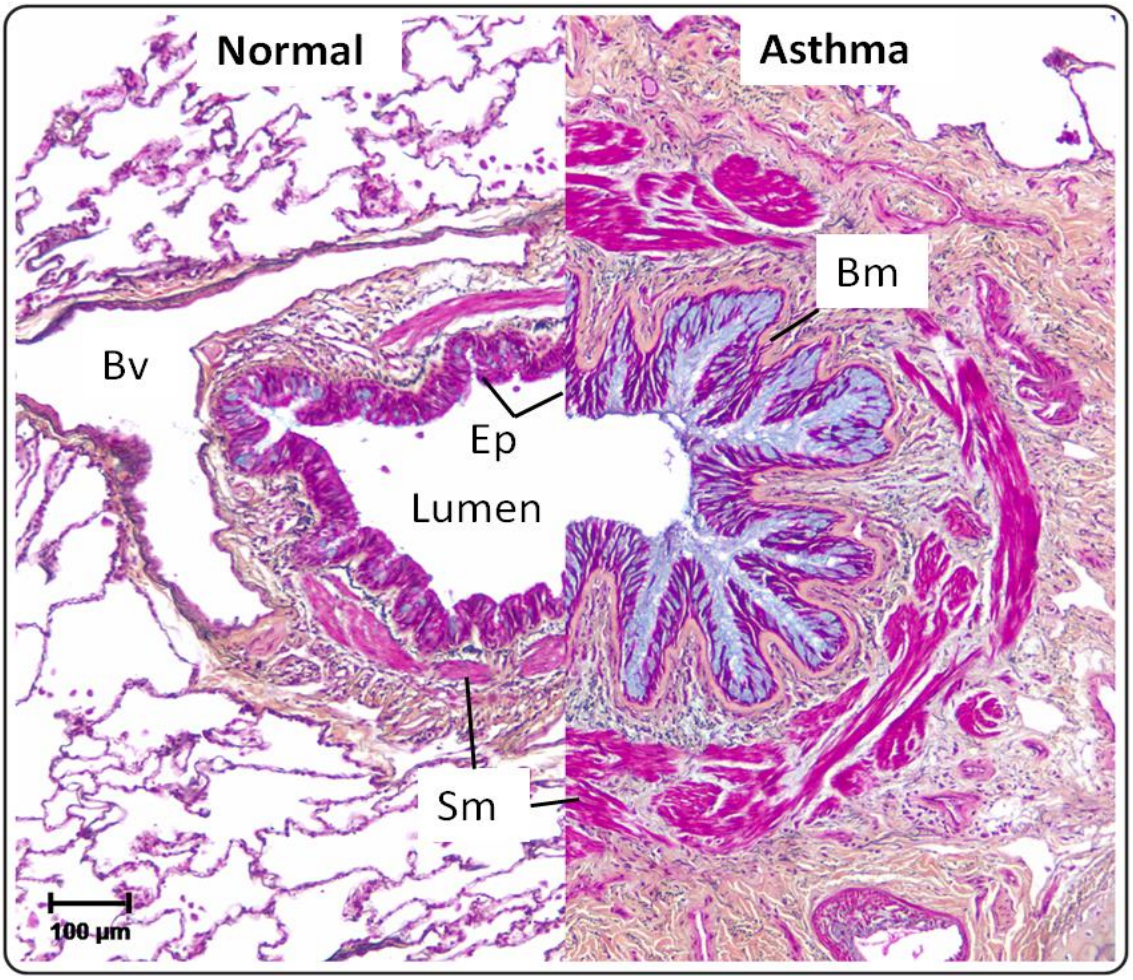


Figure 1.3. Comparison of normal and asthma airways. The airway from this asthma patient has undergone severe airway remodelling. The diameter of the airway lumen is significantly reduced. The airway epithelium has undergone goblet cell hyperplasia resulting in increased mucin secretion (blue staining) into the lumen of the airway. In addition, we see a thickened basement membrane and hypertrophy of smooth muscle around the airway, helping contribute to airway narrowing (Wadsworth et al., 2012).

1.4 The causes of asthma

The exact causes of asthma are unclear but are thought to be triggered by a combination of both genetic and environmental risk factors (Mukherjee and Zhang, 2011). It has been established for some time that asthma has a hereditary component, a study of German children found that the risk of a child developing asthma was 2.6 fold higher when one parent had asthma compared to neither parents having a history of asthma (Dold et al., 1992). However, only recently due to major advances in the field of genetics, have we been able to study in detail genetic variations which contribute to the onset of asthma. The development of genome-wide association studies (GWAS) means we can now identify associations between variations in single nucleotide polymorphisms (SNPs) and asthma phenotypes. GWAS studies have identified over 50 genes associated with asthma, demonstrating the complexity of the disease. One of the most extensive GWAS studies to date was conducted by Moffatt et al., this study identified several asthma susceptibility genes, one of the most significant being present in the ORMDL3 locus as seen in ***Figure 1.4*** (Moffatt et al., 2010). The role of ORMDL3 in asthma is poorly understood but is thought to have a role in regulating sphingolipid production and the ER stress response. However, new research is giving an insight into the function of ORMDL3 in asthma. It has shown that increased ORMDL3 expression in bronchial epithelial cells is a driving factor behind *Alternaria*-induced airway hyper-responsiveness and allergen-induced loss of epithelial barrier integrity, through inducing uric acid release (Loser et al., 2017).

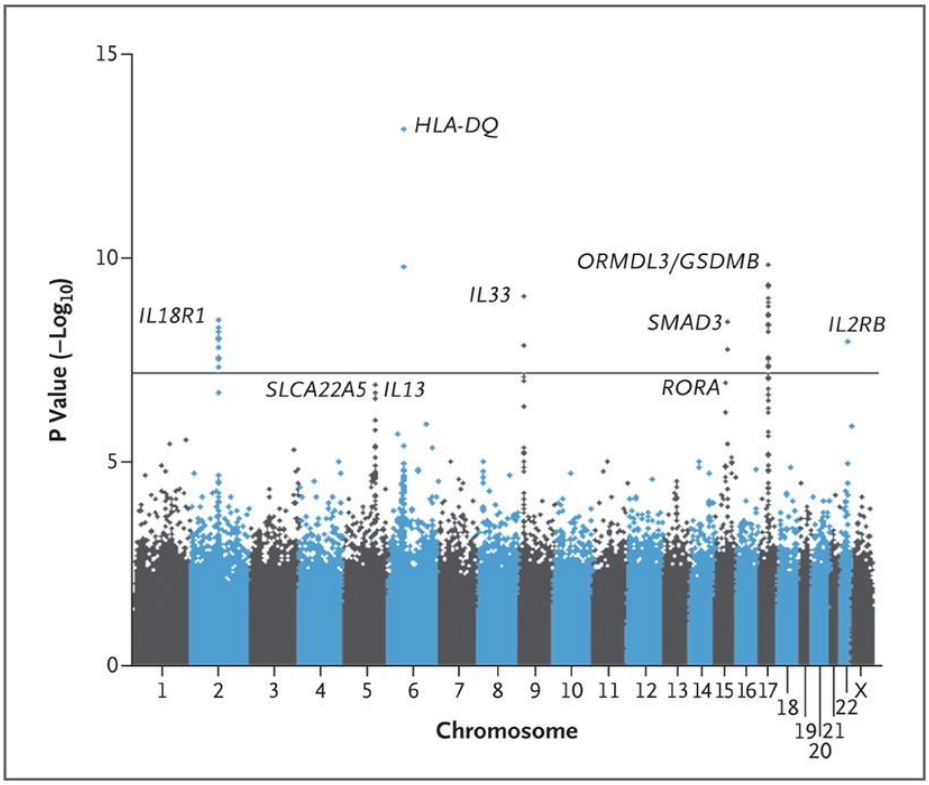


Figure 1.4. Manhattan Plot of Asthma Susceptibility Genes. The manhattan plot identifies key asthma susceptibility genes, their chromosomal position and there significance in asthma (Moffatt et al., 2010).

Additional asthma susceptibility genes are, as expected, are associated with Th2 immunity within the lungs such as the IL13, IL33 and the IL18R1/IL1RL1 loci. The IL1RL1 gene encodes for the ST2 receptor which is expressed abundantly on Th2 and mast cells. Furthermore, the IL33 gene encodes for the IL-1-like cytokine, IL-33, which is a ligand of the ST2 receptor (Schmitz et al., 2005). Airway epithelial cells have been shown to produce IL-33 in response to exposure to allergens such as house dust. Production of IL-33 can lead to the activation of type 2 innate lymphoid cells (ILC2s), which produce classic Th2 cytokines such as IL-5 and IL-13 (Lambrecht and Hammad, 2015). Alternatively, IL-33 has also been found to promote DC activation, in the process driving Th2 allergic inflammation (Besnard et al., 2011). As a result, the IL-33/ST2 receptor axis has become of great interest in respiratory diseases and additionally, IL-33 has been shown to be upregulated in both asthma and COPD (Byers et al., 2013; Prefontaine et al., 2010). Epithelial cells are thought to be a major source of IL-33, releasing the cytokine in response Rhinovirus-induced asthma exacerbations

and following exposure to cigarette smoke (Jackson et al., 2014; Liew, 2015; Pace et al., 2014). Further GWAS studies have identified urokinase plasminogen activated receptor (PLAUR) as a susceptibility gene for asthma through analysis of SNPs from asthma populations (Barton et al., 2009). The plasminogen system is an important signalling cascade responsible for regulating a number of cell functions. It is proposed that changes in normal epithelial functions such as proliferation, apoptosis and migration can be driven by increased expression of PLAUR accumulating in the progression of obstructive airway disease (Stewart et al., 2012a). The described studies only serve to highlight the significance of epithelial cells and their contribution to the onset of airway diseases such as asthma and COPD and why more research is required to derive potential treatments.

In addition to a wide range of genetic variants, there are numerous environmental factors which can disrupt airway homeostasis and drive chronic airway disease. Epithelial cells are specialised cell types, adapted to form a physical barrier which separate the environment from the body and are therefore the body's first line of defence (Vareille et al., 2011). Environmental factors including pollutants, chemical irritants, cigarette smoke, allergens and pathogens are all capable of inducing significant damage to the epithelial layer (Bascom, 1991; Lambrecht and Hammad, 2014; Mills et al., 1999; Schamberger et al., 2015). Damage to the airway epithelium can allow the passage of pathogens and other potential irritants into the underlying tissue. As a result the disruption of the susceptible epithelial layer by environmental factors propagates inflammation and airway remodelling observed in a number of respiratory diseases such as asthma and COPD (Davies, 2009). ***Figure 1.5*** illustrates the consequences of pathogens and environmental pollutants on the physiology of the airway epithelium and the underlying tissue.

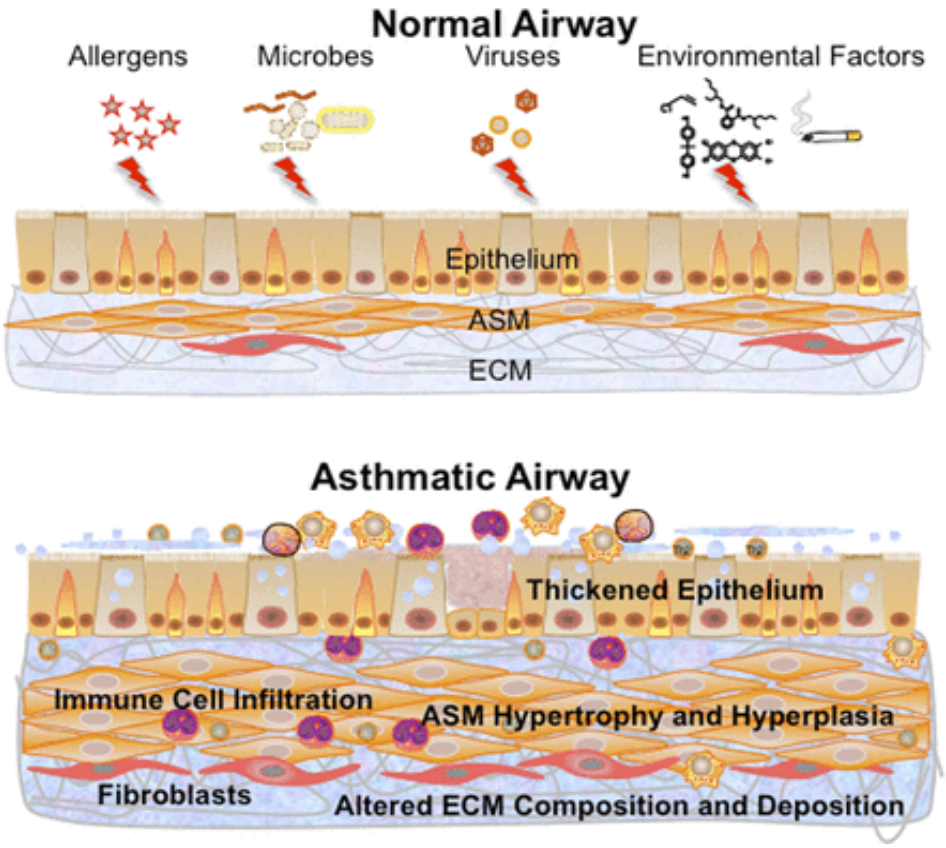


Figure 1.5. The Effects of Environmental Factors on the Airway Epithelium.
Environmental factors such as cigarette smoke, allergens, pathogens such as microbes and viruses have a profound impact on airway homeostasis by promoting immune cell infiltration, ASM hypertrophy and alteration of the epithelial composition (Prakash, 2013).

1.5 In vitro studies of asthmatic and healthy epithelial cells

The bronchial epithelium is made up of several specialised cell types including; ciliated columnar, mucus secreting goblet and clara cells. There is growing evidence to indicate that the airway epithelium in diseases such as asthma and COPD exhibit different biochemical and functional characteristics to normal airway epithelium (Kicic et al., 2006b). This has been supported by many studies which have demonstrated that airway epithelial cells isolated from patients with asthma, show distinctive gene expression profiles commonly associated with inflammation, repair and remodelling in comparison to epithelial cells derived from healthy patients (Kicic et al., 2010; Stevens et al., 2008). The differences between asthmatic and healthy epithelial cells can be

observed after their differentiation. These features often include excessive shedding and increased numbers of goblets cells with reduced quantities of ciliated cells. The consequence of goblet cell hyperplasia is mucus hypersecretion a feature commonly associated with severe asthma (Gras et al., 2012; Parker et al., 2010). In addition to goblet hyperplasia Hackett et al., demonstrated that epithelial cells from asthmatic patients display a less differentiated phenotype, distinguished through elevated numbers of basal cells as shown in ***Figure 1.6*** (Hackett et al., 2011).

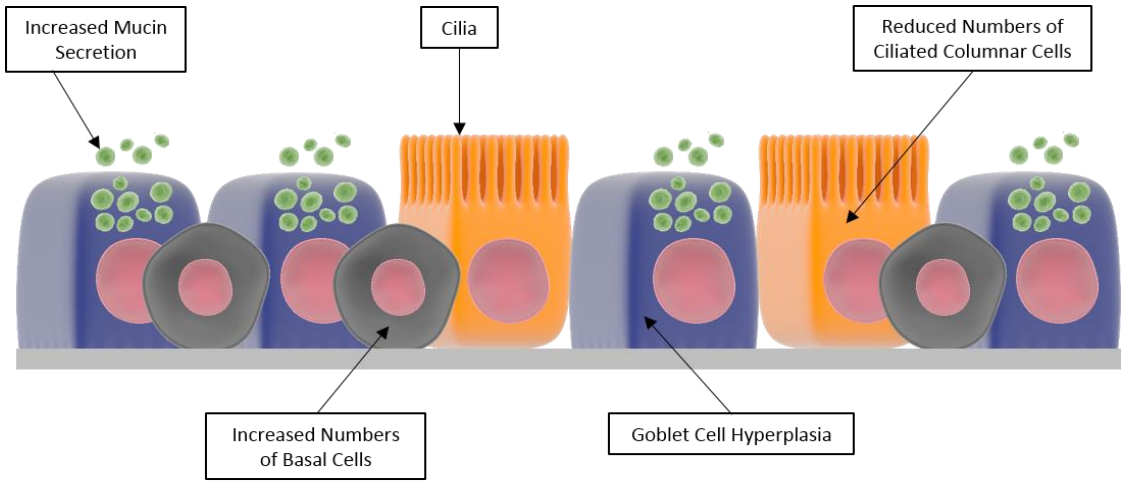


Figure 1.6. The Epithelial Layer in Patients with Severe Asthma. The airway epithelium of severe asthma patients is drastically altered in comparison to healthy individuals. The changes that manifest include goblet cell hyperplasia and consequently increased mucin secretion, reduced ciliated columnar cells and a less differentiated phenotype.

Asthmatic epithelial cells have an increased proliferative capacity in comparison with healthy cells, potentially a consequence of elevated epidermal growth factor (EGF) expression (Kicic et al., 2006a). In addition to EGF, the increased proliferation of asthmatic epithelial cells may be stimulated due to higher levels of IL-6, a molecule known to stimulate basal cell proliferation in lung cancers (Hackett et al., 2011; Yamaji et al., 2004). Interleukins mediate several functions in airway epithelial cells including immune stimulation, proliferation and airway remodelling. IL-4 and IL-13 stimulation of asthmatic bronchial epithelial cells induced significantly increased expression of the airway remodelling factor, TGF-β2. VEGF, like TGF-β2, is thought to drive epithelial

remodelling and both are significantly upregulated in epithelial cells derived from asthmatic children in comparison to healthy children (Lopez-Guisa et al., 2012).

Air-liquid interface (ALI) is a readily used technique in respiratory medicine which recreates the conditions within the lungs. HBECs are cultured on a semi-permeable membrane within a transwell. Once a confluent monolayer has formed on the membrane, culture media is kept on the basolateral membrane but can be removed from the apical surface of the cells. The exposure of the apical surface of the cells to air mimics *in vivo* conditions within the lungs. This is supported by the comparison of expression profiles of ALI cultures in submerged nutrient media and *in vivo* airway epithelia. They found that the transcriptional profile of primary ALI cultures most closely resembled that of *in vivo* airway epithelium (Pezzulo et al., 2011).

ALI cultures can be used to derive physiological data regarding primary epithelial cells, data which is difficult to establish through growing cells in monolayers on plastic substrates, like traditional *in vitro* methods. As a result, ALI cultures can be used to measure transepithelial electrical resistance (TEER), the measurement of TEER can be used to indirectly assess the formation of tight junctions and thus quantify the level of disruption in the epithelial layer. A recent study by Wawrzyniak et al., demonstrated that asthmatic epithelial cells grown at ALI have low tight junction integrity induced by Th2 cytokines, IL-4 and IL-13, and increased expression of HDAC 1 and 9 (Wawrzyniak et al., 2016). Inhibition of HDAC activity helped reverse the loss of barrier integrity in asthmatic epithelial cells through increasing tight junction formation (Wawrzyniak et al., 2016). Through culturing cells at ALI, it has been found that asthmatic epithelial cells have an inability to repair mechanical wounds. The dysregulated wound repair of asthmatic epithelial cells is likely to involve a number of factors. Evidence suggests that the plasminogen activator inhibitor-1 plays an important role in the repair process of healthy epithelial cells. However, this repair mechanisms is dysregulated due to the protein being constitutively upregulated in asthmatic epithelial cells (Stevens et al., 2008). In addition to displaying poor wound repair, studies have indicated that the bronchial epithelial barrier of asthmatic subjects is compromised. The defective epithelial layer is a result of disrupted tight junctions which allows the

passage of allergens into the airway tissue exasperating the onset of asthma (Xiao et al., 2011).

1.6 The uses of human airway epithelial cells for respiratory research

Primary epithelial cells taken from the airways of human donors have been key to furthering our understanding of airway diseases such as asthma. As discussed in previous sections, studies of epithelium have taught us a great deal about the mechanisms of airway disease. These include how environmental factors contribute to the onset of chronic respiratory diseases, how epithelial cells and damage to the epithelial layer propagate the immune response (Holgate, 2012; Lambrecht and Hammad, 2014; Mills et al., 1999; Schamberger et al., 2015). RNA sequencing projects using epithelial cells have identified numerous asthma susceptibility genes and specific single nucleotide polymorphism which contribute to poor lung function (Ierodiakonou et al., 2016; Wan et al., 2012). If we intend to develop therapies to combat airway diseases, then more research will be required into the airway epithelium.

1.7 The need for a new model of the human airway epithelium for research purposes

Epithelial cells derived from patients and grown *in vitro,* generally survive between 4-5 passages, a lifespan which is often insufficient to establish large scale experimental data such as drug screening. In addition, primary cells can be notoriously difficult to grow and make experimental planning difficult.

Engineering epithelial cells to prolong their lifespan whilst maintaining the desired characteristics of epithelial cells, would be greatly beneficial for not only enhancing our understanding of respiratory disease but potentially lead to therapeutic interventions. In addition, the procedure for isolating epithelial cells from patients (bronchoscopic biopsy) is an invasive procedure and carries a risk to patients,

especially those with severe forms of asthma. Extending the lifespan of epithelial cells would hopefully reduce the frequency of bronchoscopic procedures required and improve patient well-being.

An alternative to primary epithelial cells is using immortalised cell lines such as Calu-3 or BEAS-2B cells. The Calu-3 cell line was produced from pleural effusion of a lung adenocarcinoma, isolated from submucosal gland serous cells (Fogh, 1978). Whilst the BEAS-2B cell line was produced through immortalisation of NHBECs with AD12-SV40 virus (Reddel et al., 1988). These cells possess characteristics more suited to some aspects of research as they are more robust, easier to manipulate and cheaper than primary cells. Furthermore, primary cells can give variable results, studies of cell lines are generally more reproducible (Kaur and Dufour, 2012).

Calu-3 cells show a greater resemblance to primary cells than BEAS-2B cells when cultured at ALI. Calu-3 cells produce high TEER and a pattern of expression not that dissimilar to primary epithelial cell markers, whereas BEAS-2B cells show weak TEER and lack primary epithelial cell differentiation markers (Stewart et al., 2012b). As a result, BEAS-2B cells are unsuitable for studies which involve assessment of cell barrier properties. Calu-3 cells do form a resistive barrier but they are also an imperfect model of the airway epithelium. Calu-3 cells lack normal pairs of chromosomes 1, 13, 15 and 17, in addition, they are triploid for other unidentified chromosomes (Shan et al., 2011). Immortalised cell lines including Calu-3 cells and BEAS-2B cells provide a useful option for studying the airway epithelium, especially preliminary experiments assessing drug transporter properties of HBECs (Hamilton et al., 2001; Sakamoto et al., 2015). However, beyond these types of studies, immortalised cell lines are of limited use in progressing our understanding of airway disease mechanisms.

Mouse models have also been important in enhancing our knowledge of numerous diseases and for respiratory diseases, including asthma, this is no exception. Mouse models are particularly useful for assessing mechanical forces within the airway such as resistance and elastance in real time. However, as shown previously in ***Figure 1.2*** the composition of the airway epithelium differs to an extent between mice and humans and whilst the bronchial epithelium is not drastically different it is still a

variable that ought to be considered when choosing an epithelial model (Iwasaki et al., 2017).

