The Role of Ion Channels in Airway Mucus Hypersecretion in Asthma

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Abstract

The Ca^{2+}-activated potassium channel, $K_{Ca}3.1$, and the Ca^{2+} release-activated calcium channel, Orai, are implicated in the process of exocytic release of intracellular granules from mast cells. MUC5AC mucin is upregulated in asthmatic human airways, and is released from secretory human bronchial epithelial cells (HBECs) via exocytosis of intracellular granules. The central hypothesis tested here was that $K_{Ca}3.1$ and Orai channels modulate MUC5AC production and secretion from HBECs.

Ion channel expression was examined in primary HBECs isolated from asthmatic and healthy donors and the human airway epithelial cell line, H292, by qPCR, western blotting and patch clamp electrophysiology. MUC5AC and $K_{Ca}3.1$ expression in bronchial biopsies were analysed using immunohistochemistry. Mucin production and secretion from *in vitro* cultures were quantified by lectin assay and immunohistochemistry.

This study provides the first report of $K_{Ca}3.1$ expression and activity in primary HBECs. $K_{Ca}3.1$ mRNA was expressed to a similar extent in both asthmatic and healthy HBECs, and protein expression was evident in western blots. Asthmatic HBECs displayed significantly larger TRAM-34-sensitive $K_{Ca}3.1$ currents than healthy HBECs (P = 0.023). MUC5AC and $K_{Ca}3.1$ co-localised in approximately 50% of HBECs within bronchial biopsy samples in asthma and in health. Severe asthmatic bronchial epithelium displayed significantly higher levels of $K_{Ca}3.1$ (P = 0.001) and MUC5AC (P = 0.018) immunostaining in comparison to healthy epithelium. $K_{Ca}3.1$ and MUC5AC immunostaining in the bronchial epithelium correlated across the severities of asthma (P = 0.0211). $K_{Ca}3.1$ blockade did not inhibit amphiregulin-induced MUC5AC production and secretion from H292 cells, or IL-13-induced MUC5AC mRNA expression in primary HBECs. Despite detecting Orai protein and mRNA expression in primary HBECs, Orai currents could not be recorded. These data suggest that $K_{Ca}3.1$ expression in the bronchial epithelium in asthma has functional relevance, but is unlikely to regulate mucus production. The role of Orai channels in primary HBECs requires further investigation.

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The role of the $K_{Ca}3.1$ channel in the regulation of airway mucus hypersecretion in asthma

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Abbreviations

1-EBIO	1-ethyl-2-benzimidazolinone
Ab	Antibody
AEC	3-Amino-9-ethylcarbazole
AHR	Airway hyperresponsiveness
ALI	Air liquid interface
AREG	Amphiregulin
ASL	Airway surface liquid
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
BAPTA	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetracetic acid tetrakis(acetoxymethyl ester)
BEGM	Bronchial epithelial growth medium
bp	Base pairs
BSA	Bovine serum albumin
BTS	British Thoracic Society
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Free intracellular calcium ion concentration
CF	Cystic fibrosis
Cl^-	Chloride
COPD	Chronic obstructive pulmonary disease
Cs	Caesium
C_T	Threshold cycle
DC	Dendritic cell
DC-EBIO	5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazole-2-one
DMEM	Dulbecco's Modified Eagles Medium
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
EGF	Epithelial growth factor
EGFR	Epithelial growth factor receptor
EGTA	Ethylene glycol tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ELLA	Enzyme-linked lectin assay

ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FcεRI	Fc epsilon receptor I high-affinity IgE receptor
FEV_1	Forced expiratory volume in one second
FOXA2	Forkhead box A2
FVC	Forced vital capacity
g	Grams
GalNAc	*N*-acetylgalactosamine
Gd^{3+}	Gadolinium
GINA	Global Initiative for Asthma
GMA	Glycol methacrylate
GMFI	Geometric mean of fluorescence intensity
GWAS	Genome-wide association study
H&E	Haematoxylin and eosin
h	Hour
HBEC	Human bronchial epithelial cell
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HLF	Human lung myofibroblasts
HLMC	Human lung mast cells
HRP	Horseradish peroxidase
IC_{50}	50% inhibitory concentration
ICA-17043	bis(4-fluorophenyl)phenyl acetamide (Senicapoc)
ICS	Inhaled corticosteroids
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
I_m	Membrane current
IP3	Inositol triphosphate
IQR	Interquartile range
JAM	Junctional adhesion molecule
kDa	Kilo Daltons
K^+	Potassium

K_{Ca}	Calcium-activated potassium channel
La^{3+}	Lanthanum
LABA	Long-acting β_2 agonists
LTC_4	Leukotriene C_4
M	Molar
MAb	Monoclonal antibody
MARCKS	Myristoylated alanine-rich C kinase substrate
MC	Mast cell
µg	Microgram
mg	Milligram
Mg^{2+}	Magnesium
µl	Microlitre
ml	Millilitre
Min	Minutes
µm	Micrometre
mm	Millimetre
µM	Micromolar
mM	Millimolar
mRNA	Messenger RNA
mV	Millivolt
n	Number
Na^+	Sodium
NEAA	Non-essential amino acids
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NICE	National Institute for Health and Care Excellence
nM	Nanomolar
pA	Pico-amp
PAb	Polyclonal antibody
PAS	Periodic acid Schiff
PBS	Dulbecco's phosphate-buffered saline
PC_{20}	Provocative concentration of inhaled methacholine required to induce FEV_1 by 20%

PCR	Polymerase chain reaction
PEF	Peak expiratory flow
PGD_2	Prostaglandin D_2
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulfonylfluoride
qPCR	Quantitative real-time PCR
TSLP	Thymic stromal lymphopoietin
rh	Recombinant human
RPM	Revolutions per minute
SABA	Short-acting β_2 agonists
SCF	Stem cell factor
Sec	Seconds
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca2+ ATPase
SIGN	Scottish Intercollegiate Guidelines Network
SNARE	Soluble *N*-ethyl-maleimide-sensitive factor attachment protein receptor
SOC	Store-operated channel
SPDEF	SAM-pointed domain-containing Ets-like factor
STAT	Signal transducer and activator of transcription
STIM1	Stromal interaction molecule -1
TBS	Tris-buffered saline
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM-34	1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole
TRP	Transient receptor potential
V_{mem}	Membrane potential
VSMC	Vascular smooth muscle cell

Chapter 1

Introduction

1.1   *Asthma*

1.1.1   *The definition of asthma*

Asthma is a chronic respiratory disease affecting approximately 300 million people worldwide (Masoli et al. 2004). The prevalence of asthma is particularly high in Western societies and is a substantial burden on individuals and healthcare systems (Kay 2001). For approximately 10% of patients with asthma current therapies have poor efficacy. These patients represent a disproportionate total of healthcare costs and morbidities associated with asthma (Anon.2000).

Although there is no gold standard definition, asthma is described by the Global Initiative for Asthma (GINA) in the Global Strategy for Asthma Management and Prevention as a chronic disorder of the airways involving many different cells and cellular elements: in particular, mast cells, eosinophils, macrophages, neutrophils, T lymphocytes and epithelial cells (Bateman et al. 2008, British Thoracic Society et al. 2011, Expert Panel Report 1991, National Asthma Education and Prevention Program 2007). Chronic inflammation of the airways is associated with airway hyperresponsiveness (AHR), and this causes recurrent episodes of wheezing, chest tightness, breathlessness and coughing, particularly at night or in the early morning (Bateman et al. 2008). Episodes are often associated with widespread, variable airflow obstruction, which has the potential to revert either spontaneously or with treatment (Bateman et al. 2008).