1.8 Approaches to extend the lifespan of primary human cells

There have been many successful attempts to extend the lifespan of primary human cells. The Epstein-Barr virus latent membrane protein, LMP1, is capable of immortalising human primary B-lymphocytes via suppression of $p16^{INK4A}$ (Yang et al., 2000). A study by Garcia-Escudero et al., attempted to immortalise human olfactory ensheathing glia (hOEG) through gene transfer of BMI-1, SV40 large T antigen (TAg), a short hairpin RNA against p53 (shp53) and a catalytic subunit of telomerase reverse transcriptase (TERT). However, only the combination of BMI-1/TERT transduction was capable of prolonging the lifespan of hOEGs, whilst maintaining the cells proliferative potential, karyotype, and preventing cell senescent phenotypes developing (Garcia-Escudero et al., 2010). hTERT has been used effectively in several other studies as a mechanism of immortalising primary cell lines whilst still retaining their phenotype. Neural progenitor cells induced to express hTERT could undergo 120 population doublings whilst retaining a diploid karyotype and retaining key neural progenitor markers (Bai et al., 2004). Similarly, immortalisation of human keratinocytes by hTERT delayed senescence without majorly impacting differentiation systems (Dickson et al., 2000). However, other studies using hTERT as an immortalisation tool have found several problems. Milyavsky et al., showed that whilst prolonged expression of hTERT in fibroblasts enhanced the cells replicative potential, these cells eventually showed signs of becoming malignant clones (Milyavsky et al., 2003). Previous research by our group has also shown that hTERT expression is not an efficient method for immortalisation of HBECs. Not only do epithelial cells induced to express hTERT, lose their barrier resistance, they also show signs of major chromosomal abnormalities (Torr et al., 2016).

1.9 The potential of BMI-1 in delaying cell senescence

B cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) is a polycomb protein complex encoded by the BMI-1 gene. The 37kDa BMI-1 protein consists of three separate domains: An N-terminal RING finger domain, a central helix-turn-helix domain and a C-terminal proline-serine rich domain (Gray et al., 2016). As a polycomb group (PcG) protein, BMI-1 is a key transcriptional regulator and has been shown to have a number of important cellular functions including the renewal of haematopoietic and neural stem cells (Lessard and Sauvageau, 2003; Molofsky et al., 2005). A study of WI-38 human fibroblasts found that BMI-1 was downregulated in cells undergoing replicative senescence; furthermore, overexpression of BMI-1 extended the replicative life span of the cells. The mechanisms by which BMI-1 prolongs the replicative life span of human fibroblast cells is thought to be through the suppression of $p16^{INK4A}$ and $p19^{Arf}$ pathways. Additionally, the RING finger and the helix-turn-helix domains of BMI-1 have been shown to be essential for $p16^{INK4A}$ suppression and the delaying of senescence (Itahana et al., 2003). There is a wealth of evidence to suggest that the inhibitory mechanisms of $p16^{INK4A}$ on the pRb pathway, as seen in ***Figure 1.7***, to be a key factor which drives cell cycle arrest and senescence (Park et al., 2004). It can be easily recognised that unregulated BMI-1 expression could potentially lead to uncontrolled cell cycle progression and replicative immortality, a hallmark of cancer (Hanahan and Weinberg, 2011). It is therefore unsurprising that BMI-1 was first identified as an oncogene associated with T and B cell leukaemia's in mice (Haupt et al., 1991). Further research has linked BMI-1 expression to a wide range of cancers. Upregulation of BMI-1 expression is commonly associated with hematologic cancer such as mantle cell lymphoma and chronic myeloid leukaemia, were enhanced expression are also correlated with a more advanced-phase of the disease (Beà et al., 2001; Mohty et al., 2007).

BMI-1 is not just associated with hematologic cancers, increased expression has also been associated with highly metastatic breast cancer (Guo et al., 2011). Interestingly, BMI-1 inhibition in primary colorectal cancer cells halted tumour growth, these studies highlight the importance of BMI-1 in cell replication and demonstrate that it could

potentially be utilised to prevent cell senescence in primary cell cultures (Kreso et al., 2014).

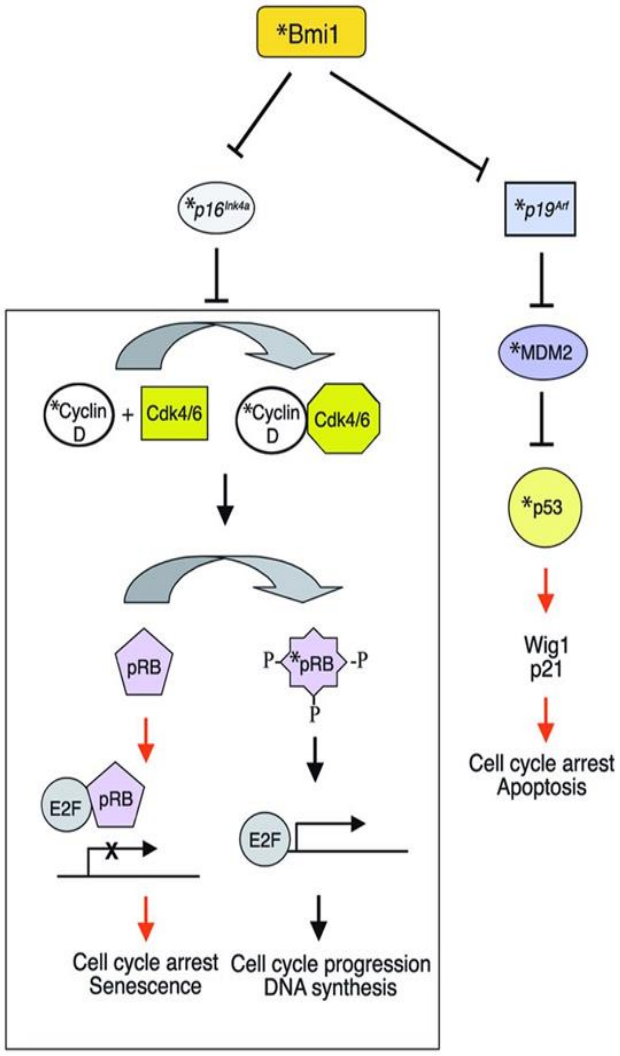


Figure 1.7. The mechanisms by which BMI1 inhibits cell senescence. The suppression of $p16^{INK4A}$ activity by BMI1 promotes the formation of CyclinD-Cdk4/6 complexes triggering the phosphorylation of Rb and the cell cycle progression. Furthermore, BMI1 inhibition of $p19^{Arf}$ promotes the degradation of p53, preventing cell apoptosis (Park et al., 2004).

To test this theory, a study conducted by Fulcher et al., involved the transduction of HBECs with both hTERT and BMI-1, they found that genetically modified HBECs continued to replicate well beyond 38 doubling events whilst control HBECs would undergo senescence before 25 population doublings. In addition, the cells maintained

a stable karyotype and differentiated morphology at ALI culture (Fulcher et al., 2009). However, research conducting by our research group showed that inducing expression of hTERT only resulted in HBECs losing their viability after an increasing number of passages and beginning to display chromosomal abnormalities as early as passage 8 (Torr et al., 2016; Walters et al., 2013). Inducing constitutive expression of BMI-1 through infecting primary epithelial cells with a lentivirus vector containing BMI-1, has shown even greater promise in prolonging the lifespan of epithelial cells. BMI-1 engineered cells appear to maintain their plasticity, the ability to differentiate when cultured in ALI and showed no evidence of chromosomal abnormalities (Torr et al., 2016). To extend on this work another group has attempted to prolong the replicative lifespan of primary diseased cell cultures once again using BMI-1. Munye et al., induced BMI-1 expression in cystic fibrosis bronchial epithelial cells through lentivirus transduction. They demonstrated that BMI-1 expression allows for extended passaging of cystic fibrosis epithelial cells and that mucociliary differentiation is possible in these cells. In addition, the study demonstrated that BMI-1 engineered HBECs can be furthered engineered to produce cell models of rare diseases such as primary ciliary dyskinesia, were patient samples are hard to acquire (Munye et al., 2017).

1.10 Aims of the MRes

The overall aim of my MRes project was to determine the effectiveness and practicality of a method for delaying cell senescence in primary human bronchial epithelial cells. Ultimately, are BMI-1 expressing HBECs suitable cell models of the airway epithelium? There are three predominant objectives from the project as shown below:

- To produce stable genetically modified BMI-1 expressing HBECs and determine the viability of these pLVX-BMI-1 HBECs.
- To determine how increased BMI-1 expression alters key epithelial cell properties such as cell growth, cell spreading and barrier formation.
- To determine the impact of upregulated BMI-1 expression on the transcriptome of HBECs.

It is hoped that these HBECs with increased replicative potential will be significant in furthering our understanding of airway disease development, the mechanisms of airway diseases and the eventual production of therapeutic interventions. Furthermore, this research has the potential to reduce the need for bronchial epithelial cells, derived from patients, through mechanism such as nasal brushes and biopsies which are invasive and potentially harmful to patients.

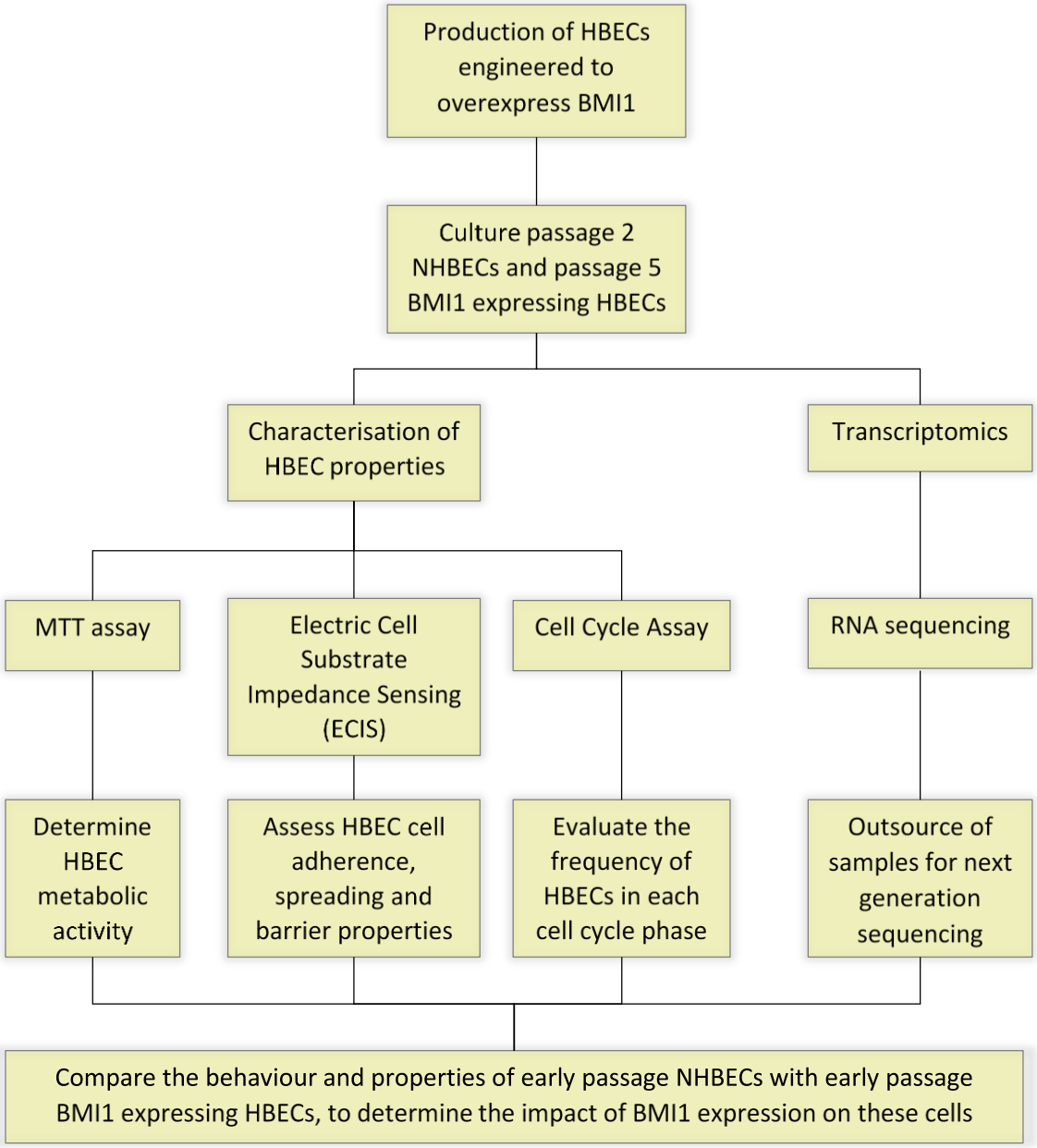


Figure 1.8. Flow chart of the characterisation process of BMI1 engineered HBECs. Here I have outlined the plan for the following chapters. Once HBECs have been engineered to express BMI1, stocks of cells will be frozen down for following experiments. RNA will be collected from NHBECs and pLVX-BMI1 HBECs and outsourced for RNA sequencing. Meanwhile, characterisation studies involving MTT assays, ECIS and cell cycle assays will be performed to determine how comparable BMI1 expressing HBECS are to NHBECs.

2 Chapter 2: Production of pLVX-BMI-1 HBECs

2.1 Introduction

In chapter 1 I outlined why there is a need for an improved cellular model of the airway epithelium. Primary HBECs undergo replicative senescence as a result of stresses induced by telomeric dysfunction, DNA damage or through alterations to key signalling pathways (Campisi and d'Adda di Fagagna, 2007). Immortalised cell lines, specifically BEAS-2B, lack key epithelial markers and barrier properties, whilst Calu-3 have major chromosomal abnormalities (Shan et al., 2011; Stewart et al., 2012b). Furthermore, recent studies have found alarming evidence that cell lines are becoming increasingly cross contaminated. Of 360 reported contaminated cell lines almost 30% of them were found to be contaminated with HeLa cells (Capes-Davis et al., 2010). Cell contamination is a serious problem which has the potential to detract the from the use of cell lines as models, the research by Capes-Davis et al., highlights the need for authenticating samples prior to experimentation.

Research by Fulcher et al., demonstrated a compromise between primary human cells and immortalised cell lines as a possible solution. Their research showed that by inducing BMI-1 expression in primary cells it is possible to extend their passaging (Fulcher et al., 2009). Additional studies have indicated that BMI-1 expression in HBECs not only extends the cells lifespan but has been shown to maintain key epithelial cell properties, including plasticity, barrier formation and karyotype (Munye et al., 2017; Torr et al., 2016).

There are multiple gene delivery methods available but in this study lentivirus transduction will be used to manipulate HBECs. Lentiviruses belong to a sub-group of the retrovirus family, along with HIV. They have evolved the ability to infect non-dividing cells as a result lentivirus vectors can be utilised to infect a wide range of tissues (Lewis et al., 1992). The viral mechanism of lentiviruses has been adapted to

allow the delivery of specific gene sequences without inducing a large immune response. The lentivirus vectors are replication defective due to the cis-acting non-coding sequence elements (RNA synthesis, packaging, reverse transcription and integration) being segregated from the trans elements encoding enzymatic, structural and accessory proteins (Cockrell and Kafri, 2007). The lentivirus vector system used in this project is a second generation, replication incompetent system. Third generation systems are available and involve two packaging plasmids, one encoding Gag and Pol and the other encoding Rev. In addition, this system is TAT independent and as a result the third generation system is considered safer although more difficult to use as it requires more individual plasmids (Dull et al., 1998).

Lentivirus transduction involves delivering the gene of interest directly into the host genome where it is permanently integrated, when the cell divides the genetic modification is passed on to future progeny. As a result, lentivirus transduction is a suitable genetic manipulation system for this project. In addition, lentivirus transduction has a high efficiency rate which can be greater than 90%, in contrast transfecting primary cells is difficult and often results in poor yields.

There are minor disadvantages of using lentivirus as a gene delivery method such as possible insertional mutagenesis and transient expression of the transgene, although these side-effects are common in other viral delivery systems such as adenovirus or the herpes simplex virus. Furthermore, these delivery methods are highly immunogenic and the vector genome is not integrated into the host genome unlike lentivirus transduction (Vannucci et al., 2013). Whilst integration of the vector into the host genome is an advantage of the lentivirus system it may potentially induce problems, especially within this project. Constitutive expression of a PcG protein such as BMI-1 over an extended period may yield unwanted side effects. Methods have been developed which have the potential to overcome this problem, these are inducible systems. Briefly, the inducible system involves infecting cells with two separate lentivirus plasmids. One plasmid encodes a repressor system and the second encodes the gene of interest (in this case BMI-1). Under standard condition the

repressor binds to the promoter (CMV promoter) of BMI-1 and blocks transcription of the gene. However, with the addition of a secondary molecule such as cumate, which can be supplemented into the culture media, inhibition of BMI-1 can be relieved. The cumate can bind to the repressor inducing a conformational change and its release from the promoter, allowing transcription of the BMI-1 gene (Mullick et al., 2006). New increasingly promising methods such as nanoparticles are also being developed which offer an alternative gene delivery system. This methodology has been developed as an exciting clinical application to alter gene expression in diseased cells. This delivery system could potentially be utilised against a range of diseases, especially cancer, as the nanoparticles can be customised to target receptors which are upregulated on cancer cells. The nanoparticle once bound to its target receptor enters the cell through endocytosis were it delivers its payload which could be DNA, mRNA or siRNA (Chen et al., 2016). Although, this system has been developed for clinical applications it could be also be exploited for other research purposes. In the future this may be a suitable alternative to lentivirus based gene delivery. There are fewer safety concerns in comparison to working with live virus. Furthermore, this technique would not elicit an immune response unlike lentivirus and other virus based delivery systems.

Aims of the chapter

1. To generate a stock of HBECs from multiple donors, genetically engineered to overexpress BMI-1.
2. To perform initial characterisation experiments to determine firstly, whether infecting HBECs with pLVX-BMI-1 is effective across multiple donors and additionally if enhanced BMI-1 expression effects basic HBEC properties.

2.2 Methods

2.2.1 BMI-1 plasmid construction and lentivirus packaging

The construction of the BMI-1 plasmid and its integration into lentivirus was performed previously by Liz Torr as described in (Torr et al., 2016).

2.2.2 Primary bronchial epithelial cell culture

Human Bronchial Epithelial Cells (HBECs) from nine donors were purchased from Lonza. Donor cell information is shown in ***Table 2.1***.

		Safety Testing		Virus Testing								
Donor	Age (Y)	Sex	Race	Viability (%)	Seeding	Doubling Time (Hrs)	HIV	HDV	HCV	Sterility Test	Mycoplasma	Smoker	Alcohol Consumption
1	56	M	C*	70	31	24	N*	N	N	N	N	Yes	Yes
2	50	M	C	75	45	21	N	N	N	N	N	Yes	Yes
3	19	M	C	91	66	16	N	N	N	N	N	No	Yes
4	43	M	C	71	60	20	N	N	N	N	N	No	Yes
7	19	F	C	83	61	28	N	N	N	N	N	No	No
8	18	F	C	85	99	31	N	N	N	N	N	No	No
9	60	F	C	82	49	19	N	N	N	N	N	No	No
11	38	M	C	83	45	35	N	N	N	N	N	No	No

Table 2.1. Lonza HBEC Donor Information. Normal HBECs, isolated during post-mortem from non-asthmatic patients, were purchased from Lonza. The table provides general donor information in addition to quality control and safety testing of the cells.

*C= Caucasian, *N= Negative

HBECs were grown in BEGM supplemented with 13mg/ml BPE (Bovine Pituitary Extract), 0.5mg/ml Hydrocortisone, 0.5µg/ml hEGF (human recombinant Epidermal Growth Factor), 0.5mg/ml Epinephrine, 10mg/ml Transferrin, 5mg/ml Insulin, 0.1µg/ml Retinoic Acid, 6.5µg/ml Triiodothyronine, 30ug/ml Gentamicin and 5ng/ml Amphotericin.

2.2.3 Immunofluorescence

Passage 2 NHBECs were grown to confluency split and seeded into an 8 chamber well slide and again grown until around 80-90% confluent. Media was removed and the wells washed 2x for 5 minutes in PBS. Cells were fixed in 1ml of 4% formaldehyde in PBS and left overnight at 4°C. Formaldehyde was removed from the cells and the cells washed 3x for 5 minutes in PBS. The slides were then blocked overnight at 4°C in blocking solution (10% goat serum, 1% BSA in PBS). Blocking solution was removed and the cells washed 3x for 5 minutes in PBS. 100µl of primary cytokeratin-14 (Chemicon) diluted 1 in 200 and 100µl of Mouse IgG1 isotype control (Sigma) diluted 1 in 1000 was added to the appropriate wells. The primary antibody and isotype control was incubated on the cells overnight at 4°C. The cells were washed 3x for 5 minutes in PBS. 100µl of Goat anti-Mouse Ig FITC secondary antibody (Sigma) was added to the appropriate wells and incubated for 30-45 minutes at 37°C in the dark to minimise bleaching. Cells were washed 3x with PBS and the chamber wells removed from the slides. Slides were mounted onto coverslips with 2 drops of Vectashield HardSet Mounting Media with DAPI and left overnight at 4°C covered to prevent bleaching.

2.2.4 Production of genetically modified HBECs

Passage 1 HBECs were thawed from cryopreservation and grown in a T75 culture flask. Once confluent, cells were split and seeded into 6 well plates at $5x10^4$ cells per well. The cells were incubated overnight ready to be infected the following day. Approximately 16 hours after splitting, media was aspirated from each well and replaced with 800µl of BEGM containing 2µg/ml polybrene, to facilitate the entry of lentivirus into the cell. 6.25µl of lentivirus stock solution (pLVX-Puro-BMI-1 or pLVX-Puro-EV) or media only was added to each well to give a transfection rate of >90%.

The plates were incubated for 6.5 hours at 37°C. For the first 2 hours the plates were rocked gently every 20 minutes to ensure good distribution of the virus. After 6.5 hours the polybrene and viral mixture was removed from the cells and replaced with 2ml of BEGM only. Cells were given 72 hours to recover and proliferate before the

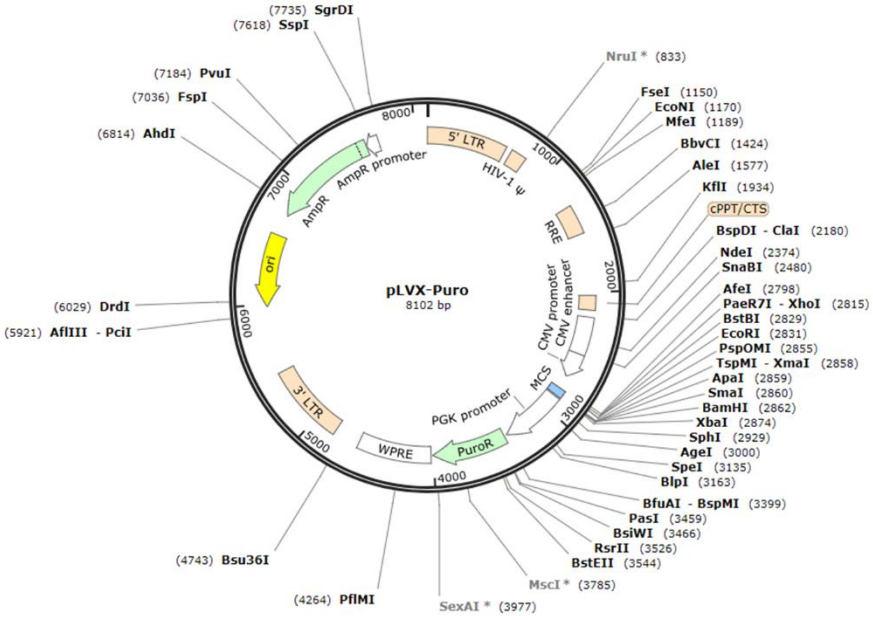


Figure 2.1. pLVX-Puro Vector. Full-length Human BMI1 was inserted into the pLVX-Puro vector using the XhoI/BamHI restriction sites. The vector contains a CMV promoter and enhancer which amplify BMI1 gene expression. In addition, the vector contains a puromycin resistance gene which can be used to select the cell populations (Clontech).

selection agent was added. After 72hrs, BEGM containing 300ng/mL of puromycin was added to each well, media containing 300ng/mL of puromycin was changed every 2-3 days. Selection of lentivirus infected cells was continued until all basal cells were dead, this was donor dependent but required between 6-10 days. Cells remaining after puromycin selection were considered puromycin resistant and hence stably infected GM cells expressing BMI-1. Once cells were approximately 80% confluent they were split and 3 wells of pLVX-Puro-BMI-1 or pLVX-Puro-EV infected cells were pooled together in order to seed a T75 culture flask. pLVX-Puro-BMI-1 and pLVX-Puro-EV cells were continued to be grown in culture with stocks frozen down at passage 4/infection passage 2 (P4/P2) and P5/P3.