AHR, airway inflammation and airway wall remodelling are characteristic features of asthma. A greater understanding of how these abnormalities interact to produce the clinical manifestations of asthma is required. Studies have recurrently reported that inflammation drives the physiological and pathological abnormalities in the airway wall, but there is increasing evidence to suggest that these aberrations occur independently (Siddiqui et al. 2008, Crimi et al. 1998, Southam et al. 2007). In fact, every cellular component of the airway wall is dysfunctional. The asthmatic airways display epithelial damage and an inability to heal, and there is an overproduction of growth factors, mucus and proinflammatory cytokines (Grainge et al. 2013, Ordonez et al. 2001, Holgate et al. 2009). Remodelling of the airway smooth muscle (ASM), involving hypertrophy and hyperplasia of ASM cells (Woodruff et al. 2004, Ebina et al. 1993), is often detected in the asthmatic airway

wall, and there is a plethora of evidence for the presence of activated inflammatory cells including mast cells, T cells, eosinophils and neutrophils (Brightling et al. 2002, Carroll et al. 2002a, Carroll et al. 2002b, Pavord et al. 2012). In addition, airway mucus hypersecretion is associated with goblet cell hyperplasia and submucosal gland hypertrophy; this is likely to have important clinical implications since chronic mucus hypersecretion is an indicator of FEV_1 decline in patients with asthma (Lange et al. 1998, Thomson et al. 2013, de Marco et al. 2006).

The extensive heterogeneity of asthma is evident in its clinical presentation. The presence and severity of abnormal airway components varies from patient to patient and results in a complex clinical appearance. According to National Institute for Health and Care Excellence (NICE) British guidelines on the management of asthma, the diagnosis of asthma is based on clinical observations. No standardised definition of the frequency, severity and types of symptoms exists. However, the guidelines report that the diagnosis of asthma is based upon the presence of more than one of the following symptoms: wheeze, breathlessness, cough, or chest tightness. The physiological symptoms of asthma are attributable to airflow obstruction, which can be detected and assessed by spirometry. Airflow obstruction is caused by contraction of hyperresponsive ASM, hypersecretion of viscid mucus, and airway wall oedema, and is targeted by current therapies.

1.1.2 *Current treatments for asthma*

The aims of pharmacological therapies for the treatment of asthma are to control the disease. Ultimately, treatments should reduce or eliminate daytime and night-time symptoms and exacerbations and improve lung function; ideally normal lung function should be restored. A stepwise approach to treatment is recommended by the British Thoracic Society (BTS) and Scottish Intercollegiate Guidelines Network (SIGN) guidelines (British Thoracic Society et al. 2011, British Thoracic Society et al. 2014). Each treatment is started according to the initial severity to achieve early control, and to maintain control this treatment is stepped up or down. Mild intermittent asthma (Step 1) is initially treated with inhaled short-acting β_2-agonists (SABA) as required, and can be stepped up (Step 2) by the addition of an

inhaled steroid for regular preventative therapy. Initial add-on therapies of long-acting β_2-agonists (LABA) are administered for increased control, and this can be combined with higher doses of inhaled steroids (Step 3). For persistent asthma exhibiting poor control (Step 4), the dose of inhaled steroids can be increased further with concomitant additional therapies, such as leukotriene receptor antagonists and supplementary LABA.

A substantial percentage of patients exhibit therapy-resistant severe asthma that responds poorly to treatment (Steps 4 and 5). The European Respiratory Society's (ERS) definition of severe asthma is poorly-controlled asthma with chronic symptoms, persistent and variable airway obstruction, episodic exacerbations and a necessity for SABA even with maximal doses of inhaled corticosteroids (Chung et al. 1999). For patients with severe asthma, reasonable control may require courses or regular doses of oral corticosteroids, typically prednisolone (British Thoracic Society et al. 2011, British Thoracic Society et al. 2014, Chung et al. 1999).

Treatments can be effective but relieve and control symptoms rather than preventing or curing the disease (Bateman et al. 2006, Milgrom et al. 2001, Pauwels et al. 1997). In consequence, novel approaches to treatment are urgently required.

1.1.2.1 *Glucocorticoids and bronchodilators*

The chronic inflammation of asthma is associated with AHR that causes episodes of wheezing, breathlessness, and airflow obstruction (Bateman et al. 2008). Inflammatory processes are also thought to contribute by interacting with other pathophysiological features, such as mucus hypersecretion and airway remodelling. Altogether these features can lead to significant reductions in airflow. Consequently, targeting the underlying airway inflammation, AHR, mucus secretion and remodelling should have clinical benefits for patients with asthma.

Glucocorticoids such as fluticasone, budesonide and beclomethasone target the chronic inflammatory component to improve pulmonary function via mechanisms of transactivation and transrepression (Barnes 1996). As inhaled therapies, the systemic presence of corticosteroids is minimised and inflammation within the

airway wall is targeted more directly. Currently, inhaled corticosteroids represent the most effective method of asthma control; they reduce exacerbations and symptoms, improve lung function, diminish eosinophilic inflammation and prevent death (Foresi et al. 2000, Suissa et al. 2000). *In vitro* studies have also suggested that glucocorticoids might be capable of exerting a direct effect on mucus production; dexamethasone suppresses baseline glycoconjugate production and expression of MUC5AC mRNA in the airway epithelial cell line H292 (Kai et al. 1996). However, most evidence demonstrates that glucocorticoids do not directly target features such as mucus hypersecretion, but improve the pathophysiology of asthma by limiting inflammation and defective lung function (Rogers 2005).

Despite the efficacy of glucocorticoids, which provide good control in low doses in many patients with asthma, a significant proportion of patients exhibit steroid-resistant severe asthma (Anon.2000, Moore et al. 2007). Resistance or insensitivity to the anti-inflammatory actions of glucocorticoids is indicated by the need for higher doses of glucocorticoids to achieve disease control, and in some cases regular oral doses of glucocorticoids are required. But, even in this instance, a small number of patients exhibit no clinical improvement following high doses of oral corticosteroids (Bousquet et al. 2010). This implies that not only is inflammation unlikely to be the sole driver of asthma, but also that the heterogeneity of asthma prevents broad-spectrum treatment of the disease with glucocorticoids. Asthma phenotypes are driven by a vast array of genetic and acquired factors, and these in turn are thought to dictate glucocorticoid sensitivity (Boardman et al. 2014). In addition, steroid-resistant patients differ from sensitive patients in clinical features such as their degree of AHR and longer duration of symptoms (Carmichael et al. 1981), and steroid resistance is associated with severe asthma (Barnes 2013). Consequently, glucocorticoid insensitivity represents a significant problem in the clinic and demonstrates that new targets for biological intervention must be identified, as well as a greater understanding of endotypes and phenotypes of asthma.

With the exception of glucocorticoid-insensitive patients, improved disease control can often be achieved by higher doses of glucocorticoids (British Thoracic Society et al. 2011, British Thoracic Society et al. 2014). However, higher doses are

associated with adverse side effects (Moore et al. 2007, Bumbacea et al. 2004, Chung et al. 2009). Reducing doses of inhaled corticosteroids to lessen the adverse effects is aided by concomitant treatment with bronchodilators such as β_2-agonists and theophylline (Pauwels et al. 1997, Greening et al. 1994, Evans et al. 1997, Lofdahl et al. 1999). Bronchodilators can be administered without corticosteroids as part of a step-down relief treatment; mild intermittent asthma responds to inhaled SABA (British Thoracic Society et al. 2011) such as salbutamol and terbutaline, which relieve bronchoconstriction by rapidly relaxing ASM (British Thoracic Society et al. 2011, British Thoracic Society et al. 2014, Baldwin et al. 1994, Nials et al. 1993, Finney et al. 1985).

LABA, such as formoterol and salmeterol, reduce exacerbations and improve symptoms by bronchodilation (Nials et al. 1993, Johnson 1995, Ullman et al. 1988, Pizzichini et al. 1996). B_2-adrenoceptor agonists do not directly reduce inflammation or mucus secretion but inhibit anti-IgE-induced ASM contraction in human airways (Johnson 1995, Ullman et al. 1988, Pizzichini et al. 1996, Gorenne et al. 1995). In comparison with SABA, LABA improve long-term control when used as add-on therapy to inhaled corticosteroids (Anderson et al. 1991, Newnham et al. 1993, Palmqvist et al. 1992, Wong et al. 1992). LABA are not used as a monotherapy as this may be harmful (Jonas et al. 2011).

Glucocorticoids exhibit efficacy in asthma and improve disease control, but are associated with adverse events and cannot resolve steroid-resistant severe asthma. Due to the inability of glucocorticoids and β_2-adrenoceptor agonists to specifically target features such as mucus hypersecretion, new approaches to therapies are necessary.

1.1.2.2 Alternative anti-inflammatory treatments for asthma

Non-steroidal therapies such as omalizumab and methotrexate have shown efficacy in asthma. Omalizumab, an antibody with specificity for free IgE, inhibits MC activation and the inflammatory cascade of moderate-to-severe allergic asthma (Zietkowski et al. 2010, Rodrigo et al. 2011, Djukanovic et al. 2004). Methotrexate, a folic acid antagonist, is used to reduce the use of oral corticosteroids (Domingo et

al. 2009, Marin 1997, Aaron et al. 1998), and is thought to act by reducing the recruitment of polymorphonuclear cells by LTB_4 and IL-1 (Calderon et al. 1991) and by reducing serum concentrations of immunoglobulins (Corrigan et al. 2005). However, responses to both methotrexate and omalizumab are variable and only suitable for a subset of asthmatic patients (Rodrigo et al. 2011, Walker et al. 2006, Polosa et al. 2008).

Agonists of toll-like receptors (TLRs) have been examined as suppressors of inflammation and could represent potential targets for therapies. For instance, TLR-7 agonists reduce T helper (Th) 1 and Th2 cytokine production and goblet cell hyperplasia in the rat lung (Camateros et al. 2007).

Treatments targeting proinflammatory cytokines have also been investigated. Anti-IL-5 therapy reduces eosinophil inflammation and limits airway remodelling, such as extracellular matrix deposition in the basement membrane (Nair et al. 2009, Desai et al. 2012, Flood-Page et al. 2003). Mepolizumab, a monoclonal antibody against IL-5, reduces the risk of exacerbations in patients with severe eosinophilic asthma (Pavord et al. 2012, Haldar et al. 2009), but appears unsuitable for non-eosinophilic asthma.

In other studies, anti-IL-9 antibody therapies reduce bronchoconstriction in patients with mild to moderate asthma (Parker et al. 2011, White et al. 2009). Targeting IL-33, which is expressed by bronchial epithelial cells and immune cells and is associated with airway mucus production, is also being examined (Smith 2010, Schmitz et al. 2005). Genome-wide association studies (GWAS) studies highlighted the potential of thymic stromal lymphopoietin (TSLP) as a therapeutic target (Hirota et al. 2011). Blocking TSLP signalling by immunoglobulins reduced airway inflammation, Th2 cytokine release and inflammatory cell airway infiltration in mouse models of asthma (Zhang et al. 2011), and anti-TSLP immunoglobulin treatment reduced allergen-induced bronchoconstriction and airway inflammation in humans (Gauvreau et al. 2014).

Anti-IL-4 and IL-13 therapies are in clinical studies and preclinical phase studies have indicated potential clinical efficacy for these treatments (Corren et al. 2010, Wenzel et al. 2007, De Boever et al. 2014, Hodsman et al. 2013). IL-4 and IL-13 are

useful targets for asthma treatments due to their roles in eosinophil accumulation and IgE synthesis (Yang et al. 2001, Nonaka et al. 1995, Robinson et al. 1993, Del Prete et al. 1988, Kasaian et al. 2013). Furthermore, IL-13 has a prominent role in mucus production, demonstrated by animal models of asthma (Kuperman et al. 2002, Webb et al. 2000, Grunig et al. 1998, Steenwinckel et al. 2007).

Another potential therapeutic target for mucus hypersecretion includes SAM-pointed domain-containing Ets-like factor (SPDEF). SPDEF is a downstream signalling molecule of the Notch pathway, and regulator of airway mucous cell metaplasia, goblet cell differentiation, and mucin gene expression (Chen et al. 2009, Maeda et al. 2011, Gras et al. 2013).

1.1.3   *The aetiology and pathogenesis of asthma*

The precise cause of asthma is unknown but it is thought to occur via an interaction between genetic and environmental factors. Several recognisable risk factors, such as genetic predisposition and a personal or family history of atopy or asthma, have been identified and are considered during asthma diagnosis (Rees 2010); (Townshend et al. 2007, Anderson 2005, Huss et al. 2001, Rotsides et al. 2010, Limb et al. 2005, Sears et al. 2003). While asthma is attributable to genetic susceptibility, heritability and atopy, individually these factors do not account for the development of the disease. Instead, they are thought to interact with environmental factors such as allergens (including pollens, fungi, house dust mite, and animal allergens), respiratory and parasitic infections, smoking, air pollution, obesity and chemicals (Huss et al. 2001, Bener et al. 2007, Salo et al. 2008, Gent et al. 2009). In this way, the environmental factors are thought to influence the susceptibility to and severity of asthma in predisposed individuals (Huss et al. 2001, Rotsides et al. 2010, Salo et al. 2008, Gent et al. 2009).

1.1.3.1 *Asthma has an inheritable component*

A family history of asthma and atopy significantly increases the risk of the development of asthma; heritability of asthma is estimated to be as high as 95% (Sporik et al. 1990, Marsh et al. 1981, Ober et al. 2011). Accordingly, asthma is

recognised to have an inheritable component. GWAS have identified susceptibility genes for asthma, and genetic loci have been associated between multiple allergic diseases. For example, chromosome 17q21 and polymorphisms of several genes, including IL-33 and SMAD3, are associated with asthma (Moffatt et al. 2007, Moffatt et al. 2010, Binia et al. 2011, Tulah et al. 2013). The asthma susceptibility genes are involved in a variety of roles, ranging from inflammatory cell function and activation to epithelial function and secretion. Particularly large numbers of genes, such as TSLP and IL-33, are associated with epithelial cell function by GWAS studies. This has contributed to the anticipated role of dysfunctional epithelium in asthma, accompanied by aberrant innate and adaptive immune responses (Holgate et al. 2009, Bonnelykke et al. 2014, Wan et al. 2012).