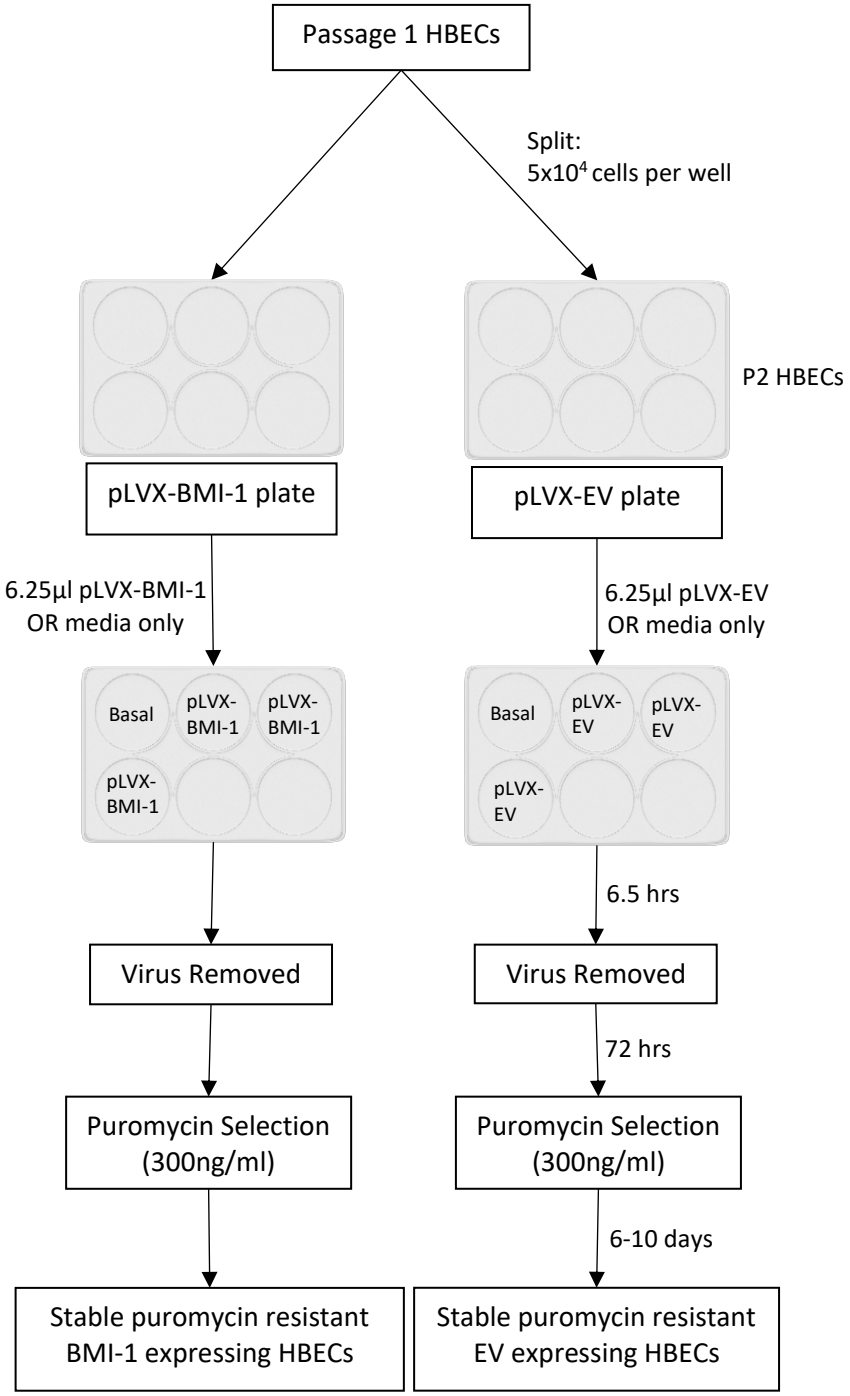


Figure 2.2. Production of pLVX-BMI-1 and pLVX-EV HBECs. Passage 1 NHBECs were split and $5x10^4$ cells seeded into wells of a 6 well plate and the cells allowed to adhere overnight. The following day passage 2 HBECs were infected with either pLVX-BMI-1 or pLVX-EV or treated with media only. After 6.5 hours virus was removed. 72 hours after lentivirus infection, cells were selected with 300ng/ml puromycin. Puromycin selection was continued until all basal cells were dead (6-10 days depending on donors). This method was repeated for all 8 HBEC donors.

2.2.5 RNA isolation

RNA was isolated using a Qiagen RNeasy Mini Kit and following the manufacturer's protocol. NHBECs and pLVX-Puro-BMI-1 cells were grown in T75 flasks until P2 and P5/IP3 respectively. Once the cells were around 80% confluent they were harvested for RNA samples. Media was aspirated from the cells and the cells washed briefly with PBS. 600µl of RLT plus buffer was added to disrupt the cells and a cell scraper used to dislodge them from the culture flask. The cell lysate was collected and stored at -80°C until required.

Following thawing of the cell lysate, the solution was homogenised by placing the lysate directly into a QIAshredder spin column with a 2ml collection tube and centrifuging for 2 minutes at 13,000rpm. 1X volume (600µl) of 70% ethanol was then added to the homogenised lysate and the solution mixed thoroughly. Up to 700µl of the cell lysate was added to a new RNeasy spin column and centrifuged for 30 seconds at 13,000rpm. As the lysate exceeded 700µl, the flow-through was discarded and the remaining sample placed into the column and centrifuged for 30 seconds at 13,000rpm. 350µl of Buffer RW1 was added to the column and centrifuged for 30 seconds at 13,000rpm. 10µl of DNase I stock solution diluted in 70µl of Buffer RDD was added directly to the RNeasy spin column membrane and incubated at room temperature for 15 minutes to degrade genomic DNA. The RNeasy spin column was washed with 350µl of Buffer RW1 and centrifuged for 30 seconds at 13,000rpm. 500µl of Buffer RPE was added to the RNeasy spin column and centrifuged for 30 seconds at 13,000rpm. This step was repeated but the column centrifuged for 2 minutes at 13,000rpm to ensure no carry over of ethanol. In addition, the column was also transferred to a new collection tube and centrifuged for a further 1 minute at 13,000rpm to ensure the column membrane was completely dry before eluting. Finally, 50µl of RNase-free water was added directly to the column membrane and centrifuged for 1 minute at 13,000rpm. The elute was collected and pipetted directly back on to the spin column membrane and centrifuged for a further 1 minute at 13,000rpm to achieve a high concentration of nucleic acid.

2.2.6 cDNA synthesis

Single stranded complimentary DNA (cDNA) was produced using Invitrogen's Superscript First-Strand Synthesis System for RT-PCR (ThermoFisher).

2.2.7 Quantitative polymerase chain reaction (qPCR)

Quantitative PCR (qPCR) is a technique which enables us to determine gene expression of a sample. There are different qPCR methodologies, in this experiment a Taqman probe was used. This involves a fluorogenic probe and a quencher attached to the 5' and 3' ends of an oligonucleotide with a gene specific sequence. Under normal conditions the quencher blocks the signal from the fluorescent reporter. The temperature of the reaction is raised to convert double stranded DNA into single stranded DNA. The temperature is then lowered to allow the annealing of the primers and probe to its target sequence. Taq DNA polymerase synthesises the new DNA strand and its 5' nuclease activity cleaves the Taqman probe. As a result, the cleaved reporter dye is separated from the quencher and a fluorescent signal is emitted. After each cycle, more fluorescent reporters are cleaved and eventually the fluorescent signal can be detected. This is known as the threshold cycle (CT), when DNA replication is in the log linear phase. The CT activation of a sample is inversely proportional to the target DNA present in that sample (Heid et al., 1996).

To determine whether BMI-1 was differentially expressed in pLVX-BMI-1 HBECs when compared to NHBECs a multiplex qPCR was performed. RNA isolated as described in section 2.2.5 was utilised to produce cDNA samples, outlined in section 2.2.6, for the following samples:

➢ **Donor 1-P2-Endogenous HBECs**	➢ **Donor 1-P5-pLVX-BMI-1 HBECs**
➢ **Donor 2-P2-Endogenous HBECs**	➢ **Donor 2-P5-pLVX-BMI-1 HBECs**
➢ **Donor 3-P2-Endogenous HBECs**	➢ **Donor 3-P5-pLVX-BMI-1 HBECs**
➢ **Donor 4-P2-Endogenous HBECs**	➢ **Donor 4-P5-pLVX-BMI-1 HBECs**
➢ **Donor 7-P2-Endogenous HBECs**	➢ **Donor 7-P5-pLVX-BMI-1 HBECs**
➢ **Donor 8-P2-Endogenous HBECs**	➢ **Donor 8-P5-pLVX-BMI-1 HBECs**
➢ **Donor 9-P2-Endogenous HBECs**	➢ **Donor 9-P5-pLVX-BMI-1 HBECs**
➢ **Donor 11-P2-Endogenous HBECs**	➢ **Donor 11-P5-pLVX-BMI-1 HBECs**

A multiplex master mix consisting of the following solutions was prepared and 15µl added to each well:

- 10µl per well – TaqMan gene expression master mix buffer (Applied Biosystems)
- 1µl per well – BMI-1 COMMD3 Hs00180411_m1 PDAR-FAM (ThermoFisher)
- 1µl per well – HPRT1 Hs01003267_m1 PDAR-HEX (ThermoFisher)
- 3µl per well – Sterile water

In addition, cDNA master mixes for each sample was produced:

- 1µl per well – cDNA sample
- 4µl per well – Sterile water

5µl of the appropriate cDNA master mix was added to the 15µl of PDAR master mix to give a final volume of 20µl per well. For each RT positive samples three replicates were performed whilst for RT negative samples one replicate was run.

The plates were run on the following amplification protocol:

Temperature	Time	No. of Cycles
50°C	2 minutes	1
95°C	10 minutes	1
95°C	15 seconds	40
60°C	1 minute	

Table 2.2 Cycle parameters for qPCR.

2.2.8 MTT assay

An MTT assay is a widely used method of assessing metabolic activity of cells and to an extent cell proliferation (Mosmann, 1983). MTT solubilised in tissue culture medium has a yellow colour. However, mitochondrial dehydrogenases of viable cells are capable of cleaving the tetrazolium ring from MTT, yielding MTT formazan crystals. Dissolving formazan crystals in isopropanol results in a purple solution which can be quantified through spectrophotometry (Morgan, 1998). More viable cells will produce more formazan crystals yielding an increasingly purple solution that absorbs more light. Therefore, the MTT assay is a good indicator of mitochondrial activity and thus cell viability.

P5/IP3 pLVX-Puro-BMI-1 and P2 NHBECs were removed from cryopreservation and cultured until they were approximately 80% confluent. Cells were split, counted and diluted in BEGM. 200ul of BEGM containing 2500 cells was placed into each well of a 96 well plate. For each cell type five replicate wells were set up. Three identical plates were produced and analysed at three different time points: 72 hours, 96 hours and 120 hours. After the appropriate elapsed time, media was removed from the wells and replaced with 200µl of filter sterilised 0.5mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) dissolved in pre-warmed BEGM. As MTT is light sensitive, the plates were covered in foil and incubated at 37°C for 4 hours. After 4 hours MTT was aspirated from the cells and replaced with 200µl of isopropanol. The solution was mixed well and placed on a rocker for 10 minutes to ensure that the MTT formazan crystals were fully dissolved in the isopropanol. The absorbance of the solution was measured at 570nm (subtracting the 690nm background) using a Flexstation 3 microplate reader.

2.2.9 Statistical analysis

The results shown are of one experiment displaying the mean and SD from the 5 technical replicates. An unpaired t test was performed on the data from each individual donor to determine whether BMI-1 expression significantly altered HBEC viability.

The mean values from each donor was combined, the median and 95% confidence limit calculated. A Mann Whitney test was performed to determine whether BMI-1 expression altered the viability of HBECs from multiple donors.

2.2.10 Cell viability and cell count

The Cell Viability and Cell Count assay is a simple method of counting cells and determining their viability. The method contains two main proponents: Acridine orange and DAPI. Acridine orange is an organic compound which can permeate the cell membrane and interact with DNA and RNA via intercalation. As a result, when acridine orange is placed in a cell suspension it interacts with all cells giving a total cell count. DAPI, unlike acridine orange, is incapable of penetrating the cell membrane, therefore cells stained with DAPI are non-viable cells. The method also provides an automated "rough estimate of cell size and cell aggregation properties". The cell viability assay should complement the MTT assay which is more a measure of metabolic activity (https://chemometec.com/cell-counting-assays/cell-viability-cell-count-assays/).

P2 NHBECs and P5/IP3 pLVX-BMI-1 HBECs were grown to confluency and split for ECIS experiments as in Section 3.2.1. In order to gain a correct cell count for these experiments a Cell Viability and Cell Count Assay was performed on the Chemometec NC-3000. The Cell Viability and Cell Count Assay were performed using two very similar methods which detect the same dyes: Acridine Orange and DAPI. The first method was using a Via-1 Cassette which is lined with Acridine orange and DAPI. Cells in suspension are transferred into the Via-1 Cassette through capillary action via compression of the piston. The cell suspension dissolves and mixes with the Acridine Orange and DAPI as it enters the cassette chamber. The Via-1 Cassette was finally loaded and read in the Chemometec Nucleocounter NC-3000. The second method involved mixing the cell suspension with a solution of Acrimide Orange and DAPI (Solution 13 - Chemometec) in a 19:1 dilution. 30µl of the solution was added to an NC-Slide A2 and the slide read in the Chemometec Nucleocounter NC-3000.

2.3 Results

2.3.1 Lentivirus transduction is an efficient genetic manipulation system.

The aim of this chapter was to produce stable genetically modified HBECs from multiple donors. Eight HBEC donors were successfully infected to produce puromycin resistant, BMI-1 expressing HBECs. In parallel, I attempted to produce pLVX-EV HBECs from the same donors. We successfully produced 16 stable epithelial cell lines (8 pLVX-BMI-1 and 8 pLVX-EV) which were puromycin resistant. However, cells expressing the EV quickly underwent cell senescence without the proliferative advantage of BMI-1. As shown in ***Figure 2.3***, pLVX-EV HBECs from donor 9 underwent senescence immediately after infection, HBECs from donor 7, donor 8 and donor 11 stopped replicating one passage after infection, whilst cell senescence occurred two passages after infection for HBECs from donor 2 and donor 3. Only pLVX-EV HBECs from donor 1 and donor 4 continued to replicate to passage 5, were RNA samples were collected. I intended to use pLVX-EV HBECs as an additional control group alongside NHBECs for RNA sequencing and characterisation experiments. However, as only RNA samples from two donors were collected from pLVX-EV HBECs this was abandoned.

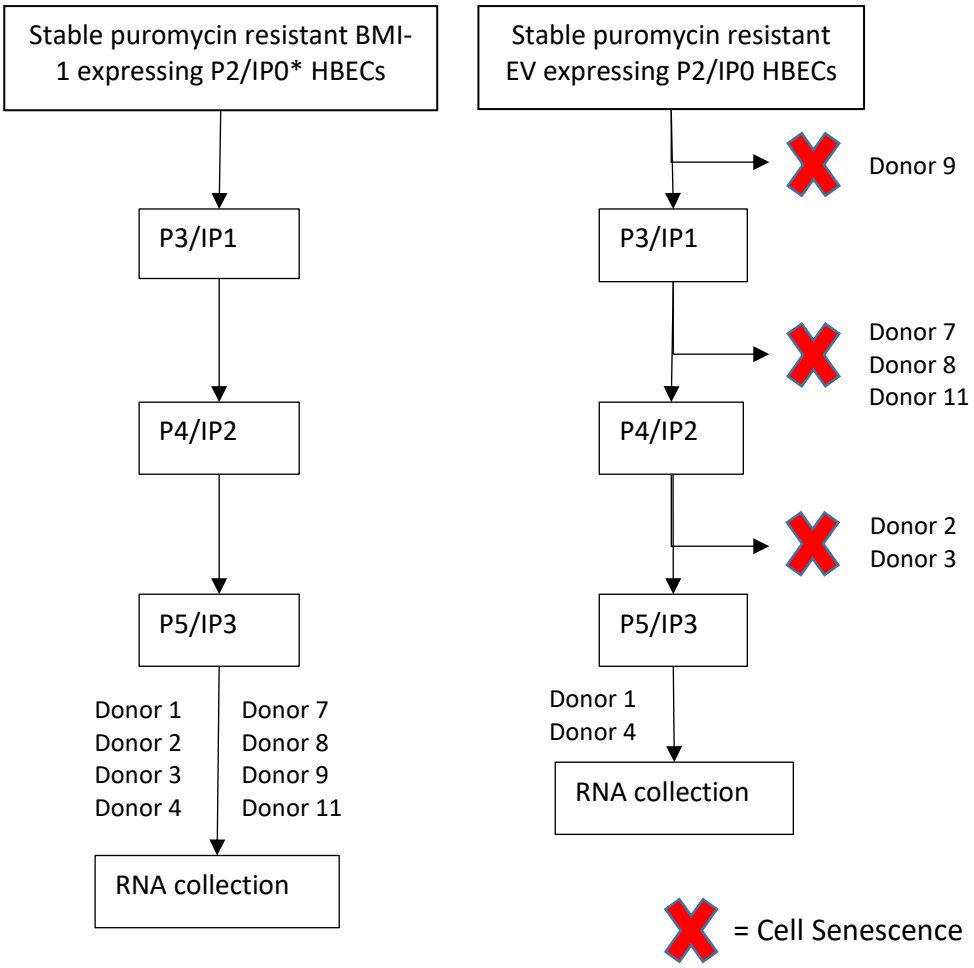


Figure 2.3. Overview of the production of pLVX-BMI-1 and pLVX-EV cells. HBECs which had been infected and selected for using puromycin, were grown to confluency and then split. pLVX-BMI-1 HBECs from 3 wells were combined and seeded into a T75 flask, the same was done for pLVX-EV HBEC. pLVX-BMI-1 HBECs from donors 1, 2, 3, 4, 7, 8, 9 and 11 were all successfully grown to passage 5 and RNA samples were collected from confluent T75 culture flasks. We attempted to grow pLVX-EV HBECs to passage 5; however, as shown above donor 2, 3, 7, 8, 9 and 11 HBECs underwent cell senescence before samples could be collected, we were only able to collect passage 5 pLVX-EV HBEC RNA samples for donors 1 and 4.

2.3.2 Morphology of HBECs

Basal cell cultures of NHBECs can differ slightly between donors but generally have a similar phenotype. As shown in ***Figure 2.4***, basal cultures of NHBECs consist of a large percentage of small basal epithelial cells with the occasional abnormally shaped cell with a fried egg-like morphology can be observed.

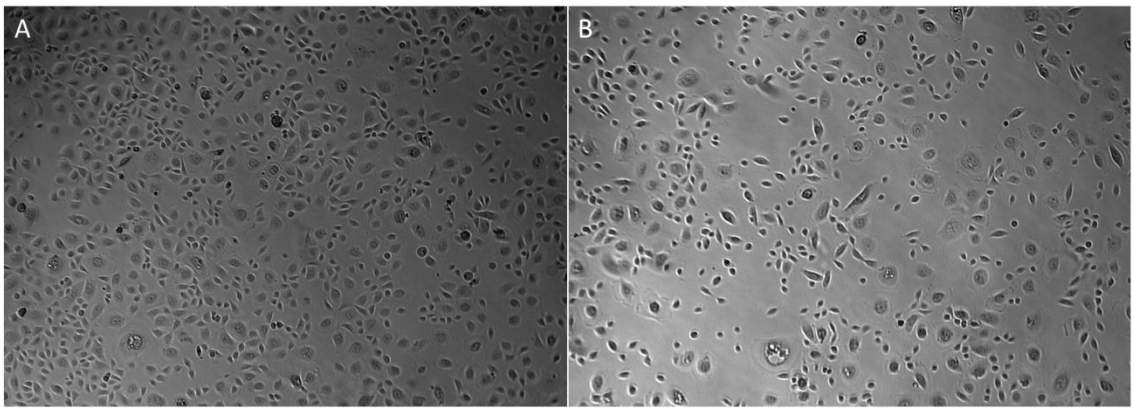


Figure 2.4. Morphology of low passage NHBECs. **A)** Donor 3 passage 3 HBECs. **B)** Donor 4 passage 3 HBECs. Magnification= x5.

Additionally, I performed immunofluorescence on NHBECs staining for a traditional epithelial marker cytokeratin-14. In ***Figure 2.5*** and ***Figure 2.6*** the distribution of cytokeratin-14 around the cytosol of donor 3 and donor 4 NHBECs respectively can be seen. Research previously completed by our research group has shown that BMI-1 expressing HBECs show the same distribution of cytokeratin-14 around the cytosol, a good example that BMI-1 expressing HBECs express key epithelial markers.