1.1.3.2 Allergen sensitisation and viral infections in the development of asthma

Viral infections and allergen sensitisation are risk factors for the development of asthma, particularly when they occur in combination, and when the infection is severe (Saglani 2013, Bardin et al. 1992). This does not occur in everyone. In an immature immune system viral infections can trigger the development of an asthma phenotype in genetically susceptible individuals (Dakhama et al. 2005, Jackson et al. 2012, Sigurs et al. 2010). As shown by murine models, viral infections may lead to the development of deviant adaptive responses, and subsequently predispose individuals to viral-induced asthma exacerbations (Kaiko et al. 2013, Krishnamoorthy et al. 2012).

Sensitisation to allergens can increase the risk of developing lower respiratory wheezing illness in response to viral infections (Saglani 2013, Jackson et al. 2012, Sigurs et al. 2010). This implies that allergen sensitisation also causes a predisposition to asthma.

Both allergen sensitisation and viral infection can increase the likelihood of developing asthma and may have a synergistic effect. For instance, a combination of virus infection and allergen exposure is associated with increased prevalence of allergic asthma, a greater exacerbation rate, and a persistence of the disease into

adulthood (Murray et al. 2004, Sigurs et al. 2010, Murray et al. 2006).
Furthermore, lower respiratory viral infections increase the risk of asthma, and
allergic sensitisation induces more severe viral-induced illness (Jackson et al.
2012, Sigurs et al. 2010).

1.1.3.3 Environmental triggers are risk factors for asthma

A wide range of environmental and occupational triggers are considered to
contribute to asthma aetiology, indicated by the variations in the temporal and
geographic prevalence of the disease (Marks 2006, Altzibar et al. 2014).
Environmental factors can induce aberrant reactions to common stimuli, thus
creating an asthmatic phenotype. However, such factors may also influence the
expression of genes associated with the asthmatic phenotype (Blumenthal 2005).
Importantly, only those who are susceptible to the disease develop asthma; the
presence of the trigger alone is not sufficient to induce asthma in everyone
exposed (Altzibar et al. 2014, Yucesoy et al. 2011). Consequently, preventing
exposure to potential triggers may reduce the risk of asthma (Merget et al. 2001,
Merget et al. 2000). In conclusion, an underlying genetic susceptibility interacts
with different environmental, allergen and viral stimuli, leading to the
development of asthma.

1.1.4 *Promoting the asthmatic condition in susceptible individuals*

Viral infections, allergen sensitisation and other environmental factors create a
predisposition to asthma, and various stimuli can interact with the asthmatic
condition in genetically susceptible individuals. These interactions lead to reduced
lung function and exacerbations, and aggravate the underlying airways disease.

Viral infections are common precipitants of severe asthma exacerbations (Bardin
et al. 1992, Phan et al. 2014, Murray et al. 2004, Tan 2005). In mice, rhinovirus
infection exacerbates house dust mite-induced lung disease (Phan et al. 2014). In
asthmatic patients there is a greater risk of developing a cold, and this culminates
in frequent asthmatic symptoms (Tan 2005). This is caused by viral damage to
airway mucosa which is already suffering from pre-existing abnormalities. Viral

infections not only initiate aberrant immune and inflammatory reactions, but in the asthmatic airways can induce the release of mediators already present in large quantities, cause IgE-driven cytokine release, or instigate mucous cell metaplasia and epithelial damage (Bardin et al. 1992, Kaiko et al. 2013, Satkunanathan et al. 2014, Xatzipsalti et al. 2008, Khan et al. 2008, Grayson et al. 2007). Inflammation is amplified and sustained for longer, causing airway obstruction and severe symptoms in predisposed individuals (Bardin et al. 1992, You et al. 2006). This involves epithelial cells and inflammatory cells such as granulocytes, dendritic cells and Th2 cells, and the secretion of cytokines and mediators (Satkunanathan et al. 2014, Khan et al. 2008, Bochkov et al. 2013).

In patients with asthma, environmental stimuli can initiate an acute decline in lung function, or cause a persistence of symptoms and a prolonged drop in peak expiratory flow (PEF). During such episodes, intervention with a course of oral corticosteroids may be required. In acute exacerbations, exposure to stimuli such as allergens, cold air, exercise, pollutants, stress or infections results in AHR and rapid bronchoconstriction (Murray et al. 2006, Strauss et al. 1977, Sandberg et al. 2004, Delfino 2002, Beasley et al. 1988). The bronchoconstrictor response of AHR leads to narrowing of the airways and reduced airflow, and requires bronchodilator therapy. Bronchoconstriction can be caused by IgE-dependent and -independent pathways depending on the stimuli. Airway inflammation can augment AHR and the susceptibility to bronchospasm (Frick et al. 1988, Eggleston 1988). Thus, airflow obstruction is affected by airway inflammation (Holgate et al. 1985, Cushley et al. 1985, Flint et al. 1985, Mehlhop et al. 1997).

1.1.5   *The pathophysiology of asthma*

The interaction of characteristic pathophysiological features affects the clinical manifestations and severity of the asthma, as well as how the disease responds to treatment. Distinguishing features of the asthmatic airway wall have been identified by post-mortem analysis of asthmatic subjects who died from an asthma attack (Ebina et al. 1993, Dunnill 1960, Messer et al. 1960), in bronchial biopsy specimens, and in *in vivo* studies of different severities of asthma (Kirby et al. 1987, Bradley et al. 1991). Airflow obstruction and resulting respiratory symptoms of

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asthma vary over time and in severity. For example, relatively symptom-free periods can be punctuated by exacerbations, or chronic daily symptoms can occur with or without intermittent exacerbations. This airflow obstruction is attributable to AHR, airway inflammation, wall remodelling, and mucus hypersecretion.

1.1.5.1 Airway hyperresponsiveness

Airflow obstruction is caused by AHR to stimuli that would not evoke a bronchoconstrictor response in healthy airways. AHR in response to challenges such as histamine, exercise or methacholine is a defining feature of asthma (Holgate et al. 2008, Burrows et al. 1989). However, AHR is associated with other conditions such as chronic rhinosinusitis rather than asthma alone (Turktas et al. 2002, Bucca et al. 1995). AHR is identified by an exaggerated response of the ASM to a broad range of stimuli, resulting in rapid bronchoconstriction. This results in a reduced cross-sectional area of the airway lumen, and leads to increased airflow resistance (Hartley et al. 1982).

AHR is mediated by structural changes of the airway wall, particularly those involving changes in ASM. For instance, asthmatic ASM have a hyperresponsive nature *in vitro*, and ASM mass is increased in patients with asthma, particularly in severe disease (Woodruff et al. 2004, Schellenberg et al. 1984, de Jongste et al. 1988, Carroll et al. 1993, Kuwano et al. 1993).