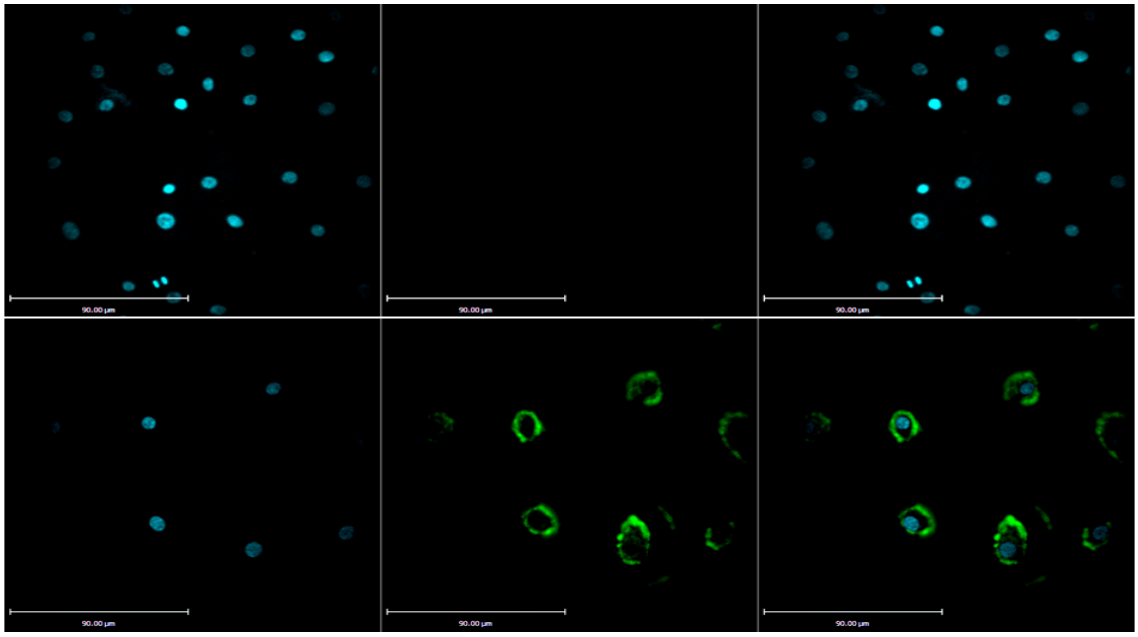


Figure 2.5. Cytokeratin-14 distribution amongst Donor 3 NHBECs. Donor 3 passage 3 NHBECs viewed at x10 magnification. Top Panel: Istotype control. Bottom panel: Cytokeratin-14. Left: DAPI (blue). Centre: (Cytokeratin-14). Right: Merged.

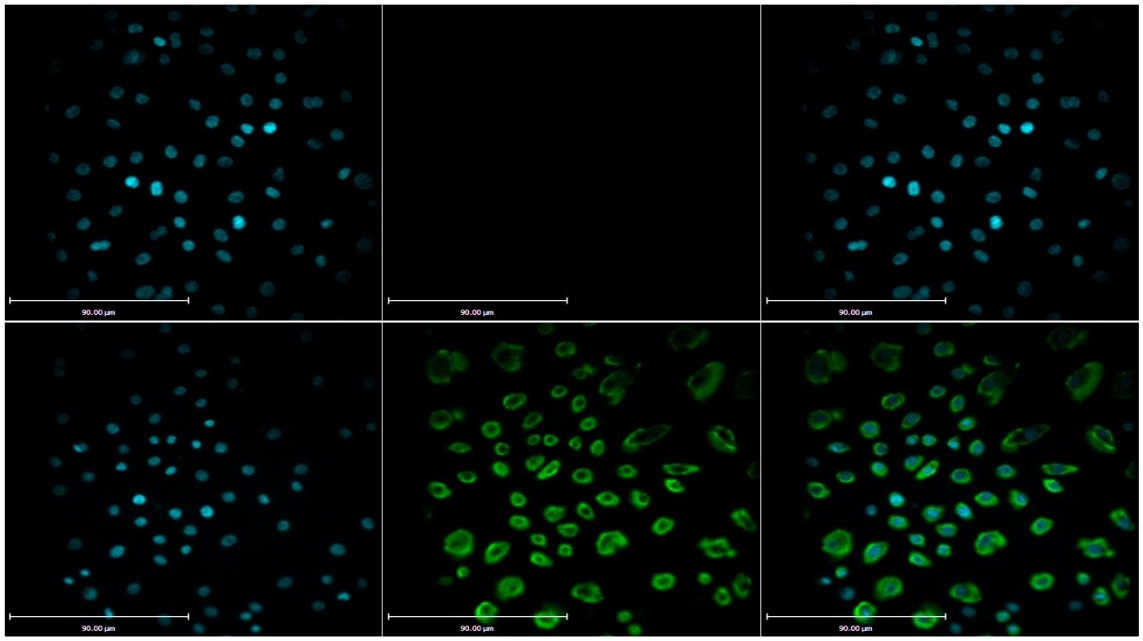


Figure 2.6. Cytokeratin-14 distribution amongst Donor 4 NHBECs. Donor 4 passage 3 NHBECs viewed at x10 magnification. Top Panel: Istotype control. Bottom panel: Cytokeratin-14. Left: DAPI (blue). Centre: (Cytokeratin-14). Right: Merged.

2.3.3 pLVX-EV HBECs do not have a proliferate advantage over NHBECs

As a control, HBECs were infected with an empty vector and the cells treated identically to our pLVX-BMI-1 HBECs. Infection of HBECs with the empty vector was clearly successful as these HBECs survived puromycin selection whilst basal cells died within 6-10 days. However, pLVX-EV HBECs did not obtain the enhanced replicative potential observed in pLVX-BMI-1 HBECs. As expected pLVX-EV HBECs started to undergo cell senescence shortly after lentivirus infection. As shown in ***Figure 2.7***, the morphology of the HBECs expressing the empty vector began to change with a reduction in small basal cells and a greater proportion of large apoptotic cells.

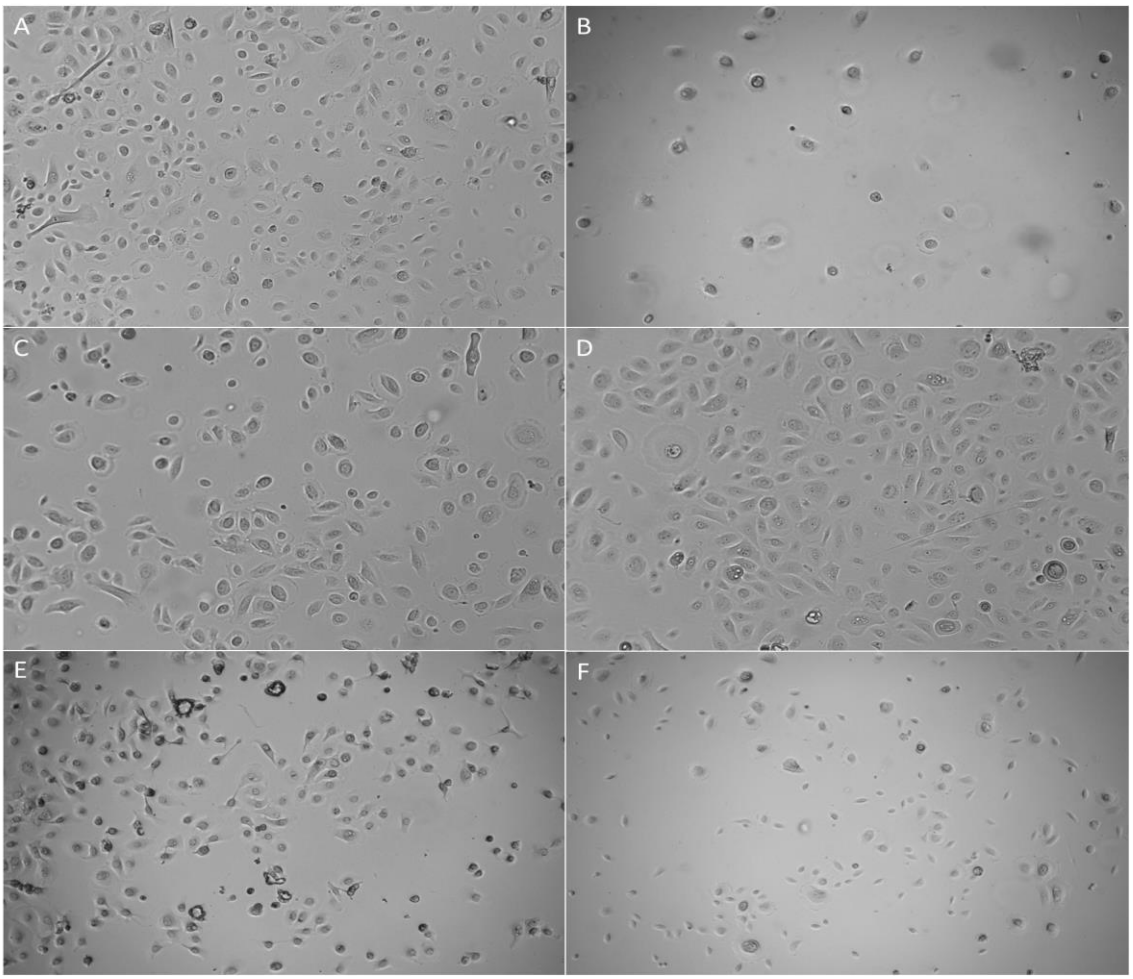


Figure 2.7. Cell senescence of pLVX-EV HBECs. pLVX-EV HBEC morphology before being discarded. **A)** Donor 2 P4/IP2 pLVX-EV HBECs. **B)** Donor 3 P4/IP2 pLVX-EV HBECs. **C)** Donor 7 P3/IP1 pLVX-EV HBECs. **D)** Donor 8 P3/IP1 pLVX-EV HBECs. **E)** Donor 9 P2/IP0 pLVX-EV HBECs. **F)** Donor 11 P3/IP1 pLVX-EV HBECs. Magnification= x5.

The images in ***Figure 2.7*** are of pLVX-EV HBECs which were still not confluent after 10 or more days of culture, as a result these cells were discarded.

2.3.4 pLVX-BMI-1 HBECs possess a similar morphology to NHBECs

The morphology of HBECs across the 8 donors was not drastically altered by inducing BMI-1 expressing in HBECs. In pLVX-BMI-1 HBECs again we see predominantly small basal cells with a small percentage of fried egg-like cells as seen in ***Figure 2.8***.

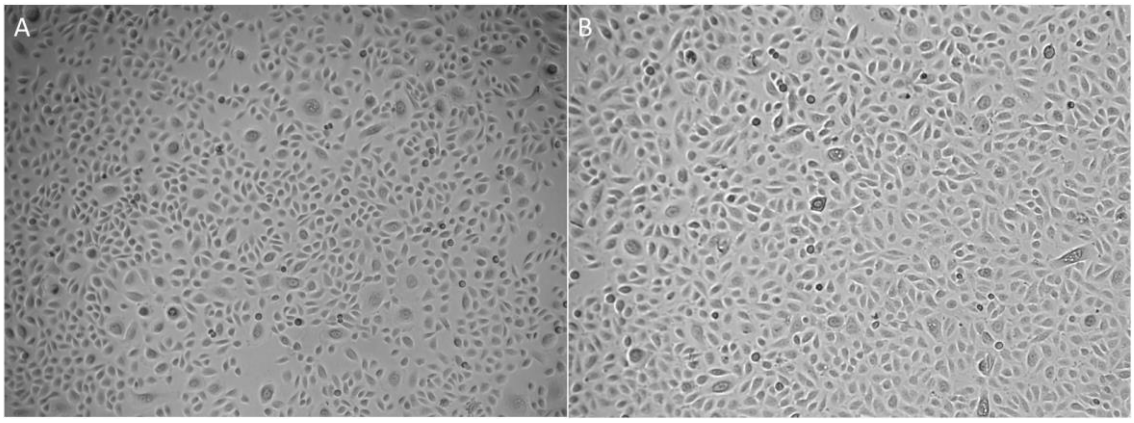


Figure 2.8. pLVX-BMI1 HBEC morphology. Brightfield images of passage 5/Infection passage 3 pLVX-BMI-1 HBECs. A) Donor 3 pLVX-BMI1 HBECs. B) Donor 4 pLVX-BMI1 HBECs. Magnification= x5.

2.3.5 Comparing BMI-1 mRNA expression in pLVX-BMI-1 HBECs and NHBECs

Due to the methodology utilised for producing pLVX-BMI-1 cells, specifically puromycin selection, we are confident that our HBECs infected with lentivirus express our vector containing BMI-1 and puromycin resistance. To confirm that BMI-1 was upregulated in our pLVX-BMI-1 HBECs in comparison to NHBECS, a multiplex qPCR was performed. **Figure 2.9** displays the amplification plot from the multiplex qPCR and it is immediately evident that there are two distinct groups. The eight samples which are positive at around 26-28 cycles are pLVX-BMI-1 HBEC cDNA samples, whilst the samples at approximately cycle 30-31 are NHBEC cDNA samples. I had hoped to correct for variation in cDNA levels by detecting the expression of a housekeeper

gene, HPRT1. However, the HPRT PDAR used in the qPCR had appeared to have expired as samples were only positive beyond cycle 34 whilst others were negative. As a result, I cannot quantify the level of BMI-1 mRNA upregulation in these cells but I hope to rectify this using the RNA sequencing data.

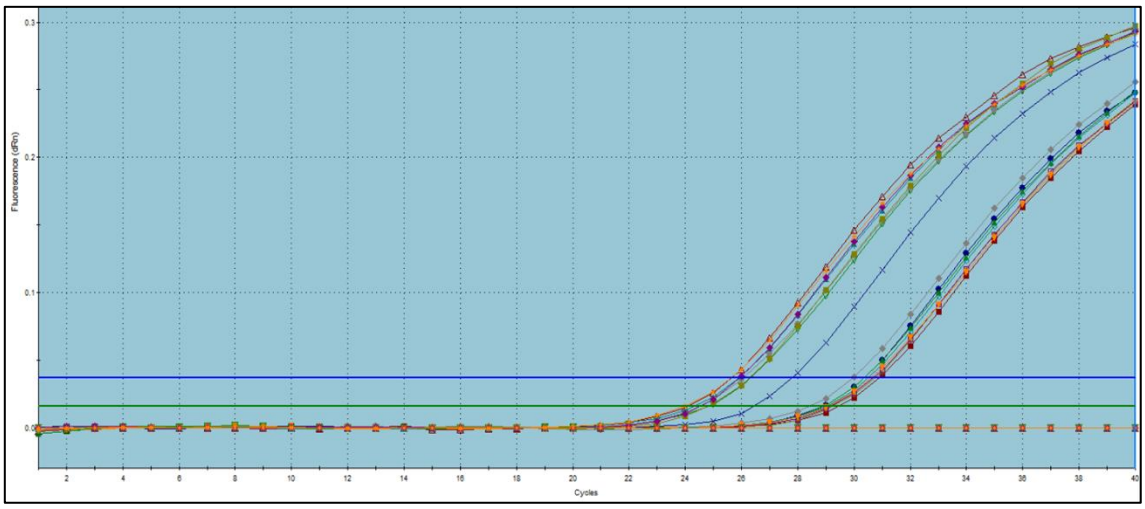


Figure 2.9. Amplification plot of BMI-1 expression. cDNA produced from 8 NHBECs and 8 pLVX-BMI1 HBECs RNA samples were run in multiplex qPCR. Unfortunately, our housekeeping gene, HPRT1, was positive at a significantly later cycle or negative all together.

2.3.6 BMI-1 upregulation in HBECs increases metabolic activity.

Our research group has previously shown that inducing BMI-1 expression in two HBEC donors, specifically donor 1 and donor 4, increases the viability of the cells (Torr et al., 2016). I was able to replicate these findings by genetically engineering normal HBECs from the same donors to express BMI-1 and demonstrate these manipulated HBECs were more viable than NHBECs in Furthermore, HBECs induced to express BMI-1 through lentivirus transduction maintained an increased viability until passage 15 (Torr et al., 2016).

The results of our MTT viability assay are supported by the finding from our previous research group. It has been demonstrated that inducing BMI-1 expression in donor 1, donor 3, donor 4 and donor 8 significantly increases HBEC viability after 72 hours of incubation, as seen in ***Figure 2.10*** ($P<0.0001$).

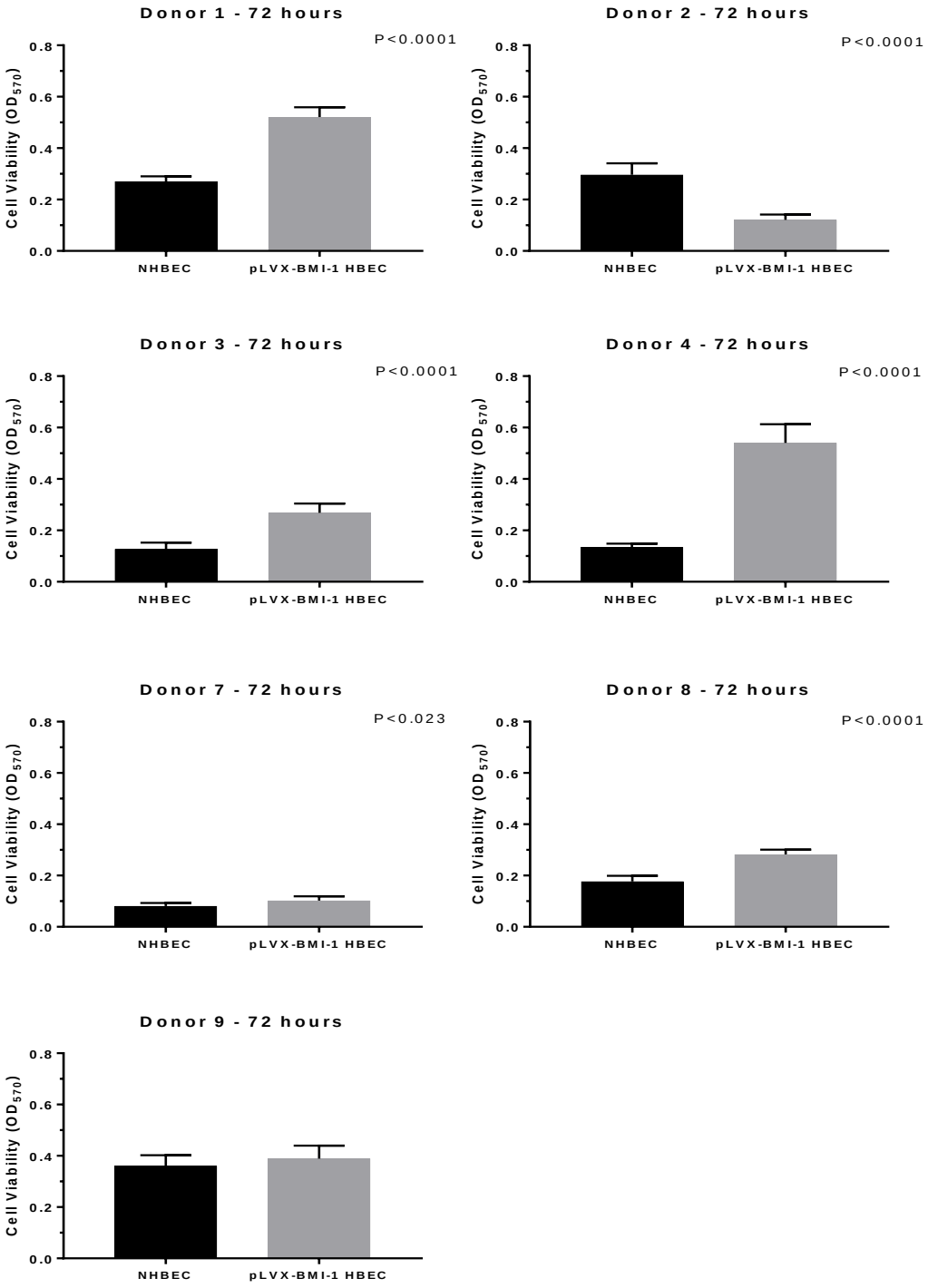


Figure 2.10. The Effects of BMI-1 Expression on HBEC Viability after a 72-hour incubation. Passage 3 NHBECs and passage 6 pLVX-BMI-1 HBECs cultured for 72 hours before the addition of MTT. Each graph represents a single experiment displaying the mean and SD of 5 technical replicates. An unpaired t test was used to determine the significance of BMI-1 expression.

Inducing BMI-1 expression in donor 7 HBECs also increases viability ($P<0.023$). These results support the previous findings by our research group, which showed viability to be increased in donor 1 and donor 4 HBECs expressing BMI-1. However, there is some heterogeneity in the viability of pLVX-BMI-1 HBECs. BMI-1 expression in donor 9 HBECs had very little impact on the viability of the cells, whilst donor 2 pLVX-BMI-1 HBECs were shown to be significantly less viable in comparison to NHBECs ($P<0.0001$).

At 96 hours, the same pattern of viability across NHBECs and pLVX-BMI-1 HBECs can be observed. Inducing BMI-1 expression in HBECs from donors 1 ($P<0.0001$), donor 3 ($P<0.0001$), donor 4 ($P<0.0001$), donor 7 ($P<0.0002$) and donor 8 ($P<0.0009$) significantly increases the viability of the cells as shown in ***Figure 2.11***. Again, it is evident that BMI-1 upregulation in donor 9 HBECs does not significantly alter the viability of these HBECs. Furthermore, upregulation of BMI-1 in donor 2 HBECs reduced the viability of the cells in comparison to NHBECs ($P<0.0002$).

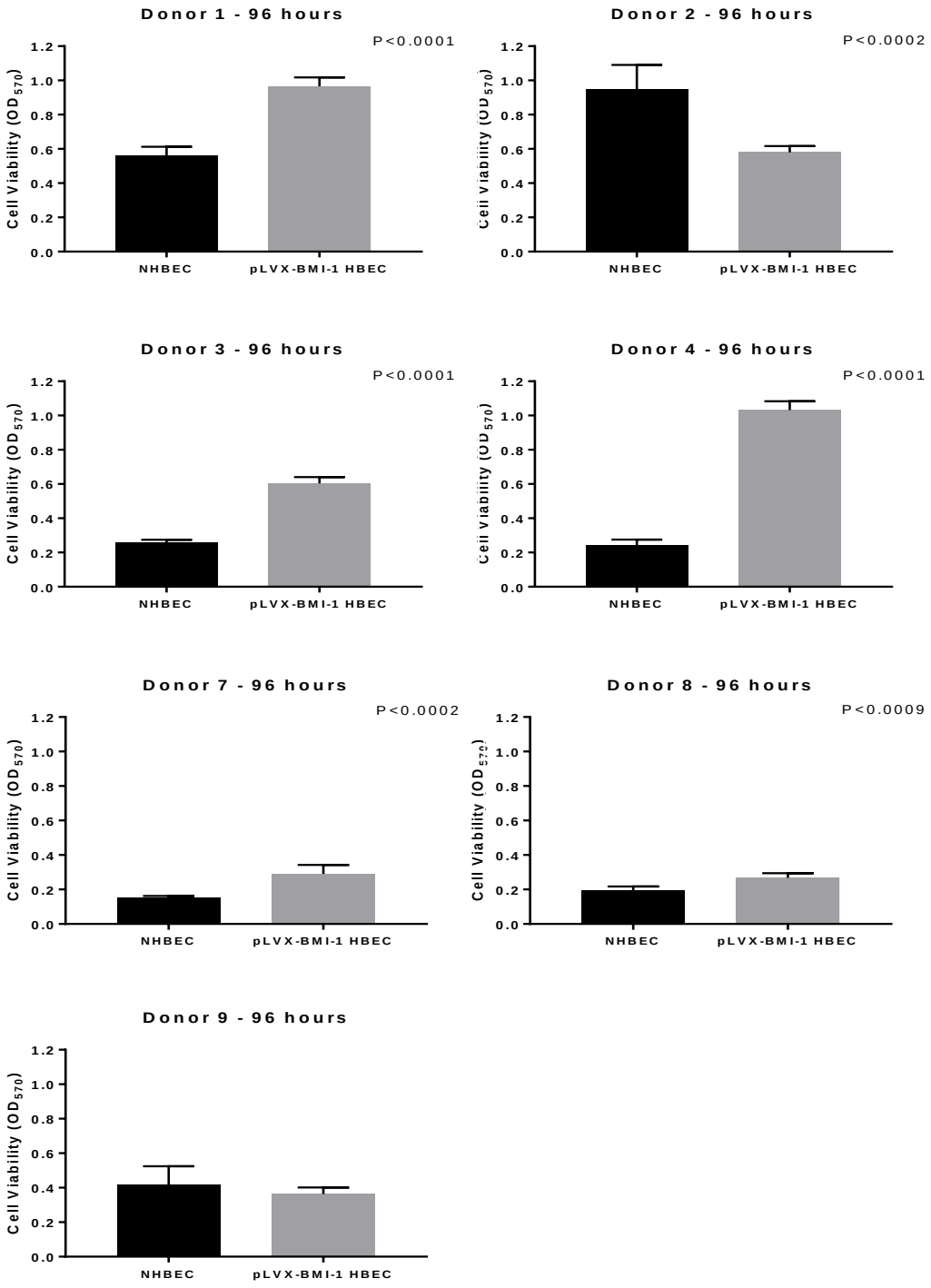


Figure 2.11. The Effects of BMI-1 Expression on HBEC Viability after a 96-hour incubation. Passage 3 NHBECs and passage 6 pLVX-BMI-1 HBECs were incubated for 96 hours before the addition of MTT. Each graph represents a single experiment displaying the mean and SD of 5 technical replicates. An unpaired t test was used to determine the significance of BMI-1 expression on viability.