1.1.5.2 Airway wall remodelling

Airway remodelling encompasses structural changes of the airway wall components that may affect the airway's functional properties. For instance, subepithelial layer thickness is associated with asthma severity (Chetta et al. 1997). These changes are not fully reversible and contribute to airflow obstruction (Niimi et al. 2000). The presence and degree of remodelling can be detected clinically by measuring non-reversible airflow obstruction (FEV_1) following bronchodilator treatment (Chetta et al. 1997, Niimi et al. 2000, Lee et al. 2002). Airway inflammation and remodelling are not necessarily directly linked (Siddiqui

et al. 2008, Crimi et al. 1998, Southam et al. 2007, Kariyawasam et al. 2007), and it is not certain whether one precedes the other (Phipps et al. 2004).

Remodelling in the asthmatic airways comprises features such as ASM hypertrophy and hyperplasia (Ebina et al. 1993), increased vascularity (Carroll et al. 1997), basement membrane collagen deposition and fibrosis (Hoshino et al. 1999, Boulet et al. 1997), altered extracellular matrix composition (Yick et al. 2012, Slats et al. 2008) and dysfunction of the airway epithelium.

1.1.5.3 Airway inflammation

Airway inflammation is a major component of asthma and is thought to interact with AHR and airway remodelling to contribute. However, a substantial proportion of patients exhibit severe airways disease which responds poorly to glucocorticoid and other anti-inflammatory (Walker et al. 2006, Polosa et al. 2008). These findings suggest that inflammation is not the sole cause of asthma. Airway inflammation results from an imbalance between immune cells, the epithelium and the immune response to certain stimuli. A wide variety of effector cells contribute to airway dysfunction in asthma.

1.1.5.3.1 Mast cells

Mast cells are primary effector cells of the inflammatory response in asthma, and when activated release a plethora of proinflammatory mediators and cytokines, such as histamine, prostaglandin (PG) D_2, leukotriene (LT) C_4, amphiregulin (AREG), IL-5 and IL-13 (Bradding et al. 2006, Bradding et al. 1994, Wang et al. 2005). These mediators have a variety of roles in asthma, such as bronchoconstriction and vasodilation, and some contribute to AHR and remodelling (Bradding 2006, Weiss 1982, Lordan 2002, Murray 1986, Fuller 1986, Wong et al. 2002, Berger et al. 1999b, Sekizawa et al. 1989, Berger et al. 2001). Furthermore, cytokines such as IL-13 augment mucus synthesis and secretion (Webb et al. 2000, Grunig et al. 1998). Activation of mast cells most classically occurs via cross-linking of high affinity IgE receptors (FcεRI) by allergens, but also

via TLRs or by IL-33 (Nigo et al. 2006, Cruse et al. 2005, Ho et al. 2007, Allakhverdi et al. 2007).

Mast cells infiltrate mucous glands, and in asthmatic airways mast cell activation and degranulation are associated with increased mucus secretion and elevated mucin gene expression (**Figure 1-1**) (Carroll et al. 2002a, Carroll et al. 2002b, Okumura et al. 2005). Accordingly, mast cells within the airway mucosa contribute to obstruction of the airway lumen by mucus plugging (Carroll et al. 2002a, Carroll et al. 2002b).

Mast cell infiltration of ASM is also strongly associated with the asthmatic phenotype and helps distinguish between asthma and other respiratory conditions and healthy controls (Brightling et al. 2002, Robinson 2004, Brightling et al. 2003). Mast cell migration is mediated by the release of stem cell factor (SCF) by ASM, which promotes mast cell chemotaxis, differentiation, proliferation, maturation, activation and survival (Berger et al. 2003, Kassel et al. 1999, Ito et al. 2012, Tsai et al. 1991, Durand et al. 1994). Mast cell localisation within asthmatic ASM facilitates the ability of histamine, leukotrienes and IL-13 to induce bronchoconstriction and enhance ASM contractility (Befus 1987, Risse et al. 2011).
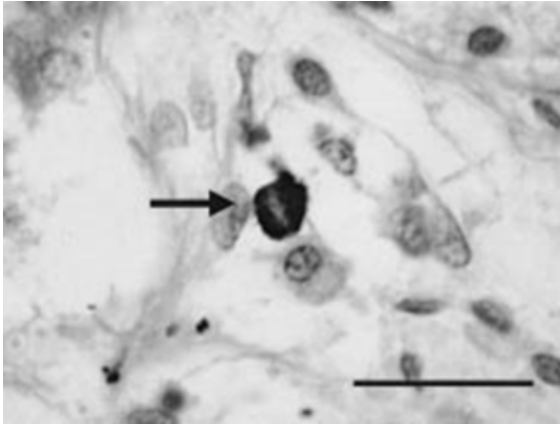
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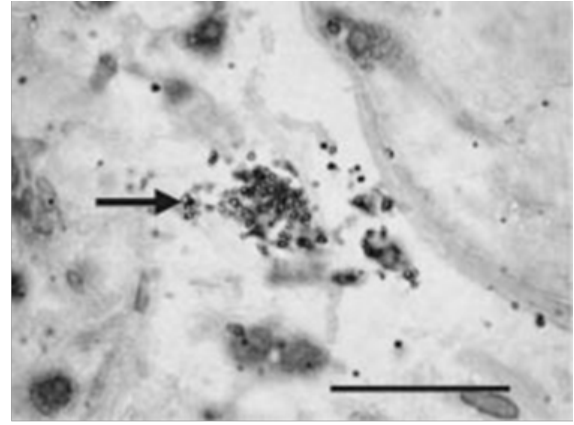
Figure 1-1: Mast cell degranulation contributes to mucus hypersecretion in asthma.

Photomicrographs of submucosal glands from (**A**) a healthy donor and (**B**) a case of fatal asthma. Gland specimens were stained with AA1 and demonstrate the presence of tryptase-positive mast cells. Mast cell degranulation is evident in the submucosal gland from a case of fatal asthma. Magnification x1000, bar = 50 μm. Images taken from (Carroll et al. 2002b).

1.1.5.3.2 Neutrophils

Neutrophils infiltrate airway mucous glands in patients with asthma (Carroll et al. 2002b); mucosal neutrophilia is a common feature of severe exacerbations in asthma (Qiu et al. 2007), and products such as neutrophil elastase stimulate mucus secretion (Jones et al. 2012, Fischer et al. 2002, Kohri et al. 2002, Voynow et al. 1999). Increased expression of IL-8, a neutrophil chemoattractant, contributes to elevated neutrophil numbers in the asthmatic airways, and is associated with loss of disease control (Maneechotesuwan et al. 2007). Airway neutrophils are not reduced by inhaled corticosteroids – studies suggest that corticosteroids prolong neutrophil survival and increase neutrophil numbers – and consequently higher number neutrophils in the airways and sputum are thought to have important clinical implications in asthma (Pallan et al. 2008, Cox 1995, Fukakusa et al. 2005).

1.1.5.3.3 Eosinophils

Eosinophils numbers are elevated during severe exacerbations of asthma (Qiu et al. 2007, Kupczyk et al. 2014, Lemiere et al. 2006), but eosinophils are not always present in the asthmatic airways; two sub-phenotypes of asthma are eosinophilic and non-eosinophilic. Consequently, eosinophils may not be a prerequisite for the clinical manifestations of the disease. Eosinophilic asthma is associated thickening of the basement membrane, atopy, IgE-mediated inflammation and corticosteroid responsiveness (Fukakusa et al. 2005, Cowan et al. 2010, Brown 1958, Kartasamita et al. 1994, Arbes et al. 2013, Balzar et al. 2007, Bettiol et al. 2002). Corticosteroid treatment of eosinophilic asthma improves FEV_1 and disease control (Cowan et al. 2010, James et al. 1995), and clinical efficacy is also seen with anti-IL-5 therapy in asthmatic patients with sputum eosinophilia (Pavord et al. 2012, Nair et al. 2009, Haldar et al. 2009).