Having looked at the effects of BMI-1 expression across individual donors I then wanted to evaluate how viability differed between NHBECs and pLVX-BMI-1 HBECs by combining the data from each donor together. Therefore, the mean viability from each donor was combined with the median and 95% confidence limit calculated, as shown in ***Figure 2.12***. At both 72 and 96 hours, the median viability is greater in pLVX-BMI-1 HBECs. At 72 hours, the slight increase in viability we can observe is not significant according to a Mann Whitney U test. However, at 96 hours the viability of pLVX-BMI-1 HBECs was significantly greater than NHBECs (P<0.049).

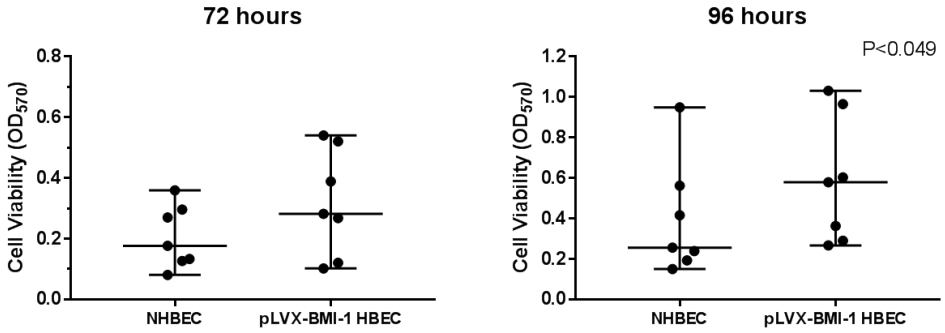


Figure 2.12. Comparing the Viability of pLVX-BMI-1 HBECs and NHBEC. Passage 3 NHBECs and passage 6 pLVX-BMI-1 HBECs (n=7) were incubated for 72 and 96 hours before the addition of MTT. A Mann Whitney U test was the statistical test used. The bottom two graphs represent the same data but presented as individual donor plots to display the effects of BMI-1 expression across donors.

2.3.7 Cell viability determined through DAPI measurements is not affected by BMI-1 overexpression

The MTT assay is a method for assessing the viability of cells through measuring the metabolic activity of a population of cells. An additional method to assess cell viability can be performed by detecting exposed cellular DNA and therefore non-viable cells using DAPI. As shown in ***Figure 2.13*** there is virtually no difference in the number of viable cells, detected through this method. In ***Figure 2.10*** and ***Figure 2.11***, BMI-1 expression significantly increases HBEC viability of five out of seven donors, measured through an MTT assay. Here an increase in viability in pLVX-BMI-1 HBECs from donor 1 and donor 7 can be seen, although this increase is no significant.

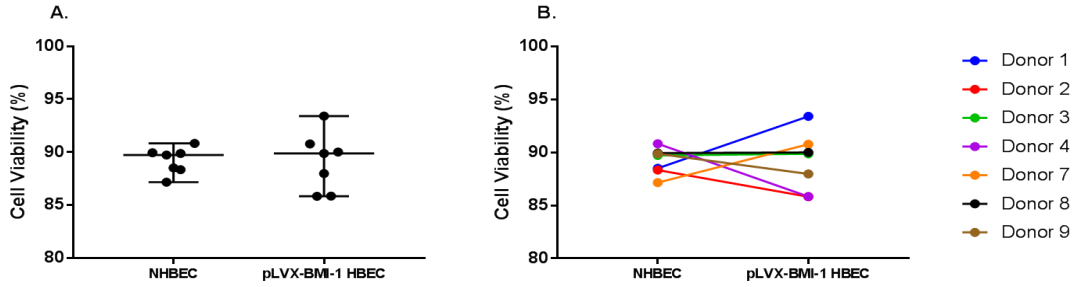


Figure 2.13. The Impact of BMI-1 Expression on HBEC Viability. **A+B)** Both graphs show the same data from passage 3 NHBECs and passage 6 pLVX-BMI-1 HBECs (n=7). **A)** The median viability and 95% confidence limit. **B)** How BMI-1 expression impacts individual donors. A Mann Whitney U test was used to determine statistical significance.

2.3.8 BMI-1 expression does not alter HBEC diameter and aggregation

In addition to assessing cell viability, basic cell phenotypes such as average cell size were also measured. It was shown that BMI-1 expression in HBECs has little impact on cell size and no significant impact on cell aggregation.

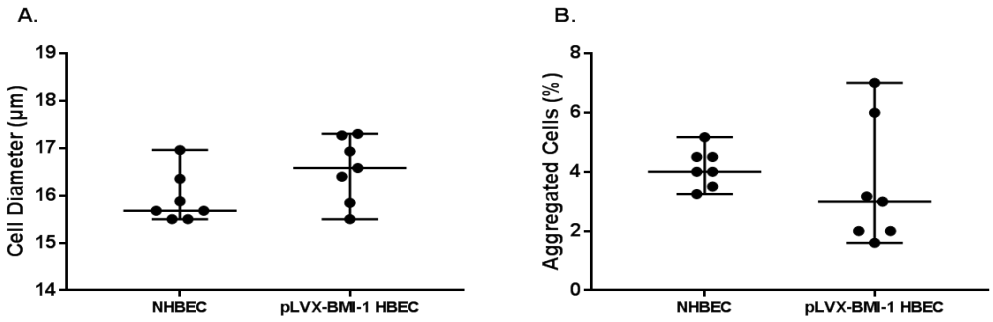


Figure 2.14. The effects of BMI-1 expression on HBEC diameter and aggregation. Passage 3 NHBECs and passage 6 pLVX-BMI-1 HBECs (n=7). **A)** Cell diameter estimated automatically using a Chemometec Nucleocounter NC-3000. **B)** Percentage of cells with 5 or more aggregated cells. Both graphs display the median and 95% confidence limit. A Mann Whitney U test was used to determine statistical significance.

2.4 Discussion

It has been shown that the pLVX-BMI-1 system is a robust method for enhancing the replicative lifespan of HBECs, having successfully produced stable BMI-1 expressing HBECs from 100% of the donors infected.

It is evident that pLVX-BMI-1 HBECs have some form of proliferative advantage as I was able to grow cells from all eight donors to passage 5 and collect RNA samples. Of the eight donors infected with pLVX-EV, only RNA samples from two donors were collected, with the other six donor pLVX-EV cells undergoing senescence. Due to the short time frame of a masters project I was not able to grow the pLVX-BMI-1 HBECs to late passages. Previously Torr et al., demonstrated that pLVX-BMI-1 HBECs can continue to proliferative beyond passage 20 (Torr et al., 2016). Future work should include determining whether or not the six additional donors produced in this project have a similarly increased replicative lifespan, to ensure that the pLVX-BMI-1 system is effective across a range of donors.

Whilst the pLVX-BMI-1 HBECs appear to have an enhanced replicative lifespan they still possess similar features to NHBECs. The morphology of BMI-1 expressing HBECs is not drastically different and these have previously been shown to maintain epithelial markers such as cytokeratin-14. It was also shown that other basic features including cell size and aggregation pattern of HBECs is not altered by BMI-1 expression.

One of the key aims for the MRes project was to replicate and build on the findings from Torr et al., were it was shown that inducing BMI-1 expression in HBECs (n=2) increases the viability of the cells (Torr et al., 2016). Our findings support this previous study, as I was able to show that pLVX-BMI-1 HBECs are more viable and metabolically active than NHBECs as seen in ***Figure 2.12***. However, the results from ***Figure 2.13*** suggest that HBECs overexpressing BMI-1 are not necessarily more viable when using a technique which measures DAPI staining.

Individually out of the seven HBEC donors manipulated to express BMI-1, five out of the seven had significantly greater MTT viability values in comparison to NHBECs. One problem between using multiple donors is that there is a large amount of heterogeneity between them. This heterogeneity is likely to be the reason we do not

see viability increase uniformly across each donor induced to express BMI-1. Factors such as the efficiency of the lentivirus transduction is likely to vary between donors. In addition, the immune response to lentivirus elements will be greatly varied amongst donors. The immune reaction to lentivirus is weak in comparison to other viral delivery methods such as adenovirus, but it still elicits an immune response (Nayak and Herzog, 2010). However VSV-G, the envelope protein used in conjugation with the packaging vector to deliver, is a viral particle that is cytotoxic to cells (Schaffer et al., 2008). A simple explanation for the result could be human error. The MTT viability score is based on cell numbers, specifically if more viable cells are present then the MTT value will be greater. As a result, an error in counting or aliquoting could influence an experiment and could explain the high values observed for donor 2 NHBECs.

I hoped to perform R2 association using the MTT data and the mRNA levels, detected from the qPCR, to determine whether BMI-1 upregulation correlated with increased cell viability. Unfortunately, due to the lack of HPRT1 expression values this could not be done. The qPCR experiment was repeated to ensure a mistake hadn't been made or a faulty aliquot of PDAR had been used, yet this yielded the same result. The HPRT1 PDAR used for the qPCR was from an old batch and had potentially expired. Unfortunately, due to the time constraints of the project I was unable to purchase a new housekeeper PDAR and repeat the experiment. For future repeat experiments, a singleplex qPCR should be considered to ensure both gene amplifications are successful. However, the preliminary RNA sequencing data confirms the upregulation of BMI-1 in pLVX-BMI-1 HBECs, as discussed in chapter 4.

3 Chapter 3: Cellular properties of human bronchial epithelial cells engineered to prevent cell senescence through overexpression of BMI-1

3.1 Introduction

Previous studies by our research group and Munye et al., 2017 assessed the effects of BMI-1 expression in primary HBECs and focused predominantly on cell phenotype changes. Importantly, they were able to demonstrate that upregulation of BMI-1 expression prolonged the replicative lifespan of not only normal but cystic fibrosis diseased HBECs. Secondly, these cells were capable of being cultured at ALI and therefore maintained the ability to differentiate into mucin-secreting goblet cells and ciliated columnar cells (Munye et al., 2017; Torr et al., 2016). A key advantage of HBECs genetically modified to express BMI-1 was that the karyotype of the cell was not altered even at high passage. Increasing the replicative potential of HBECs through hTERT resulted in multiple chromosomal abnormalities as shown in ***Figure 3.1*** (Torr et al., 2016).

The plasticity of HBECs and their ability to differentiate into specialised cell types including mucin-secreting goblet cells and ciliated columnar cells is a key feature HBECs. Other key properties of HBECs include their cell growth properties and the ability to form an epithelial barrier. The aim of this study is to determine if HBECs engineered to overexpress BMI-1 maintain key epithelial properties such as adhesion and proliferation and therefore deduce if they are comparable to NHBECs.

Previous studies have shown that BMI-1 knockdown in Ewing's Sarcoma Family of Tumours (ESFT) cells and medulloblastoma cells impacts a number of cell biology processes, which were determined through microarray analysis. One of the biological processes affected was cell adhesion, specifically cell matrix interactions (Douglas et al., 2008).

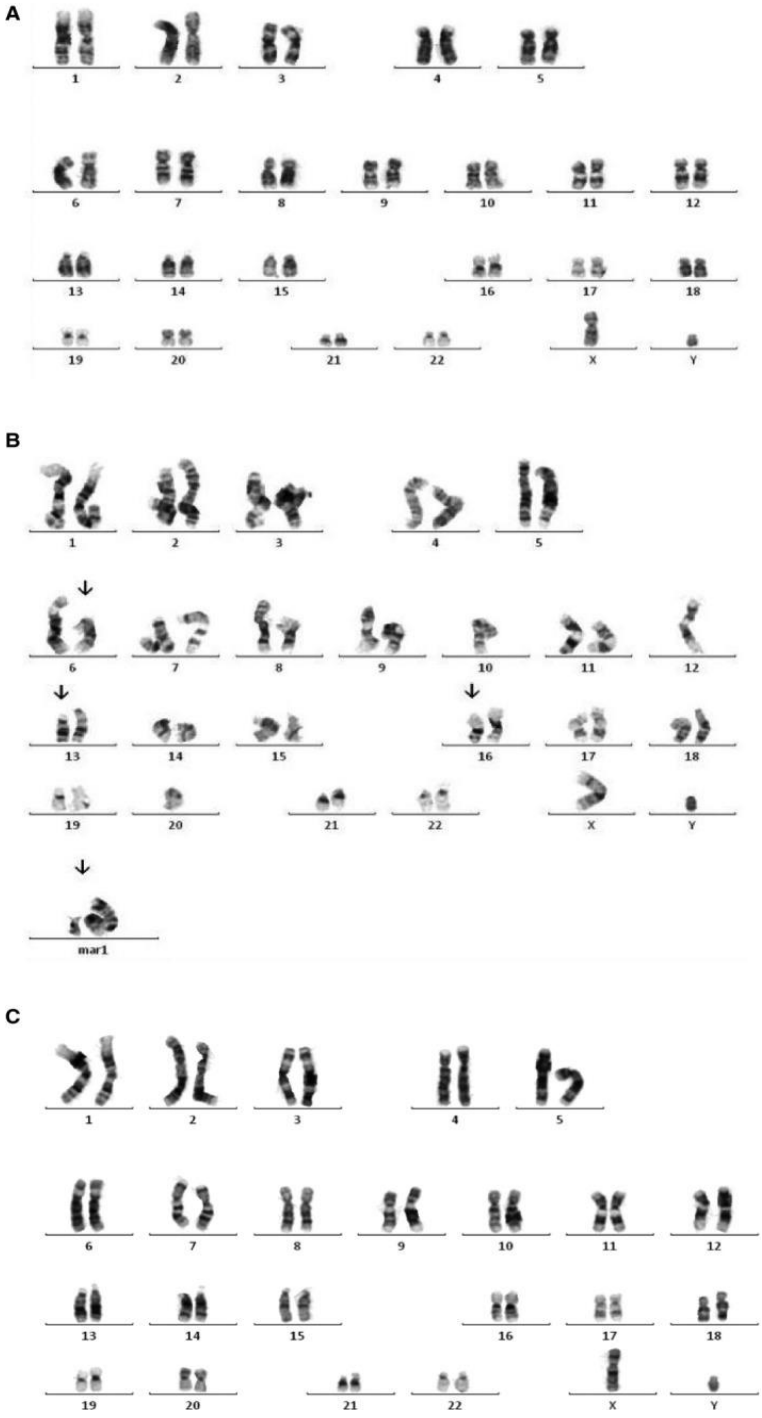


Figure 3.1. The karyotype of HBECs expressing hTERT and BMI1. **A.** Passage 3 NHBECs. **B.** Passage 10 hTERT HBECs, with abnormalities on chromosomes 6, 13 and 16. **C.** Passage 15 pFLRu-BMI1 HBECs, showing a normal karyotype (Torr et al., 2016).

Targeting and inhibiting BMI-1 activity has been shown to slow the growth of tumours from different cancers (Bolomsky et al., 2016; Srinivasan et al., 2017; Yong et al., 2016). It would therefore be anticipated that by inducing BMI-1 expression in HBECs, that these cells will potentially spread quicker than NHBECs. However, it is unclear how increased BMI-1 expression in HBECs will affect the formation of tight junctions and therefore the development of a resistive barrier, an area of great interest in respiratory research. Our research group has previously shown that BMI-1 expressing HBECs, when cultured at ALI, maintain a high TEER even at a late passage (Torr et al., 2016). These finding suggest that prolonged BMI-1 expression does not affect the ability of HBECs to form a resistive barrier.

The ability to form a resistive monolayer is a key property of epithelial cells and a characteristic that is lost in asthma patients (Holgate, 2007). Epithelial integrity is maintained through the expression and interaction of proteins involved in cell-cell contacts. Tight junctions in particular, are essential junctional complexes which regulate the paracellular permeability of the epithelial layer (Anderson and Van Itallie, 2009; Steed et al., 2010). In asthmatic epithelial cells, tight junction formation is disrupted and TEER reduced (Xiao et al., 2011). Environmental agents are known to be a driving factor in barrier dysfunction, cigarette smoke has been shown to impair cell-cell contact recovery (Heijink et al., 2012). In addition, asthma patients are also susceptible to proteolytic enzymes on the surface of pollen which are capable of degrading tight junctions (Runswick et al., 2007).

As I will discuss in greater detail in section 3.2.1, the Electric Cell-Substrate Impendence Sensing (ECIS) technique can be utilised to detect a range of cell properties, including cell adhesion, cell spreading and barrier formation (Wegener et al., 2000). By applying a small current across a cell monolayer we are able to detect subtle changes in cell behaviour including how cells adhere and divide to form a monolayer within wells. Furthermore, by applying a low frequency current to cells and measuring resistance we can assess the formation of tight junctions and the development of a confluent and resistive monolayer (Wegener, 2010). I anticipate that BMI-1, a known oncogene, upregulated in various cancer types, would promote

increased HBEC growth (Kreso et al., 2014; Song et al., 2010; Wu et al., 2011). How upregulated BMI-1 expression will influence HBEC barrier properties though is unclear.

Although the potential impact of BMI-1 on certain HBEC properties is unknown, it is strongly predicted that overexpression of BMI-1 would have a profound impact on the transcriptome and the cell cycle of HBECs. BMI-1 is a polycomb group (PcG) protein and therefore helps regulate transcription through epigenetic modifications of chromatin, including genes which regulate cell cycle progression (Sauvageau and Sauvageau, 2010). The cell cycle consists of four predominant phases; G1/G0, S, G2 and M. These phases are controlled by checkpoints to ensure correct cell division (Hartwell and Weinert, 1989; Nobury and Nurse, 1992). Cells enter the cell cycle at the gap phase (G1) before undergoing DNA replication in S-phase. G2 phase is prior to chromosome segregation which occurs in M phase. These checkpoints are closely regulated by the presence or absence of growth signals, cell size and DNA damage (Chambard et al., 2007; Elledge, 1996; Fingar et al., 2003). One of the key regulators of the G1-S checkpoint is the tumour suppressor protein, p16, as shown in ***Figure 3.2***. p16 inhibits the progression of cells through the G1-S checkpoint by inhibiting Cyclin D-CDK4 complex formation (Elledge, 1996). BMI-1 is an upstream regulator of p16, which prevents its inhibitory action on Cyclin D and CDK4 (Itahana et al., 2003). However, following studies have shown that delaying cell senescence through BMI-1 upregulation can be independent of p16. Primary normal human oral keratinocytes (NHOK) were induced to express BMI-1 through retroviral and lentiviral transduction. These cells were shown to have decreased intracellular and secreted TGF-β1 level, enhanced dephosphorylation of Smad2/3, and reduced levels of $p15^{INK4B}$ and $p57^{KIP2}$ expression. Furthermore, knockdown of BMI-1 reversed these findings. These results suggest that BMI-1 expression can delay cellular senescence through the inhibition of the TGF-β signalling pathway, at least in NHOKs (Kim et al., 2010).

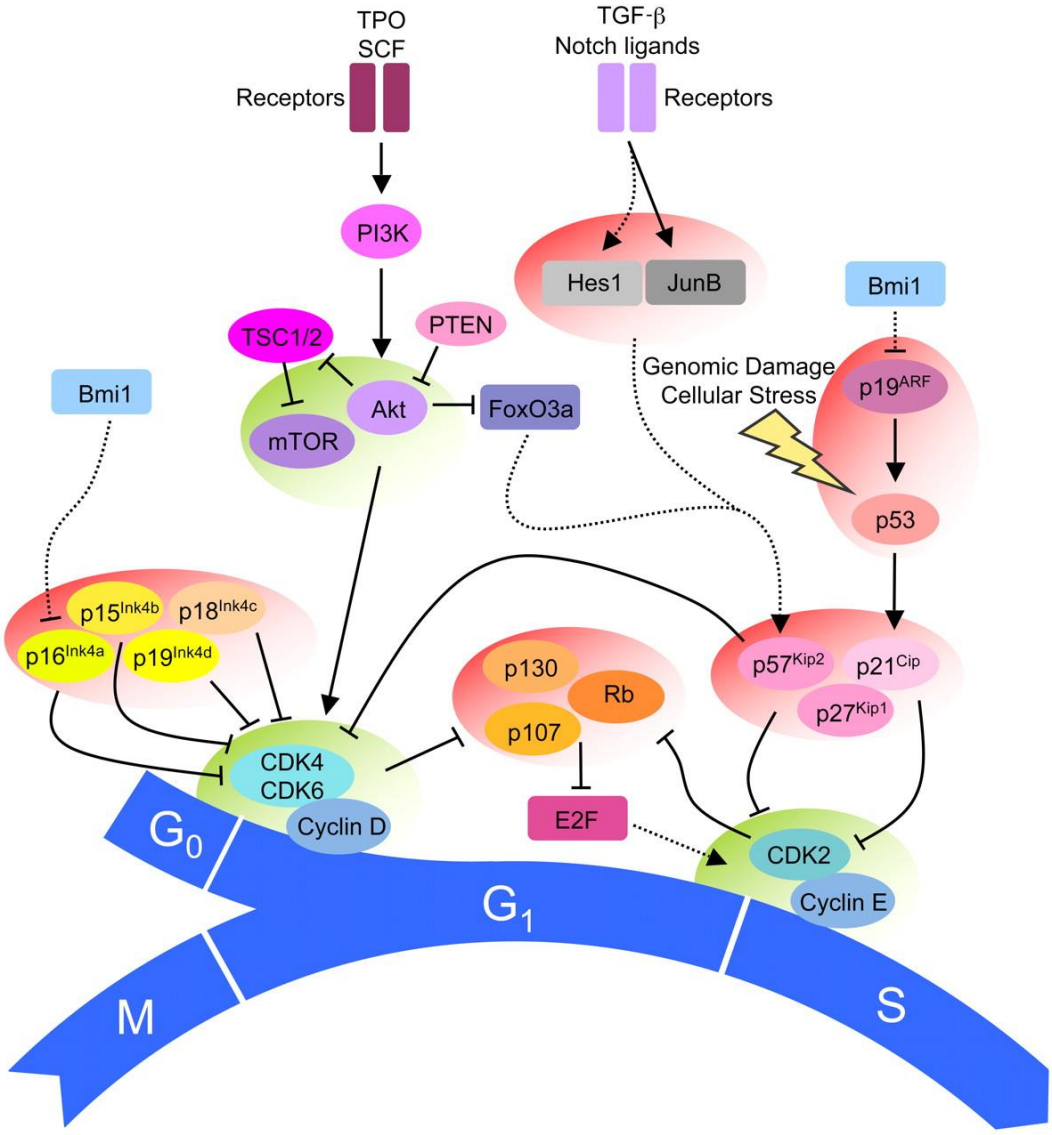


Figure 3.2. Regulation of the G1-S cell cycle checkpoint. The progression of the cell from G1 to S phase is heavily regulated by growth factors, inhibitory factors and signalling from DNA damage or stress. $p16^{INK4A}$ is a key regulator of Cyclin D-CDK4/6 complex formation and therefore blocks the transcription of E2F genes required for G1-S progression. BMI-1 inhibits the activity of $p16^{INK4A}$ and $p19^{ARF}$ promoting cell cycle progression (Pietras et al., 2011).