1.1.5.3.4 Dendritic cells

Pulmonary dendritic cells (DCs) are antigen-presenting cells responsible for regulating innate and adaptive immune responses. In healthy airways, DCs directly recognise pathogen-associated molecular patterns expressed by inherent threats

and subsequently initiate appropriate T helper (Th) 1, Th2 or Th17 responses (Lambrecht et al. 2012, Vroman et al. 2014). In asthma, this effective method of protection can be initiated in response to harmless allergens, leading to an inappropriate Th2 response (Vroman et al. 2014, Hongjia et al. 2014). DCs can also be activated by epithelial cell cytokines; thus, inappropriate responses of the airway epithelium can lead to unnecessary activation of DCs (Hammad et al. 2008, Li et al. 2010).

1.1.5.3.5 Basophils

Basophils express cell surface FcεRI and synthesise and release histamine and Th2-type cytokines (Seder et al. 1991, Schroeder et al. 2001, Min et al. 2004). Despite similarities with mast cells, basophils are thought to have distinct roles in asthma, such as promoting Th2 differentiation, B cell proliferation, and antibody generation (Ho et al. 2007, Hida et al. 2005, Denzel et al. 2008).

1.1.5.3.6 Th17 cells

Th17 cells are CD4$^+$ cells that produce cytokines such as IL-17 and tumour necrosis factor (TNF)-α, and have been identified in the airways of patients with asthma (Miossec et al. 2009, Pene et al. 2008). Their cytokines are increased in asthmatic airways and sputum levels correlate with AHR (Barczyk et al. 2003, Molet et al. 2001). Th17 cells have been associated with steroid-resistance in asthma (Barczyk et al. 2003, Molet et al. 2001, Zijlstra et al. 2012) and contribute to mediating the Th2 response (Choi et al. 2012, Wakashin et al. 2008).

1.1.5.3.7 Th2 cytokines and asthma

Th2 cells are a subclass of CD4$^+$ helper T lymphocytes identifiable by their profile of secreted cytokines (including IL-4, -5, -6, -9 and -13). These T lymphocytes are required for humoral immunity and regulate the immune response to a variety of extracellular threats. An imbalance between Th1 and Th2 cytokine profiles is found in asthmatic airways, and approximately 50% of patients with asthma

exhibit a Th2-biased response to various stimuli (Umetsu et al. 1997, Woodruff et al. 2009). Consequently, innate immune response pathways leading to the release of Th2-type cytokines are major constituents of asthma (Ober et al. 2011).

However, Th2 cells are not solely responsible for the secretion of Th2-type cytokines. For instance, type 2 innate lymphoid cells (ILC2s) express IL-5 and IL-13 (Barlow et al. 2014); activated mast cells secrete cytokines such as IL-4, -5, -6 and -13 (Bradding et al. 1994, Toru et al. 1998); eosinophils express and release IL-4 and IL-5 (Nonaka et al. 1995, Moqbel et al. 1995); and basophils are a source of IL-4 and IL-13 (Schroeder et al. 2010, Schroeder et al. 1996).

The production of Th2-type cytokines by Th2 cells and ILC2s are considered central to the events surrounding the type 2 immune response, which underlies the development of allergic inflammation. The relatively recent identification of ILC2s as a source of Th2-type cytokines has led to the reassessment of the role of both Th2 cells and ILC2s in type 2-driven inflammatory diseases such as asthma (Neill et al. 2010). Unlike group 1 ILCs, which produce IFNγ, and group 3 ILCs, which are capable of producing cytokines such as IL-17, ILC2s (or group 2 ILCs) produce type 2 cytokines (Spits et al. 2013). In response to epithelial-derived signals such as IL-25, IL-33 and TSLP, ILC2s are activated and capable of driving type 2 inflammation (Kim et al. 2013, Mohapatra et al. 2015). In the type 2 inflammatory disease chronic rhinosinusitis, ILC2s expressing the chemoattractant receptor CRTH2 were present in polyps and responded to epithelial-derived cytokines, and thus implicated a prominent role for ILC2s in mucosal inflammation (Mjosberg et al. 2011, Shaw et al. 2013). The significant role of ILC2s in allergic lung inflammation has subsequently been demonstrated in mice; ILC2-deficient mice displayed reduced levels of IgE in the serum, lower levels of type 2 cytokines and chemokines in bronchoalveolar lavage fluid, and reduced Th2 cell recruitment to the lungs in response to papain challenge (Halim et al. 2014). Thus, in addition to Th2 cells, varieties of immune cells contribute to Th2-driven inflammation and are likely to play important roles in asthma.

The Th2-phenotype is clinically significant because this subset of patients respond to corticosteroids. In contrast, non-Th2-type asthma responds poorly to current treatments (Woodruff et al. 2009). Th2-driven asthma is associated with elevated

serum IgE levels and peripheral blood eosinophil counts, increased basement membrane thickness and larger quantities of epithelial mucin stores (Woodruff et al. 2009). Elevated serum IgE in Th2-driven asthma is thought to contribute to the development of atopy, a hypersensitive reaction to different allergens driven by Th2-type cytokines and deficient in a Th1 response (Bellanti 1998). Atopy influences a predisposition to allergic asthma and involves an unnecessary production of IgE antibodies due to the isotype-switching of B cells. This results in the activation of inflammatory cells such as mast cells (Holgate et al. 2008, Burrows et al. 1989).

The recruitment of inflammatory cells by Th2-type cytokines such as IL-9 and IL-13 plays a major role in increased mucus production in asthma (Robinson et al. 1993, Umetsu et al. 1997, Woodruff et al. 2009, Robinson et al. 1992). For instance, IL-9 upregulates Muc5ac mRNA and numbers of periodic acid-Schiff- and Alcian blue-positive cells in murine airways (Reader et al. 2003); IL-13 induces mucus production in mice (Kuperman et al. 2002); and animal models lacking active IL-13 display impaired mucus production (Webb et al. 2000, Grunig et al. 1998).

1.1.5.4   *The endotypes and phenotypes of asthma*

The complexity and heterogeneity of asthma make the therapeutic response highly variable. Despite the efficacy of current treatments in mild-moderate disease, severe asthma often responds poorly and requires higher doses of corticosteroids, and this is associated with adverse effects. Consequently, identifying therapeutic agents suitable for specific sub-groups of patients with asthma should provide improved, tailored control of the disease. Rather than administering treatments according to clinical measures alone, which can vary greatly, elucidating the underlying pathogenesis driving different phenotypes of asthma should provide more effective and targeted treatments. An essential route to this personalised treatment involves characterising different endotypes and phenotypes of asthma to ascertain which treatments will be most effective.

Linking asthma phenotypes with systemic heterogeneous features by analysing large numbers of highly characterised asthmatic patients has led to the

identification of several phenotypic groups. Asthma phenotypes form clusters, each of which exhibit certain prominent characteristics (Haldar et al. 2008, Moore et al. 2010). For example, Haldar et al. identified that a subgroup within the primary-care population with early-onset atopic asthma, airway dysfunction, eosinophilic airway inflammation and symptoms was associated with more frequent asthma exacerbations requiring corticosteroid intervention and previous hospital attendance. Subgroups within the refractory asthma population were also identified (Haldar et al. 2008): one cluster of refractory asthma displayed early-onset disease and symptoms with minimal eosinophilic inflammation, whereas another cluster was found to have eosinophilic inflammation but with few symptoms and late-onset disease. In this way, cluster analysis of asthma phenotypes is capable of separating phenotypically distinct groups of asthmatic patients, and may be useful in the future for targeted therapy, as demonstrated in trials of anti-IL-5 therapy (Haldar et al. 2009).

Cluster analysis has also supported the notion that asthma heterogeneity is driven by specific cellular and molecular mechanisms. If underlying mechanisms or endotypes, such as Th2-high asthma (Woodruff et al. 2009), were linked to discrete phenotypes, this could allow each group of patients to be treated with tailored therapies rather than a broad spectrum approach. The research group U-BIOPRED are currently investigating links between markers and mechanisms of inflammation with different asthma phenotypes, and analysis of airway gene expression in patients from the Severe Asthma Research Program (SARP) has identified key associations between biomarkers such as fractional exhaled nitric oxide (FeNO) and gene expression with specific asthma phenotypes. For example, a subject cluster with high FeNO was described as the "most severe" group and exhibited bronchoalveolar lavage eosinophilia, lower lung function, and higher expression of genes related to TNF signalling compared to healthy control subjects (Modena et al. 2014). In a study analysing endobronchial tissue gene expression in patients with varying severities of asthma, a patient cluster classed as Th2-high was found to be mutually exclusive to a Th17-high cluster (Choy et al. 2015). Since multiple mechanisms exist for each clinical characteristic, such as the complex role of airway inflammation in asthma and airflow obstruction, links between endotypes and phenotypes will likely provide a logical method of identifying which

molecular mechanisms are driving the disease, and which treatments are suitable for which subgroup of patients.

1.1.5.5 Mucus secretion

Asthmatic airways respond inappropriately to certain triggers, leading to not only AHR, but also mucus secretion. Unlike AHR, mucus hypersecretion cannot be reversed rapidly by treatment; excessive mucus secretions must first be removed from the airways before normal airflow can be restored (Daviskas et al. 2007, Daviskas et al. 2005). Of the various pathophysiological features of asthma, complete or partial occlusion of the airway lumen by mucus plugs is an invariable attribute of fatal cases of asthma (**Figure 1-2**) (Aikawa et al. 1992, Bullen 1952, Houston et al. 1953, Durward et al. 2000, Kuyper et al. 2003). However, mucus hypersecretion is also a characteristic feature of stable disease and in non-fatal asthma during exacerbations (Cutz et al. 1978, Wanner 1979, Fahy 2002).

Chronic mucus hypersecretion is an indicator of FEV_1 decline in patients with asthma, and is associated with disease severity (Lange et al. 1998, Thomson et al. 2013, de Marco et al. 2006). This association with severity suggests that excessive mucus production is likely to be associated with frequent exacerbations, inadequate control, and faulty repair mechanisms of the airway epithelium, and thus plays an important role in asthma pathophysiology. With thickening of the airway wall and AHR, mucus secretions contribute to airway narrowing and can eventually completely block the lumen (James et al. 1995).

Patients with severe asthma exhibit higher levels of mucus within their central and peripheral airways in comparison to healthy controls (Aikawa et al. 1992). However, goblet cell hyperplasia and abnormal mucin gene expression are also features of mild and moderate asthma (Ordonez et al. 2001). Even during asymptomatic periods the asthmatic bronchial epithelial mucosa displays goblet cell hyperplasia, mucus plugging, abnormal cilia, and degranulated mast cells (Cutz et al. 1978). Sputum production is characteristic in asthma and is elevated during acute exacerbations (Openshaw et al. 1989), but also in patients with stable disease (Cutz et al. 1978, Wanner 1979, Fahy 2002).

Particularly when combined with ASM contraction, mucus hypersecretion and airflow obstruction by intraluminal mucus makes asthma a dangerous disease due to its susceptibility for sudden exacerbations (Janssen et al. 2006, Fahy et al. 2010). As a result, a greater understanding of the mechanisms driving mucus hypersecretion is required for targeted therapeutic advances.
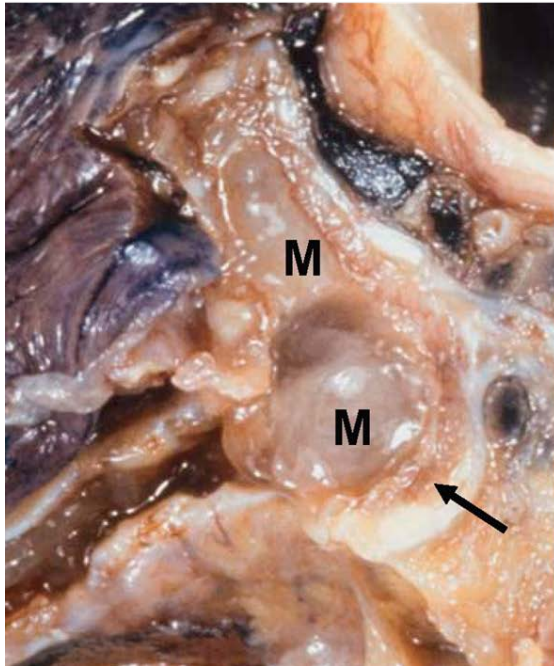
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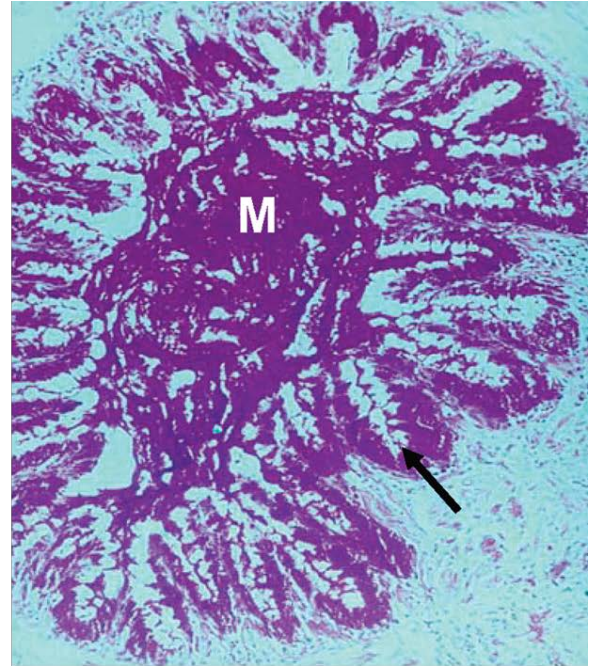
Figure 1-2: Mucus obstruction of the airways is a characteristic feature in asthma.

(**A**) Mucus plugs (M) completely block the airways of a patient who died of an acute severe asthma attack. Arrow points to the longitudinal section of an intrapulmonary bronchus. (**B**) Histological image of mucus occlusion and bronchoconstriction in a transverse section of an intrapulmonary airway of a patient who died of an acute severe asthma attack. Arrow indicates airway epithelium folds caused by smooth muscle contraction. Images taken from (Rogers 2007).