A previous study has shown that knockdown of BMI-1 in HeLa cells results in a significant increase in the proportion of cells in G1/G0 phase and a significant reduction of cells in S phase when compared to non-modified HeLa cells (Chen et al., 2011). I would anticipate that by overexpressing BMI-1 in HBECs we would see the

opposite effect with significantly fewer cells in the G1/G0 phase. Numerous studies have demonstrated that decreasing BMI-1 expression is a driving factor in the induction of cellular senescence in various cell types but especially neural and haematopoietic stem cells (Bracken et al., 2007; Lessard and Sauvageau, 2003; Molofsky et al., 2005; Park et al., 2003; Wang et al., 2012). Reduced BMI-1 expression has also been linked to the cellular senescence of primary epithelial cells from the biliary (Sasaki et al., 2006).

Aims of the chapter

1. To determine the impact of BMI-1 overexpression on key epithelial cell properties including cell adherence, cell spreading and the barrier resistance of confluent HBEC monolayers.
2. To gain an insight into the mechanisms by which BMI-1 expression provides HBECs with a proliferative advantage and delays cell senescence, whilst maintaining cell plasticity.

3.2 Methods

3.2.1 Electric cell-substrate impendence sensing (ECIS)

ECIS is a real-time and label-free method for studying the activity of cells grown in tissue culture (Wegener et al., 2000). The ECIS method involves growing cells in special culture chambers, over opposing gold electrodes. A small alternating current (AC) is applied (~1uA) between the electrodes and the potential is measured. Cells have insulating properties which resist the electrical flow resulting in higher potential between electrodes. As the cell monolayer changes the cellular impedance is recorded and from this the resistance and capacitance are calculated using Ohm's Law.

$Z=V/I$

Measuring impedance through this method and across a range of frequencies can give us an insight into a number of cell properties including cell adherence, cell growth and tight junction formation. At a low frequency of 400 Hz the current generally flows between adjacent cells. Therefore, measuring the impedance at this frequency reveals information about tight junction formation and the permeability of the resistive barrier. When measuring at high frequencies of around 32 kHz the current flows more readily through the insulating cell membrane and hence provides information on the formation of the cell monolayer, therefore, cell adherence and growth (Szulcek et al., 2014).

The aim of the project is to directly compare genetically modified HBECs expressing BMI-1 to NHBECs to determine if key properties of primary human airway cells remain unaltered. The ECIS technique allows us to sensitively measure many essential HBEC properties including cell attachment and spreading, in addition to barrier formation. As a result, ECIS is an ideal tool for determining how BMI-1 expression alters gross HBEC behaviour.

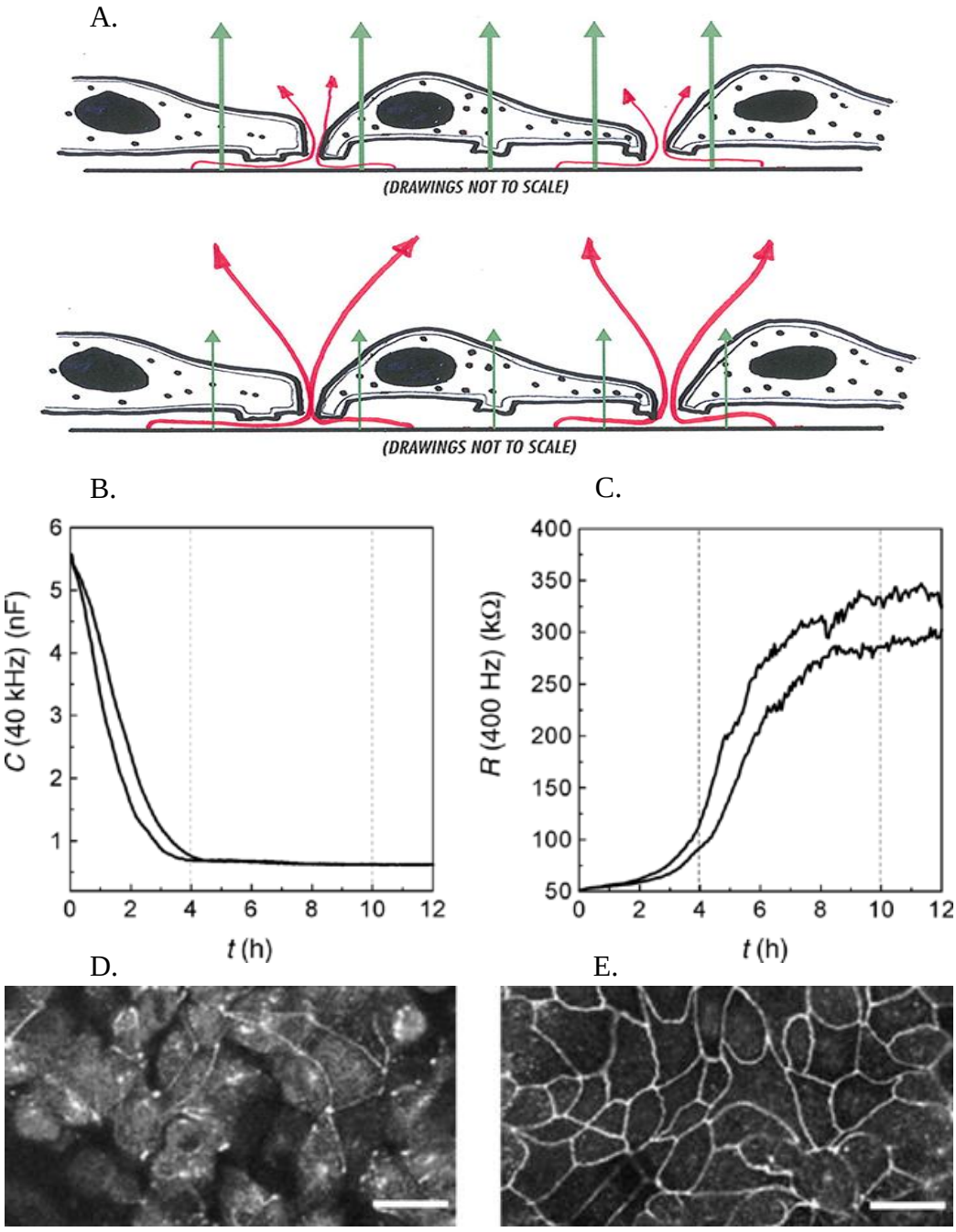


Figure 3.3. Mechanisms of ECIS. **A.** At low frequency (400 Hz) current flow between cells and gives a reading of tight junction formation and at high frequency (32 kHz) current flows through the cells providing information of cell monolayer formation. **B.** Capacitance decreases as cells become confluent **C.** Resistance increases as cells divide and become confluent, forming tight junctions in the process. **D.** MDCK cell monolayer after 4 hours. **E.** MDCK monolayer after 10 hours (Adapted from http://www.biophysics.com/ecis-theory.php and (Wegener, 2010)).

Following the methodology outlined by Szulcek et al., 8W10E arrays (IBIDI) were treated with 200µl of 10mM L-cysteine dissolved in sterile water for 15 minutes at room temperature. After 15 minutes L-cysteine was aspirated from the wells and the wells washed 3x with 300µl of sterile water. 200µl of PureCol Type 1 Bovine Collagen Solution (Advanced Biomatrix) diluted 1 in 100 in sterile water was added to each well of the arrays and the arrays incubated at 37°C overnight. The following day, collagen was aspirated from the wells and the wells washed 3x with BEGM. P2 NHBECs and P5/IP3 pLVX-BMI-1 HBECs which had previously been seeded into a T75 culture flask at $2.5x10^5$ cells per flask and grown to confluency were split and counted. $2.25x10^5$ cells were diluted in 1.2ml of BEGM (Szulcek et al., 2014).

400µl of pre-warmed BEGM was added to each well of the arrays and the arrays inserted into the ECIS instrument. The arrays were stabilised and then removed from the instrument. Media was aspirated from each well and 400µl of BEGM containing $7.5x10^4$ cells was added to each of the appropriate wells.

The arrays were again placed back into the ECIS instrument and run at multiple frequencies (including the specific frequencies of 400 Hz and 32 kHz) for 72 hours continuously.

3.2.2 Statistical analysis

To quantify the impact of BMI-1 expression on HBEC properties area under the curve (AUC) values were determined for each donor, the values were combined and the median and 95% confidence limit calculated. A Mann Whitney U test was used to determine statistical differences between the two independent groups.

To detect whether BMI-1 expression altered HBEC properties over time, normalised resistance and capacitance values were collected at specific time-points. The mean and SEM were calculated in order to perform a two-way ANOVA with Sidak's multiple comparisons test, $p<0.05$ was considered to be significantly different.

Resistance and capacitance data from NHBECs and pLVX-BMI-1 HBECs were normalised against media only wells.

3.2.3 Two-step cell cycle assay

The two-step cell cycle assay is a method of determining which cell cycle phase a population of cells are in through quantification of DNA content. Cells in the G1/G0 phase of the cell cycle have one set of chromosomes whilst cells in G2/M phase possess two sets of chromosomes. Furthermore, cells undoing DNA replication in S phase have variable quantities of DNA (between one and two sets of chromosomes). As a result, when permeabilised cells are stained with DAPI, the intensity of DNA staining produced is significantly greater for cells in the G2/M phase than cells in the G1/G0 phase. In addition, cells that have a DNA staining intensity between G1/G0 and G2/M are considered to be in S phase, as the DNA is in the process of being replicated (https://chemometec.com/cell-cycle-comparation-image-cytometry-vs-flow-cytometry/).

Passage 2 NHBECs and passage 5/infection passage 3 pLVX-BMI-1 HBECs were seeded into T25 culture flasks at $1x10^5$ cells per flask and grown until around 60-70% confluent, ensuring that the cells did not become over confluent and enter cell cycle arrest. Cells were split and counted. $4x10^5$ cells were transferred into a new universal tube and centrifuged at 400g for 5 minutes. The pellet was washed in 2ml PBS and centrifuged once more at 400g for 5 minutes. The pellet was resuspended in 100µl of Lysis Buffer (Solution 10 - Chemometec) containing 10µg/mL DAPI (Solution 12 - Chemometec) and incubated at 37°C for 5 minutes. 100µl of stabilization buffer (Solution 11 – Chemometec) was added to the solution and 30µl of sample added to an NC-Slide A2. The slide was loaded into the Chemometec Nucleocounter NC-3000 and a Two Step Cell Cycle Assay run.

The data was exported as an FCS file and analysed using FlowJo software. The cell cycle distribution for 10,000 cells was automatically quantified using the cell cycle-modelling algorithm, Watson Pragmatic.

3.3 Results

3.3.1 Cells overexpressing BMI-1 show identical cell adhesion, cell spreading and barrier resistance properties to NHBECs

To determine whether BMI-1 upregulation alters the behaviour of HBECs, pLVX-BMI-1 HBECs and NHBECs from 6 donors were cultured simultaneously on an ECIS array. Over 72 hours the impedance of the circuit was monitored and used to calculate resistance and capacitance which was then utilised to determine the impact of BMI-1 expression. ***Figure 3.4A*** and ***Figure 3.4B*** show the raw profiles of HBEC resistance at 400 Hz and capacitance at 32000 kHz. In ***Figure 3.4C*** and ***Figure 3.4D*** displays the same resistance and capacitance data normalised to the control media.

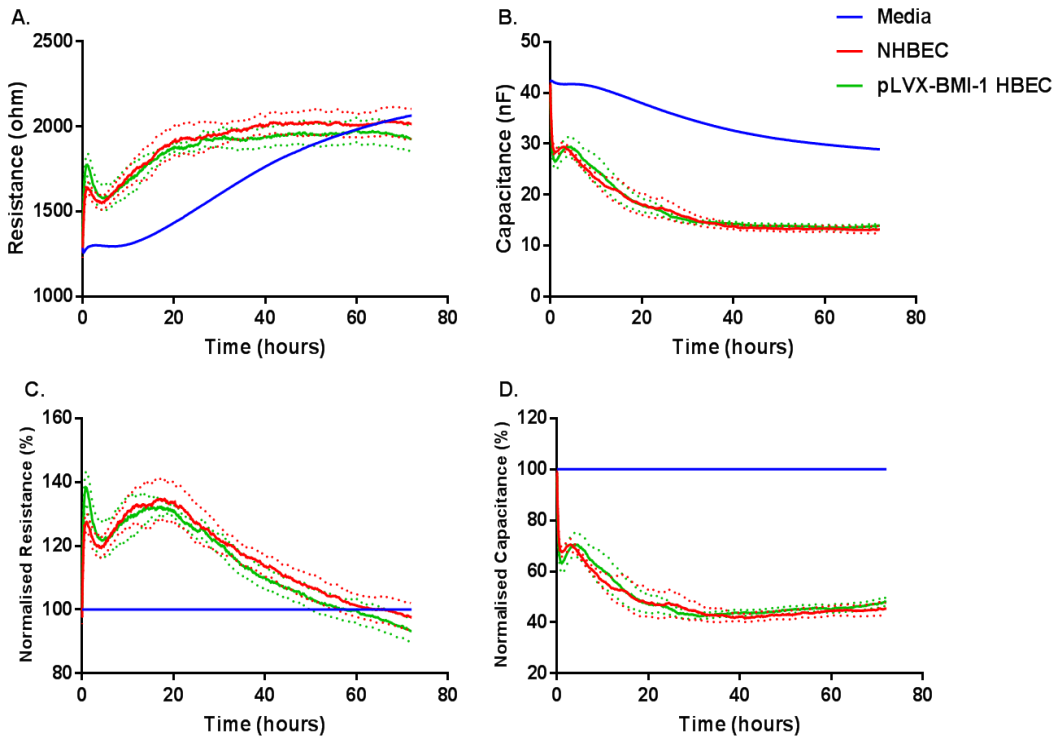


Figure 3.4. Raw Resistance and Capacitance Profiles of NHBECs and pLVX-BMI-1 HBECs. Resistance at 400 Hz (barrier formation) and capacitance at 32 kHz (cell adherence and spreading) of passage 3 NHBECs (red) and infection passage 4/passage 6 pLVX-BMI1 HBECs (green). The lines represent the mean from 6 HBEC donors and the SEM. **A.** Raw resistance profiles. **B.** Raw capacitance profiles. **C.** Resistance normalised to 100% of the control, BEGM media (blue). **D.** Capacitance normalised to 100% of the control, BEGM media (blue).

Resistance profiles at 400 Hz increase for the first 20 hours in both pLVX-BMI-1 HBECs and NHBECs as cells adhere and begin to spread with tight junctions developing. Beyond 20 hours resistance plateaus as a confluent and resistive barrier is formed. A similar pattern can be seen in the capacitance profile at 32 kHz, capacitance decreases quickly for the first 20 hours as cell adhere to the electrode, divide and spread to form a confluent monolayer. After 20 hours capacitance plateaus as the cell monolayers become confluent. Observing the raw resistance and capacitance profiles of both pLVX-BMI-1 and NHBECs no obvious differences can be distinguished between the two groups.

To assess in greater detail the effects of BMI-1 expression on HBEC growth and barrier properties, resistance and capacitance values were assessed at 12 hour intervals over the 72 hours. The mean values from each donor were combined and the SEM of the two groups calculated and compared as shown in ***Figure 3.5***. A two-way ANOVA with multiple comparisons test was performed and confirmed there was no significant differences between the groups at the 12 hour intervals. Interestingly, at around 36 hours resistance in both cell lines decreases steadily, this can also be observed in ***Figure 3.4***. It is unclear what is causing this loss of resistance, one possibility is that the cells become overgrown within the array. After around 36-48 hours in culture the cells potentially undergo contact inhibition, changing the behaviour of the cells as observed by the loss of resistance.

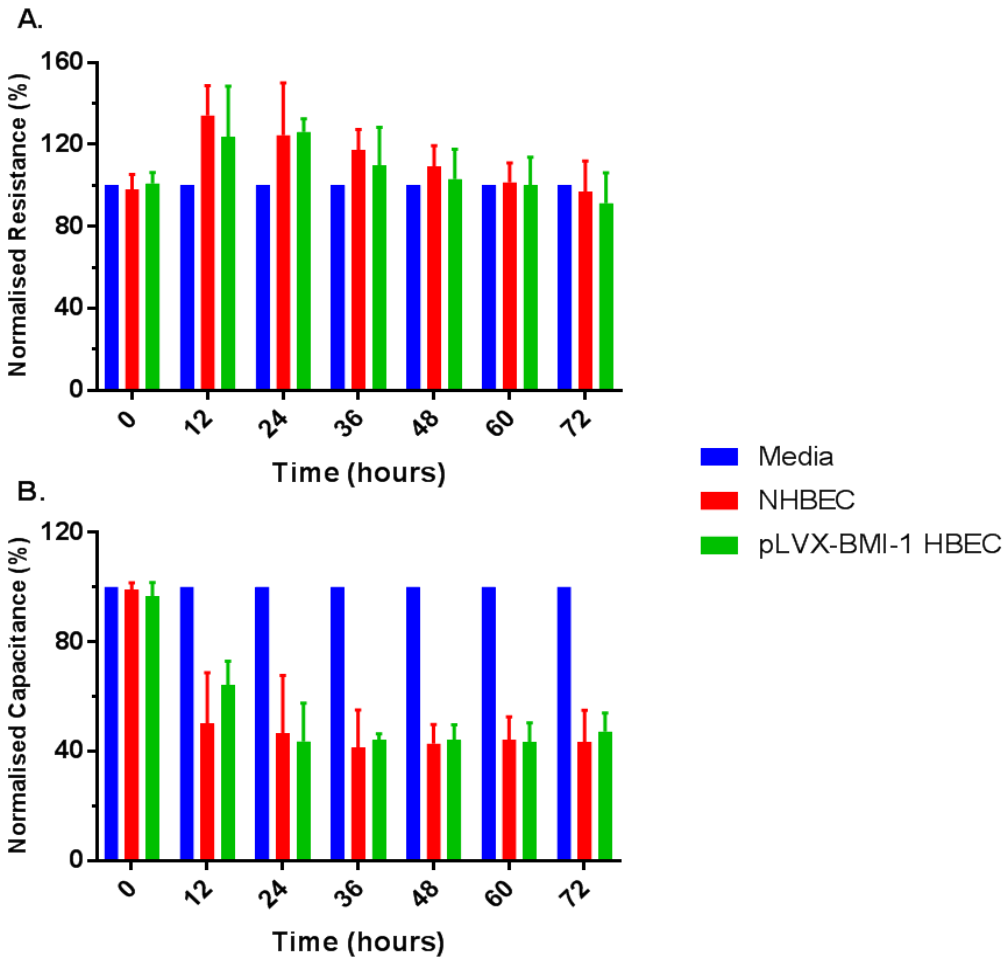


Figure 3.5. The effects of BMI-1 expression on resistance and capacitance over 72 hours. **A)** Normalised resistance profile at 400 Hz over 72 hours. **B)** Normalised capacitance profile at 32 kHz over 72 hours. The graphs display the median and 95% confidence interval from 6 donors. A two-way ANOVA was performed on normalised data with a Sidak's multiple comparisons test.

To attempt to quantify any differences between NHBECs and pLVX-BMI-1 HBECs the AUC was calculated for each donor and the two groups compared as shown in ***Figure 3.6***. We can observe that over the 72 hours there is some heterogeneity in resistance between donors, although BMI-1 expression does not have a significant impact on either resistance or capacitance.

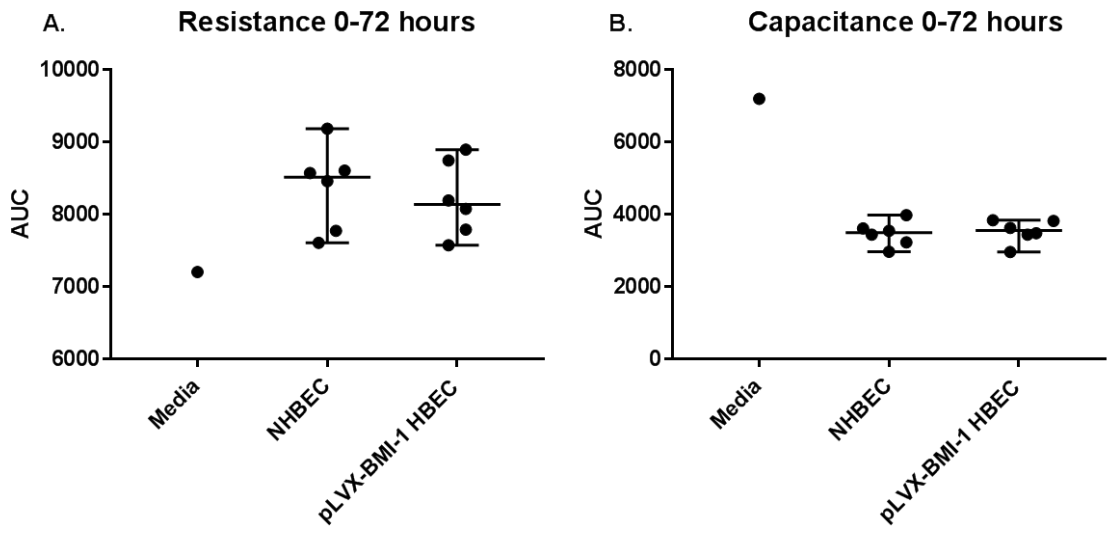


Figure 3.6. Overall effects of BMI-1 expression on HBEC properties. A+B) Total AUC from resistance and capacitance profile over 72 hours. Graphs shows the median AUC values and 95% confidence interval from 6 donors. A Mann Whitney U Test was used to determine the difference between NHBECs and pLVX-BMI1 HBECs.

However, as displayed in ***Figure 3.3*** the most significant timepoint is between 0-12 hours as the cells adhere and grow to form a confluent monolayer. As a result, the AUC values from the resistance profile at 400 Hz and the capacitance profile at 32 kHz were calculated between 0-6 and 6-12 hours as seen in **Figure 3.7**. Some slight changes in resistance and capacitance can be observed but there are no significant differences between pLVX-BMI-1 HBECs and NHBECs, as determined by a Mann Whitney U test.

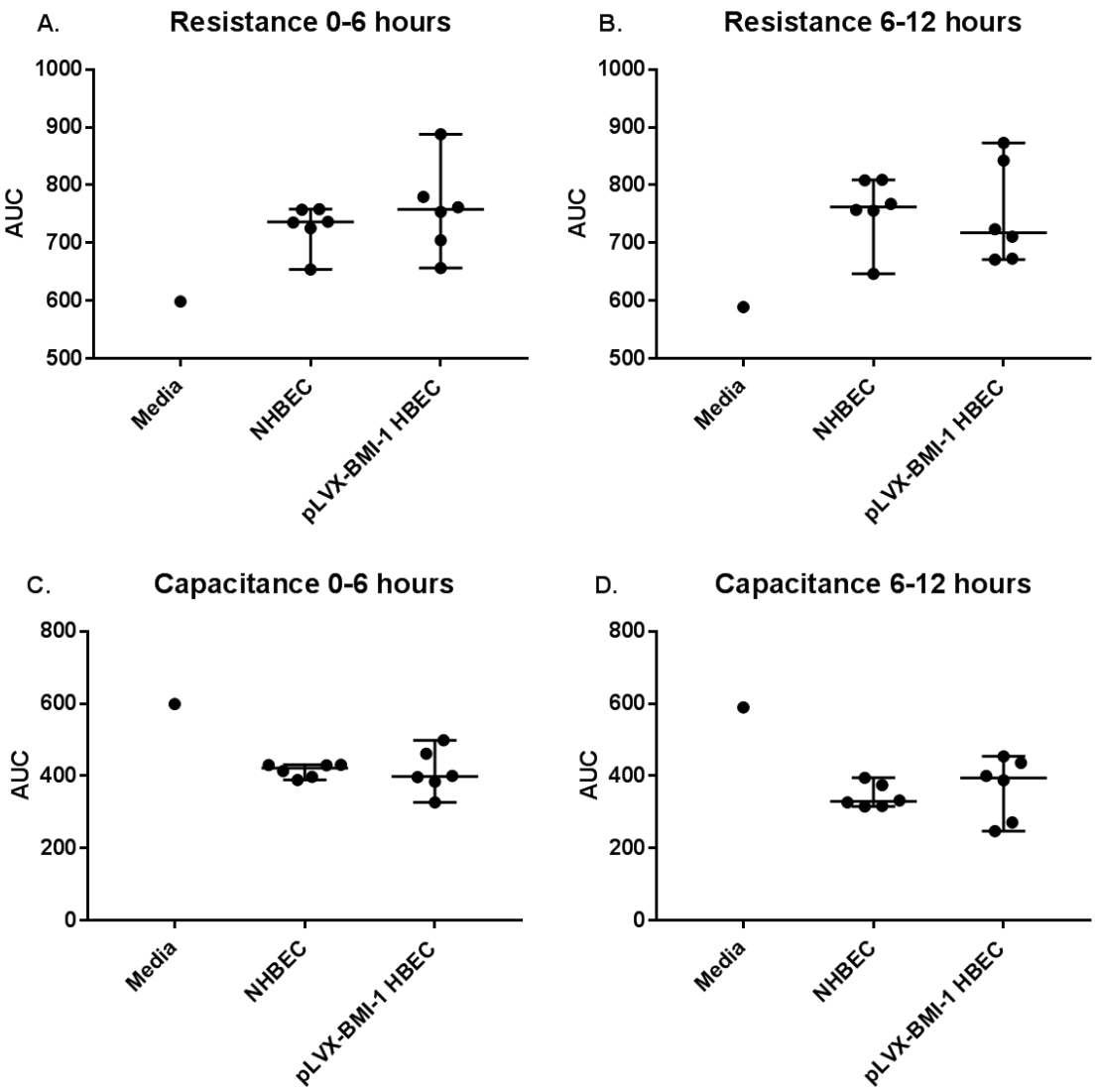


Figure 3.7. The effects of BMI-1 expression on cell spreading and barrier formation in HBECs. **A)** AUC for resistance profile at 400 Hz between 0-6 hours. **B)** AUC for resistance profile 400 Hz between 6-12 hours. **C)** AUC for capacitance profile at 32 kHz between 0-6 hours. **D)** AUC for capacitance profile at 32 kHz between 0-6 hours. Graphs show the median AUC values and 95% confidence interval from 6 donors. A Mann Whitney U Test was used to determine the difference between NHBECs and pLVX-BMI1 HBECs.

3.3.2 BMI-1 expression in HBECs promotes the progression of cells through the G1-S checkpoint

The results of the cell cycle assay, like the MTT assay from the previous chapter, were variable depending on donor. Generally, a small reduction in the mean frequency of BMI-1 expressing HBECs in the G1/G0 phase and a slight increase in pLVX-BMI-1 HBECs in the G2/M phase can be observed when compared to NHBEC. This suggest that there is a reduced frequency of BMI-1 expressing HBECs that exist in a quiescent state and that pLVX-BMI-1 HBECs progress through the cell cycle with less regulation.

In ***Figure 3.8*** there is a large decrease of 17% of donor 3 pLVX-BMI-1 HBECs in the G1/G0 phase, however, due to variability between repeats this was not statistically significant. In donor 2 and 8 pLVX-BMI-1 HBECs there was a reduction of around 12% and 10% of cells in the G1/G0 phase respectively. In addition to the reduced frequency of cells in G1/G0, an increasing proportion of cells in either the S or G2/M phases was observed. In donors 2 and 3 pLVX-BMI-1 HBECs, a steep increase of around 15% in the proportion of cells in the G2/M phase can be seen. For donor 8 pLVX-BMI-1 HBECs, a small increase of around 5% in the frequency of cells in both S phase and G2/M phase can be observed. In donor 7 pLVX-BMI-1 HBECs there is the same shift from cells in G1/G0 to G2/M phase, again around 5%.

BMI-1 expression in donor 1 HBECs resulted in almost no change in cell cycle distribution. Donor 9 pLVX-BMI-1 HBECs have a greater proportion of cells in the G1/G0 phase and a reduced frequency of cells in G2/M phase, the opposite trend to what is observed in other donors.

Of the seven donors tested, in only one donor did we see that BMI-1 expression significantly impacted the cell cycle phase distribution of HBECs. The frequency of pLVX-BMI-1 HBECs from donor 4 in the G1/G0 phase was reduced by over 20% in comparison with NHBECs ($P<0.0005$), whilst the frequency of pLVX-BMI-1 HBECs in the G2/M phase was increased by around 10% when comparing to NHBECs ($P<0.05$).

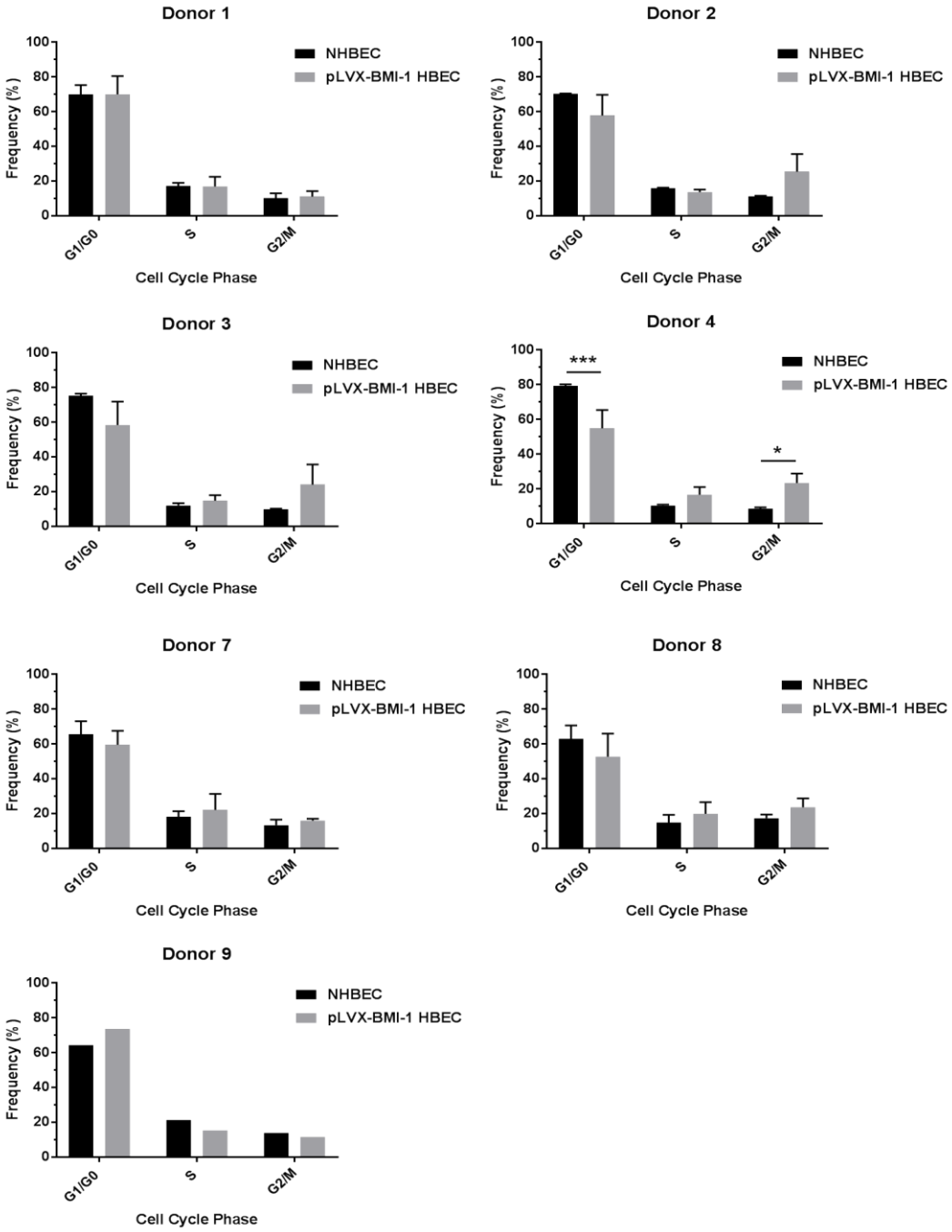


Figure 3.8. The effect of BMI-1 expression on cell cycle distribution amongst HBEC donors. Graph shows the mean and SD of passage 3 NHBECs (black) and passage 6 pLVX-BMI1 HBECs (grey). Donor 1, 2, 3, 4, 7, 8 (n=3), Donor 9 (n=1). Two-step cell cycle assay (Chemometec) with cell cycle phase frequency calculated using the Watson Pragmatic algorithm. A two-way ANOVA and Sidak's multiple comparison test was used to determine the effect of BMI-1 expression on cell cycle phases (*P<0.05, ***P<0.001).

When the cell cycle distribution from each donor is combined, as shown in ***Figure 3.9***, we can observe a similar trend. There is a significant reduction of around 10% in the frequency of pLVX-BMI-1 cells in the G1/G0 phase when compared to NHBECs ($P<0.01$). Furthermore, a similar increase in frequency of pLVX-BMI-1 cells in the G2/M phase ($P<0.05$) but virtually no change in the frequency of cells in S phase is seen.

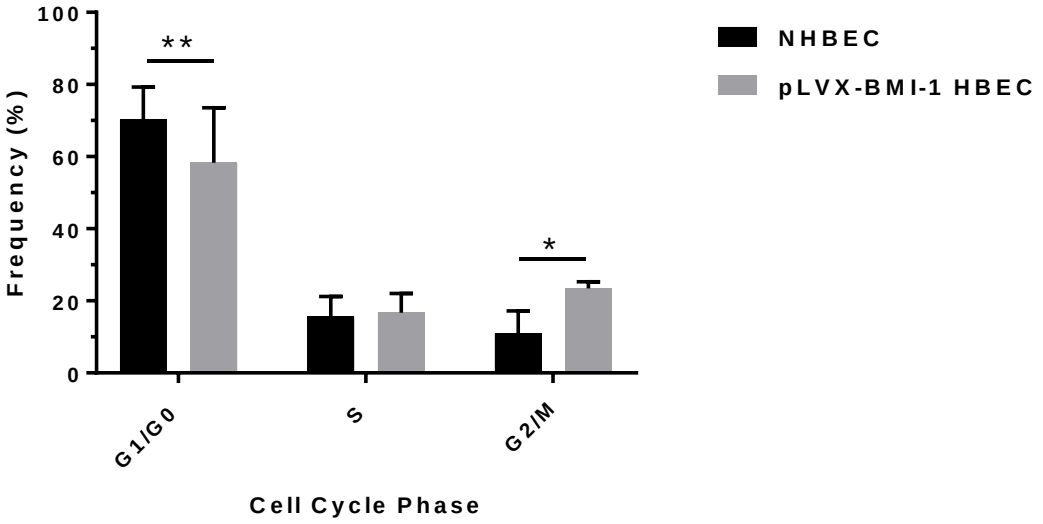


Figure 3.9. The effect of BMI1 expression on the cell cycle distribution of HBECs. The median and 95% confidence interval of passage 3 NHBECs (black) and passage 6 pLVX-BMI-1 HBECs (grey) n=7. Two-step cell cycle assay (Chemometec) with cell cycle phase frequency calculated using the Watson Pragmatic algorithm. A two-way ANOVA and Sidak's multiple comparison test was used to determine the effect of BMI-1 expression on cell cycle phases (*$P<0.05$, **$P<0.01$).

3.4 Discussion

The main objective of this masters project was to determine whether pLVX-BMI-1 HBECs are suitable models of primary human epithelial cells. The ECIS method which provides sensitive real time information about cell adherence, cell spreading and barrier properties, hence it is an ideal technique to assess the suitability of BMI-1 expressing HBECs across multiple important epithelial cell features (Wegener et al., 2000). The results from our experiments suggest that pLVX-BMI-1 HBECs would make excellent models of primary epithelial cells. It was shown that there was no significant difference between BMI-1 expressing and NHBECs in cell adherence, cell spreading and barrier properties.

Wegener et al., outlines how we can apply low frequency currents to cells and measure the formation of tight junctions and the development of an resistant barrier (Wegener, 2010). Following their methodology, a 400 Hz frequency current was applied to the cells, large variation particularly in the first 12 hours can be seen from the resistance AUC values in ***Figure 3.7***. However, whilst there is variation in the AUC values from the resistance profiles of NHBECs and pLVX-BMI-1 HBECs, these differences were modest and not statistically significant. By calculating the AUC for the resistance and capacitance profiles from each donor this allowed us to perform statistical analysis and therefore determine whether NHBECs and pLVX-BMI-1 HBECs possessed significantly different properties. I performed a Mann Whitney U test on the AUC values from each donor both over the full 72 hours and at individual time-points (E.g. 0-6hrs). However, I was unable to find significant differences between NHBECs and pLVX-BMI-1 HBECs when it came to barrier resistance. As discussed previously in the chapter the ability of HBECs to form a resistive barrier is one of their distinct characteristics. Therefore, if our pLVX-BMI-1 HBECs are to be suitable models of primary epithelial cells they must form a resistant monolayer comparable to NHBECs. Our results indicate that undifferentiated pLVX-BMI-1 HBECs cultured *in vitro* do form resistant monolayers in an almost identical manner to NHBECs.

It was unclear how increased BMI-1 expression could potentially alter HBEC behaviour. Microarray analysis of BMI-1 knockdown cells demonstrated that these cells would have altered cell to cell interaction and cell to cell matrix interactions (Douglas et al., 2008). However, as shown in ***Figure 3.5***, capacitance, an accurate indicator of monolayer formation and therefore cell adherence and cell spreading, is not significantly different between NHBECs and pLVX-BMI-1 HBECs (Szulcek et al., 2014).

ECIS, due to its ability to detect sensitive changes over multiple parameters in real time, is a particularly interesting technique. However, the ECIS method, involving growing undifferentiated cells in a monolayer on a gold electrode, is not typical of *in vivo* conditions. ALI culture is a more representative technique involving differentiated cells, exposed to air to recreate lung-like conditions. A previous study has shown that 16HBE14o- cells when cultured using the ECIS technique and ALI it was found that the cells behave differently. Using the ECIS technique, 16HBE14o- cells produce a low ECIS resistance profile, indicating poor barrier formation, whilst TEER measurements from ALI culture were comparably high (Heijink et al., 2010). Therefore, it would be useful to compare in future how TEER compares between pLVX-BMI-1 HBECs and NHBECs when cultured at ALI. Immunofluorescence is another alternative technique that could have been utilised to assess HBEC barrier properties during this study. By staining for adhesion and tight junction markers such as E-cadherin, ZO-1 or claudins, it is possible to assess epithelial barrier properties. A study of asthma subjects demonstrated that the epithelial cells from these patients had reduced recruitment of ZO-1 to the membrane and therefore compromised barrier properties in comparison to healthy individuals (Holgate, 2007). Whilst the ECIS technique provided valuable information on barrier properties, it may have been suitable to perform it in parallel with immunofluorescence.

Despite these limitations, ECIS is still a relevant technique which can provide reliable real-time information on cell behaviour. Our results indicate that pLVX-BMI-1 HBECs behave in a very similar manner to NHBECs, including comparable cell adherence, cell spreading and tight junction formation properties.

BMI-1 is a known inhibitor of both $p16^{INK4a}$ and $p19^{ARF}$ which help regulate cell cycle progression. Increasing expression of BMI-1 is thought to relieve the inhibition of cyclin D and Cdk4/6 complex formation, resulting in Rb hyperphosphorylation and the release of the E2F transcription factor which transcribes genes involved in cell cycle progression (Park et al., 2004). As a result, I anticipated that by increasing BMI-1 expression in HBECs, a significant decrease in HBECs in the G1/G0 phase would occur. However, of the seven donors we tested only one had significantly reduced cells in the G1/G0 phase. In four other donors we did see a decrease of cells in the G1/G0 phase but these were not significant.

A previous study has shown that knockdown of BMI-1 in HeLa cells significantly increases the proportion of cells in the G1/G0 phase, in addition to reducing the frequency of cells in S phase (Chen et al., 2011). Our data to an extent supports these findings. A large decrease in the frequency of pLVX-BMI-1 HBECs in G1/G0 phase can be seen. However, we do not find an increase in pLVX-BMI-1 HBECs in S phase, instead we see an increase in cell in the G2/M phase. This was not anticipated based on previous literature. As previously described by Park et al., BMI-1 interacts with $p16^{INK4A}$, a regulator of the G1-S checkpoint, and is therefore thought to act predominantly on the G1-S checkpoint (Park et al., 2004). A possible explanation for increased numbers of pLVX-BMI-1 HBECs in the G2/M phase would be BMI-1 expression having less of an impact on the other cell cycle checkpoints. As a result, BMI-1 expression promotes the progression of cells through the G1/S checkpoint but premature entry into S phase and an accumulation of DNA damage cells may halt cells at the G2/M checkpoint.

However, there is also evidence to suggest that BMI-1 expression can reduce G2/M checkpoint activation, induced by etoposide, by reducing ataxia-telangiectasia-mutated (ATM) protein kinase activation (Wei et al., 2015). ATM activation is capable of inducing cell cycle arrest at G1-S, intra-S and G2-M checkpoints in response to DNA double-strand breaks (Lee and Paull, 2007). These studies suggest that BMI-1 expression has the potential reduce activation of multiple cell cycle checkpoints. Therefore, it is unclear why an increase in frequency of pLVX-BMI-1 HBECs in the G2/M phase is observed.

Critically, it has been demonstrated that BMI-1 expression in HBECs significantly reduces the proportion of cells in the G1/G0 phase and a putative mechanism has been provided for how increased BMI-1 expression results in enhanced proliferation and delayed cell senescence.

4 Chapter 4: Final Discussion

The objective of the project was to produce a sustainable primary human airway epithelial cell model for research purposes. These cells would hopefully be representative models of primary airway epithelial cells, maintaining key phenotypes whilst gaining an enhanced replicative lifespan. As discussed in chapter 1 and chapter 2 some of the limitations of using primary airway cells and airway cell lines in research have been considered. Whilst primary airway epithelial cells have been influential in progressing our knowledge of not only asthma but multiple respiratory diseases, the early onset of cellular senescence in primary cell cultures *in vitro* is problematic. Consequently, it is difficult to use primary airway cells in large studies which require cells to be passaged multiple times *in vitro*. As a result, there is a need to produce a cell model, representative of primary airway cells, which could be utilised in large studies such as drug toxicity testing or CRISPR-Cas9. In addition, using these cells in smaller studies will hopefully reduce the need for regular bronchoscopy of control subjects and patients which are associated with invasiveness and risk to the individual.

This project has demonstrated that inducing BMI-1 expression in HBECs increases their metabolic activity and enhances their replicative lifespan by promoting the progression of the cell through the G1-S cell cycle checkpoint. However, BMI-1 expression did not significantly alter key epithelial cell properties including cell adhesion, cell spreading and barrier formation. Furthermore, preliminary RNA sequencing data suggested that a mere 2.3% of genes are significantly altered by BMI-1 expression in HBECs.

This study has yielded eight stably modified pLVX-BMI-1 cell lines which were produced from eight HBEC donors. Other studies have used BMI-1 to prolong the lifespan of NHOKs, cystic fibrosis diseased epithelial cells and adipose tissue-derived stromal cells. Furthermore, the key phenotypes of these cells were retained despite overexpression of BMI-1 (Kim et al., 2010; Munye et al., 2017; Tátrai et al., 2012). These studies, in addition to this project, support the potential use of BMI-1 in capturing the phenotype of primary cells across various cell types.

Primary human cells can be difficult to culture *in vitro* and this is a factor which must be considered when selecting a cell line, especially if an experiment is limited by time and resources. HBECs induced to express BMI-1 were shown to have greater metabolic activity and a proliferative advantage over NHBECs, as a result these cells appeared more robust and easier to culture. In order to compare how similar BMI-1 expressing HBECs were to NHBECs we required characterisation data from both groups of HBECs. However, due to difficulty culturing NHBECs from donor 11, I was unable to gather characterisation data from these cells and therefore determine how they might differ from BMI-1 expressing HBECs.

Due to the time limitations of a masters project I was unable to grow our pLVX-BMI-1 HBECs to late passage and perform further characterisation studies. Our research group previously showed in two donors that BMI-1 expressing HBECs possess a normal karyotype and form a resistive barrier up to passage 15 (Torr et al., 2016). However, it is likely that prolonged expression of a PcG protein such as BMI-1 will have consequences on cell homeostasis. This is highlighted by numerous studies which link increased BMI-1 expression with poor prognosis in multiple cancer strains (Du et al., 2010; Li et al., 2010; Liu et al., 2008; Qin et al., 2009; Wang et al., 2008).

Lentivirus based delivery appears to be the most suitable gene delivery system currently available. hTERT is another readily used system for delaying cell senescence but its effectiveness in the literature has been mixed. Some studies have shown that hTERT expressing cells have an enhanced lifespan but still maintain key cell markers (Bai et al., 2004; Dickson et al., 2000). However, contrasting studies have indicated that hTERT expression results in significant changes to cell behaviour. One study showed that hTERT immortalisation is effective during the first 150 population doublings (PDLs) as these cells behave in a similar manner to primary cells. However, beyond this point hTERT immortalised cells started to show symptoms of becoming malignant clones (Milyavsky et al., 2003). Our research group found similar results when utilising hTERT to immortalise HBECs. HBECs expressing hTERT lost the ability to form a resistive barrier and showed major chromosomal abnormalities as early as passage 10. These problems were not observed in HBECs induced to overexpress BMI-1 through lentivirus transduction (Torr et al., 2016).

Genetic modification of cells can drastically alter cell behaviour. As a result, techniques have been investigated that can prevent primary cell senescence without the need for genetic manipulation. Rho-associated protein kinase (ROCK) is a downstream effector of the GTPase Rho and is known to regulate a number of cell functions including cell motility, cell morphology and gene expression (Amano et al., 2010). Y-27632 is a selective ROCK inhibitor which has been shown to promote the survival of human embryonic stem (hES) cell by preventing apoptosis (Watanabe et al., 2007). In addition, Y-27632, when combined with fibroblast feeder cells, has been shown to induce indefinite proliferation of both normal and tumour epithelial cells. Cells immortalised with the rock inhibitor and feeder cells displayed a more normal cell phenotype in comparison to hTERT immortalised cells (Liu et al., 2012). As a result, a combination of rock inhibitors and feeder cells supplemented into the epithelial cell culture potentially offers a viable alternative to genetic manipulation.

The predominant aim of this project was to determine how comparable BMI-1 expressing HBECs are to NHBECs. The results from this study indicate that passage 5 pLVX-BMI-1 HBECs behave remarkably like NHBECs. The ECIS technique was the ideal tool for assessing just how comparable these cells are. In relation to cell adherence and cell spreading, measured through capacitance values, no significant difference could be found over the 72 hour run. More importantly there was no significant difference between the groups during the key period of cell adherence and spreading in the first 12 hours of the run (Szulcek et al., 2014). The literature suggests that BMI-1 expression would significantly impact certain cell behaviours such as the development of cell-cell matrix interactions (Douglas et al., 2008). However, these differences did not manifest in our *in vitro* cultures. Before concluding that BMI-1 expression in HBECs has little impact on cell adherence and spreading, I believe it would interesting to repeat these ECIS experiments at later passages such as passage 10 or 15. As a result it could be determined if prolonged BMI-1 expression effected cell growth.

Perhaps more importantly it was shown that pLVX-BMI-1 HBECs possess similar barrier properties to NHBECs. Much like our capacitance profile, there was no significant difference in barrier resistance, assessed using resistance measurements, between the two groups over the 72 hours, nor in the first 24 hours as a confluent layer develops

and tight junctions form. These results suggest that pLVX-BMI-1 HBECs retain the ability to form a resistive barrier through the formation of tight junctions. It is possible that overexpression of BMI-1 could alter HBEC barrier properties with increasing passages although it has been previously shown by our research group that pLVX-BMI-1 HBECs retain a high TEER when cultured at ALI, even at a late passage (Torr et al., 2016). Regardless, the data gathered across a range of donors indicates that undifferentiated passage 5 pLVX-BMI-1 HBECs have very similar epithelial barrier properties to passage 2 NHBECs when cultured *in vitro*.

Similar work which aimed to prolong the lifespan of primary epithelial cells using BMI-1 have found equally promising results. Munye et al., also replicated the findings that BMI-1 expression in NHBECs does not affect their karyotype or their plasticity. In addition, they showed that BMI-1 expressing HBECs have the same cilia beating properties when compared to NHBECs (Munye et al., 2017).

In this project I also gained an insight into how BMI-1 expression can provide HBECs with a proliferative advantage and help delay senescence. Using a two-step cell cycle assay it was shown that the frequency of cells in the G1/G0 phase was significantly reduced in HBECs overexpressing BMI-1. In addition, the frequency of these cells in the G2/M phase was significantly higher in comparison to NHBECs. It is likely that BMI-1, through inhibiting the function of $p16^{INK4A}$, promotes the progression of HBECs through the G1-S cell cycle checkpoint (Park et al., 2004). This is the main pathway through which BMI-1 expression is thought to prevent the onset of cellular senescence, although there is evidence to suggest BMI-1 can prolong cellular lifespan through the TGF-β signalling pathway in a p16 independent manner (Kim et al., 2010).

One of our key goals for the project was to determine how BMI-1 overexpression altered the transcriptome of HBECs. RNA sequencing would have allowed us to determine at a molecular level the effects of BMI-1 expression on HBECs. RNA was isolated from NHBECs and pLVX-BMI-1 HBECs and outsourced for sequencing. Unfortunately, the analyses of the data received from the sequencing process is still being worked on and the deadline for the masters has come too early to present a full chapter of RNA sequencing data. Consequently, I am unable to investigate the true

extent of BMI-1 expression on the HBEC transcriptome, but I can briefly present some preliminary data.

The initial CuffDiff analysis identified 427 genes out of 18858 genes assayed to be differentially expressed ($q<0.05$); q represents the false discovery rate (FDR) and is adjusted from the p value. As a result, only 2.3% genes were differentially expressed in BMI-1 expressing HBECs when using a FDR of 5%. Of the 427 differentially expressed genes identified, 231 were upregulated and 196 were downregulated. BMI-1 was one of the genes found to be significantly upregulated in pLVX-BMI-1 HBECs, with a 2.6442 log2 fold change ($q<0.004121$).

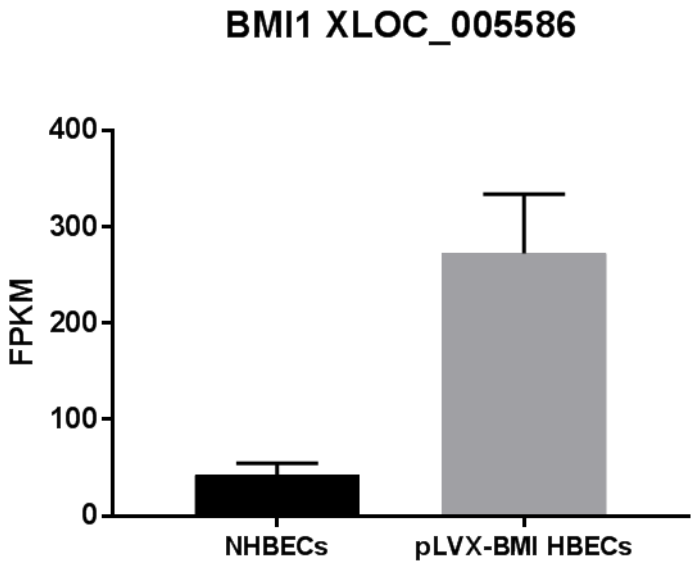


Figure 4.1. BMI-1 upregulation in pLVX-BMI1 HBECs. A bar chart of the FKPM values within the BMI-1 locus, showing the median and 95% confidence intervals, n=8 ($q<0.004121$).

Of the 427 differentially expressed genes, the top five most upregulated and the top five most downregulated genes have been shown below, in addition to a few notable genes.

Gene name	q value	Log2 (Fold change)	FKPM (NHBEC)	FKPM (pLVX-BMI-1 HBEC)	Function	Reference
HAS2 (Hyaluronan Synthase 2)	0.004121	4.20816	0.047679	0.88128	Synthesis of HA and involved in cell proliferation	(Zhang et al., 2016)
DENND2A (DENN Domain Containing 2)	0.004121	3.86786	0.037001	0.540211	Vesicle-mediated transport	(Yoshimura et al., 2010)
TSPAN18 (Tetraspanin 18)	0.004121	3.68679	0.027140	0.349501	Embryo development	(Garcia-Espana et al., 2008)
WSCD1 (WSC Domain Containing 1)	0.004121	3.50111	0.058153	0.658440	Sulfotransferase activity	(Xie et al., 2013)
DMBT1 (Deleted In Malignant Brain Tumors 1)	0.004121	3.37998	0.033602	0.349822	Involved in host defence	(Mollenhauer et al., 2000)

Table 4.1. The top 5 most upregulated genes in BMI-1 expressing HBECs. The genes are ordered by q value and Log2 (fold change).

Gene name	q value	Log2 (Fold change)	FKPM (NHBEC)	FKPM (pLVX-BMI-1 HBEC)	Function	Reference
Y_RNA (RNY)	0.004121	-6.98595	39.928	0.314991	Assist in initiating DNA replication	(Kowalski and Krude, 2015)
HEATR6 (HEAT Repeat Containing 6)	0.004121	-5.96820	456.204	7.28707	Novel gene associated with breast cancer	(Sinclair et al., 2003)
SPINK7 (Serine Peptidase Inhibitor, Kazal Type 7)	0.004121	-5.48888	1.58618	0.035321	Serine peptidase inhibitor	(Cheng et al., 2009)
ATP12A (ATPase H+/K+ Transporting Non-Gastric Alpha2 Subunit)	0.004121	-5.30671	3.60547	0.091093	H+/K+ ATPase	(Streif et al., 2011)
PROS2P (Protein S Beta Pseudogene)	0.004121	-4.81732	3.68052	0.130542	Regulates blood coagulation	(Edenbrandt et al., 1990)

Table 4.2. The top 5 most downregulated genes in BMI-1 expressing HBECs. The genes are ordered by q value and Log2 (fold change).

Gene name	q value	Log2 (Fold change)	FKPM (NHBEC)	FKPM (pLVX-BMI-1 HBEC)	Function	Reference
TGFBI (Transforming growth factor-beta-induced)	0.004121	0.801385	567.654	989.291	Extracellular matrix binding	(Nummela et al., 2012)
E2F1 (E2F Transcription Factor 1)	0.007319	1.70518	0.673224	2.19518	Transcription Activator	(Bertoli et al., 2013)
CDHR3 (Cadherin Related Family Member 3)	0.015141	1.62997	0.37547	1.16211	Cellular receptor for Rhinovirus-C	(Bochkov et al., 2015)
PLAUR (Plasminogen Activator, Urokinase Receptor)	0.044467	0.70129	36.1976	58.8558	Urokinase plasminogen activator surface receptor	(Barton et al., 2009)

Table 4.3. Genes of interest which are differentially expressed due to BMI-1 expression. The genes are ordered by q value.

One of the genes found to be significantly upregulated in BMI-1 expressing HBECs was E2F1 ($q<0.007319$). The E2F1 is a member of the E2F family of transcription factors which are required for the transcription of genes which promote the progression of cells through the G1-S checkpoint (Bertoli et al., 2013). The upregulation of E2F1 supports our data in Section 3.3.2, were it was demonstrated that the frequency of cells in the G1/G0 phase was significantly reduced in HBECs expressing BMI-1. This data suggests that BMI-1 expression prolongs cell lifespan through the Rb-E2F pathway.

Previous studies which have used BMI-1 expression to prolong cell lifespan have found that the mechanisms by which BMI-1 delays cell senescence can be independent of $p16^{INK4A}$ (Douglas et al., 2008). BMI-1 expression in NHOKs is thought to delay senescence through the inhibition of the TGF-β signalling pathway. This study showed that BMI-1 expression resulted in decreased intracellular and secreted TGF-β1 (Kim et al., 2010). However, no significant difference in the expression of TGF-β1 was observed, only TGFβI, which was significantly upregulated in BMI-1 expressing cells.

Another gene found to be upregulated in BMI-1 expressing cells was CDHR3, a known childhood asthma susceptibility gene (Bochkov et al., 2015). Furthermore, PLAUR, also a known asthma susceptibility gene, was found to be upregulated in BMI-1 engineered HBECs (Barton et al., 2009). One of the rationale for performing RNA sequencing was to determine if BMI-1 impacted the expression of key genes associated with airway disease. As shown in this preliminary data, BMI-1 expression can impact the expression of known asthma susceptibility genes including PLAUR and CDHR3. This highlights why this information should be taken into consideration before choosing a cell model to study a specific gene of interest.

As the preliminary RNA sequencing data has shown there are some relevant asthma genes affected by BMI-1 expression. Fortunately, there are alternatives to constitutive BMI-1 expression as discussed in earlier chapters. Our research group is currently working on a second-generation system for prolonging cell lifespan. This is an inducible system were BMI-1 expression can be switched on and off at will. The advantage of this method is that BMI-1 expression can be withdrawn when not needed.

However, this system is still in its preliminary staged and has not yet been perfected. For instance, it is not known how effective the switch system is, does BMI-1 expression revert to endogenous levels in the absence of cumate? If cumate is withdrawn how quickly does BMI-1 expression reduce to standard levels? These are key questions which must be addressed before this can become the preferred genetic manipulation system.

There is also the nanoparticle delivery system which has been developed in recent years but like the inducible lentivirus system, it is also unperfected. If this system is to become a readily used technique in research certain issues must be addressed such as ensuring endocytosis of nanoparticles by target cells, enhancing endosomal escape and increasing delivery efficiency (Chen et al., 2016).

From the evidence available, HBECs engineered to overexpress BMI-1 could be utilised for many experimental techniques. BMI-1 expressing HBECs could be utilised in assessing drug interactions, drug efficacy and drug toxicity testing. Currently cell lines

such as Calu-3 and Beas-2B cells are used as models of the airway epithelium but as previously eluded to, these cell lines are unsuitable models of primary airway epithelial cells (Shan et al., 2011; Stewart et al., 2012b). The evidence suggests that HBECs engineered to overexpress BMI-1 are better representative models of primary airway epithelial cells. Furthermore, these cells have the replicative lifespan and robustness to be utilised in drug toxicity testing. In addition, these cells could also be used in other long-term experiments such as CRISPR-Cas9. This method can take up to 6 weeks to complete and is therefore very difficult to complete in primary epithelial cells due to their short lifespan. However, the enhanced replicative lifespan of BMI-1 expressing HBECs and their increased robustness, means that techniques such as CRISPR-Cas9 are a feasible option.

In summary, the work completed so far suggests that utilising BMI-1 to extend the lifespan of HBECs is an extremely promising method. Our work suggests that HBECs engineered to overexpress BMI-1 behave in an almost identical manner in basal culture. These results potentially pave the way for these airway epithelial cell models to be used for future studies into airway disease and in the development of potential therapeutics.

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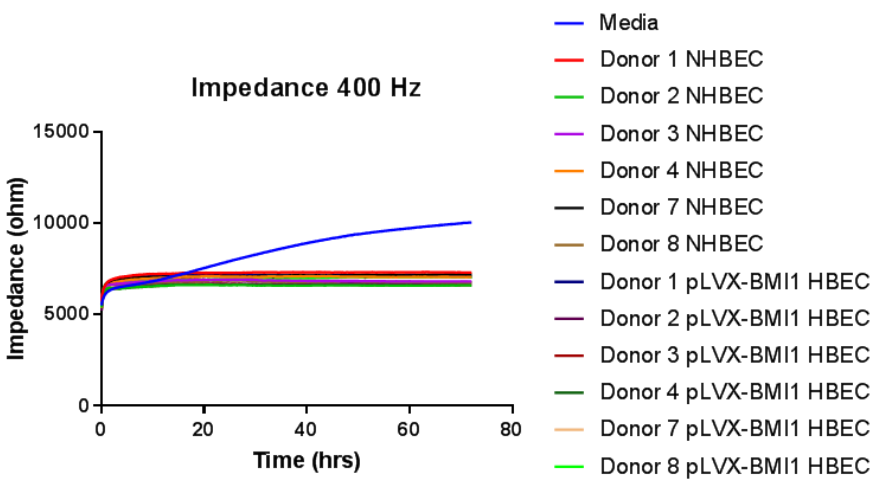
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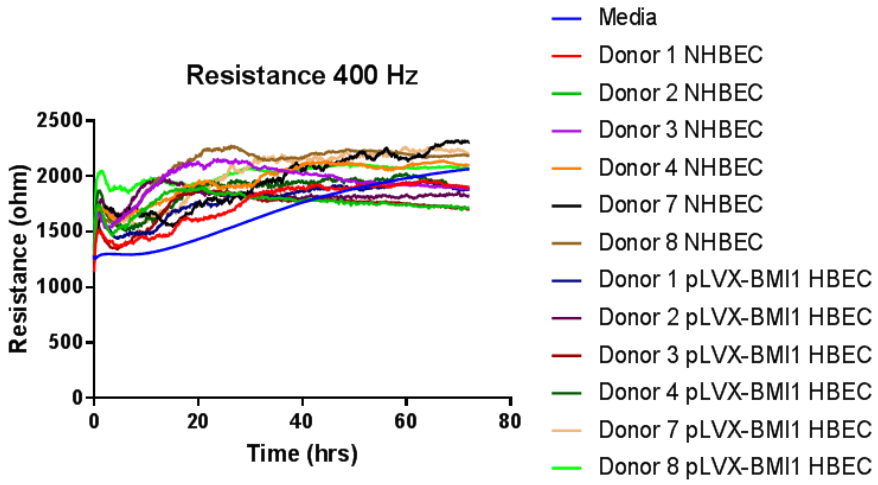
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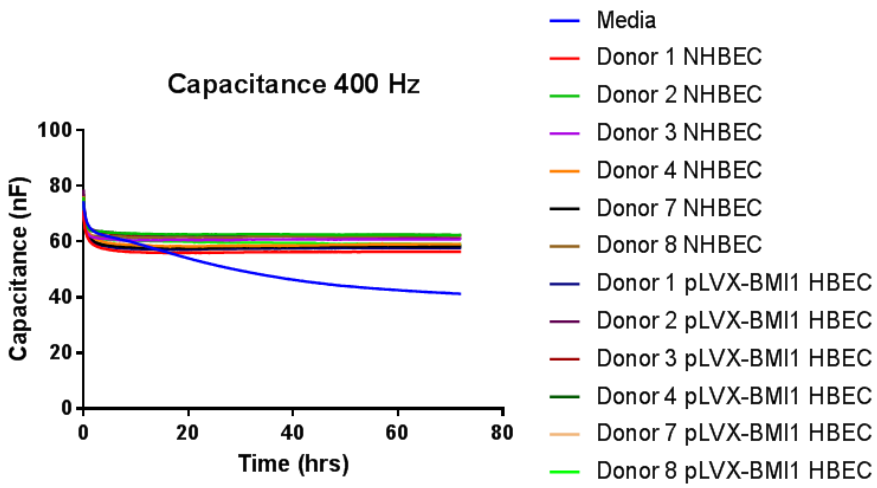
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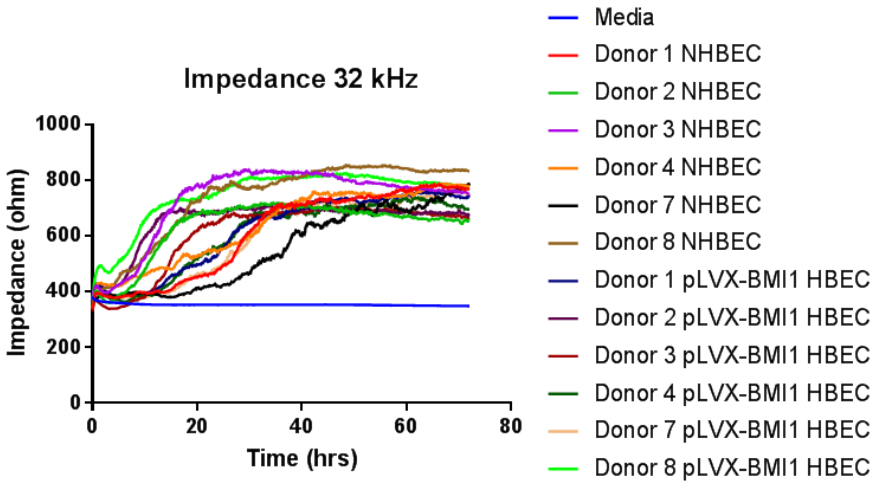
Supplementary Figure 6.1. Raw Impedance Profile. Impedance at 400 Hz. Passage 3 NHBECs and passage 6 pLVX-BMI1 HBECs (n=6).



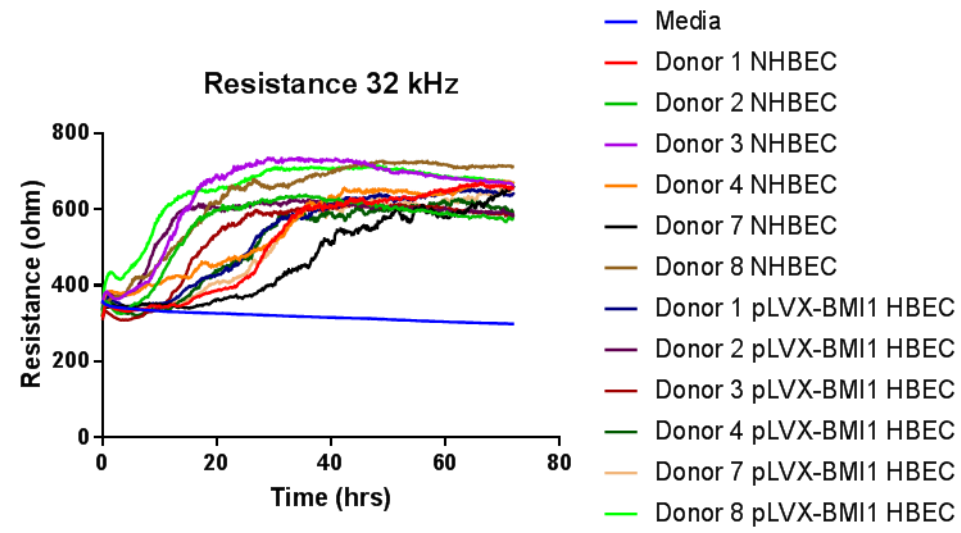
Supplementary Figure 6.2. Raw Resistance Profile. Resistance at 400 Hz. Passage 3 NHBECs and passage 6 pLVX-BMI1 HBECs (n=6).



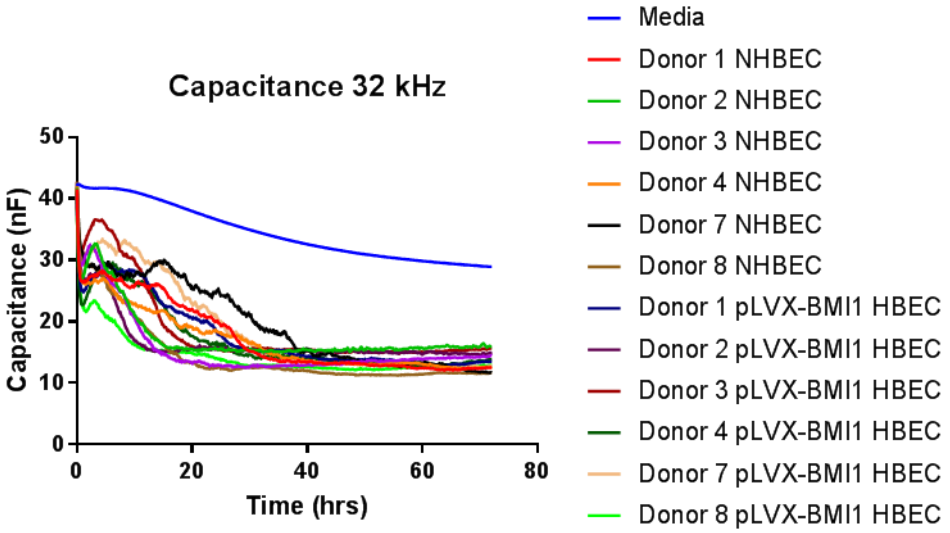
Supplementary Figure 6.3. Raw Capacitance Profile. Capacitance at 400 Hz. Passage 3 NHBECs and passage 6 pLVX-BMI1 HBECs (n=6).



Supplementary Figure 6.4. Raw Impedance Profile. Impedance at 32 kHz. Passage 3 NHBECs and passage 6 pLVX-BMI1 HBECs (n=6).



Supplementary Figure 6.5. Raw Resistance Profile. Resistance at 32 kHz. Passage 3 NHBECs and passage 6 pLVX-BMI1 HBECs (n=6).



Supplementary Figure 6.6. Raw Capacitance Profile. Capacitance at 32 kHz. Passage 3 NHBECs and passage 6 pLVX-BMI1 HBECs (n=6).