1.1.5.5.1 Mechanisms driving mucus hypersecretion in asthma

The precise mechanisms driving mucin hypersecretion in asthma are unclear, but a variety of cell types contribute to the hypersecretory phenotype. Mast cells and neutrophils are capable of infiltrating airway submucosal glands in asthmatic patients (**Figure 1-1**) (Carroll et al. 2002b). Mast cells, when activated, release proinflammatory molecules and mediators such as amphiregulin, serine proteases, IL-13, IL-4 and prostaglandins, which are associated with mucous cell secretion and mucus synthesis (Bradding et al. 2006, Baginski et al. 2006, Li et al. 2012, Kim et al. 2007, Stebbins et al. 2010). Similarly, neutrophil elastase has been associated with upregulated mucin expression (Fischer et al. 2002, Kohri et al. 2002, Voynow et al. 1999, Dwyer et al. 2000).

Remodelling of the airways also contributes to mucus hypersecretion. In particular, goblet cell hyperplasia and submucosal gland hypertrophy are associated with the hypersecretory phenotype of asthma; excessive mucus implicates an enhanced secretory capacity of the airway epithelium (Ordonez et al. 2001, Dunnill 1960, Aikawa et al. 1992). Consequently, an increase in the number of mucus-secreting cells contributes to mucus hypersecretion.

In addition, mucus hypersecretion is caused by the ability of secretory cells to secrete larger quantities of mucus (Aikawa et al. 1992, Dunnill et al. 1969, Fanta 1985, Busse et al. 2001, Sheehan et al. 1995). This is supported by findings of larger intracellular mucin stores in goblet cells isolated from asthmatic subjects compared to normal subjects (Ordonez et al. 2001). This could be driven by increased over-activation of the secretory mechanisms, or inappropriate stimulation. For instance, overexpression of IL-13 in animal models induces excessive airway mucus secretion and goblet cell hyperplasia, and higher levels of IL-13 are found in the lungs of asthmatic patients (Kuperman et al. 2002, Wills-Karp 2004, Zhu et al. 1999).

Finally, pathological mucus production in asthma is also associated with abnormal mucin gene expression. For example, analysis of relative mucin mRNA copy numbers in homogenates of endobronchial biopsies showed elevation of MUC5AC expression in asthma in comparison with healthy controls (Ordonez et al. 2001).

Further insight into these structural and functional changes is required to provide new therapeutic targets.

1.1.5.5.2 Insufficient therapies target mucus hypersecretion in asthma

Excessive mucus production is a hallmark of asthma, but therapeutically reducing this pathophysiological feature has not been achieved (Gras et al. 2013). Current therapies target airway mucus hypersecretion in other diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), as well as in asthma. Ideally, therapies aim to restore normal airflow and clear obstruction. Mucus hypersecretion is a common phenotype of these diseases, but the underlying pathophysiological mechanisms driving aberrant mucus secretion are not consistent across the different disease groups. As a result, existing therapies are not necessarily useful in asthma.

Glucocorticoids target underlying inflammation of asthma and improve lung function (**Chapter 1, 1.1.2**). This is thought to indirectly attenuate mucus hypersecretion by targeting responsible effector cells and their ability to release secretagogues such as IL-13 (Richards et al. 2000). However, studies report minimal efficacy of glucocorticoids on the hypersecretory phenotype in asthma. For instance, dexamethasone has no suppressive effect on IL-13-induced goblet cell hyperplasia in mice or *in vitro* cultures of human bronchial epithelial cells (HBECs) (Kibe et al. 2003, Kanoh et al. 2011). Similarly, inhaled beclomethasone dipropionate improves lung function but does not treat mucus hypersecretion in mild or moderate asthma (Fahy et al. 1998). Conflicting evidence has shown that glucocorticoids (dexamethasone, budesonide and fluticasone) inhibit TGF-α- and poly-IC-induced mucin production (Takami et al. 2012); consequently, the role of glucocorticoids in targeting mucus hypersecretion is unclear.

In CF and COPD, treatments such as mucolytics improve mucus clearance from the airways and reduce mucus stasis. Hypertonic saline aids mucus clearance in CF by improving airways hydration (Donaldson et al. 2006, Elkins et al. 2006), but is unsuitable for use in asthma because it causes bronchoconstriction in asthmatic patients (Anderson 2010). Concomitant administration of hypertonic saline with

amiloride has been examined in CF; amiloride is thought to sustain airway surface hydration by inhibiting Na^+ absorption by the epithelial sodium channel ENaC (Knowles et al. 1990). However, no therapeutic improvement was seen in clinical trials (Knowles et al. 1990). Dornase alfa, a recombinant human DNase I enzyme, promotes mucus clearance in CF by reducing mucus viscosity (Brandt et al. 1995, Hubbard et al. 1992). The efficacy of this treatment is due to high molecular weight extracellular DNA in mucus, which contributes to the abnormal viscoelasticity of CF mucus secretions (Brandt et al. 1995, Hubbard et al. 1992). However, mucus secretions of patients with asthma typically exhibit lower concentrations of DNA (Sheehan et al. 1995, Fahy et al. 1993, O'Donnell et al. 1998), so this treatment is unsuitable for asthmatic patients.

A summary of existing and potential therapies targeting airway mucus hypersecretion in asthma and other hypersecretory diseases are listed in **Table 1-1**. These treatments aim to decrease mucus production and improve mucus clearance by targeting the synthesis machinery and preventing release of mucins from secretory cells.

Therapy	Mechanism of action	Purpose	Stage in development
Glucocorticoids	Target inflammatory mediators that may induce/contribute to mucin accumulation, formation of mucus plugs, and goblet cell hyperplasia (Fahy et al. 2010, Evans et al. 2009, Innes et al. 2009, Rogers 2004).	Indirect decrease in mucus production	Available treatment
Inhaled mannitol	An osmotic agent - it improves mucus hydration, decreases mucus adherence to epithelium, and stimulates mucociliary and cough clearance (Daviskas et al. 2013). Improves mucus viscoelasticity and sputum rheology.	Improved mucus clearance	Efficacy in asthma, CF and bronchiectasis (Daviskas et al. 2007, Daviskas et al. 1999, Robinson et al. 1999, Daviskas et al. 2008, Daviskas et al. 2010, Bilton et al. 2013).
$\beta2$-adrenoceptor antagonists	Attenuates mucous cell metaplasia and mucin production (Nguyen et al. 2008). However, concomitant administration with β_2-agonists may be unsuitable.	Decreased mucus production	Asthmatic animal model studies suggest that this may have therapeutic potential (Nguyen et al. 2008).
BIBW 2948 BS, inhibitors of the epidermal growth factor receptor (EGFR)	EGFR activation is implicated in upregulation of mucin synthesis in airway epithelial cells (Okumura et al. 2005, Burgel et al. 2001, Casalino-Matsuda et al. 2006, Wang et al. 2014). BIBW 2948 BS	Decreased mucus production	No significant reduction in the size of airway epithelial mucin stores or levels of mucin gene expression reported in clinical trials (Woodruff et

	causes EGFR internalisation (Woodruff et al. 2010, Nadel et al. 2001).		al. 2010, Nadel et al. 2001).
MANS (peptide corresponding to N-terminal of myristoylated alanine-rich C kinase substrate; MARCKS)	MARCKS is required for mucin release from HBECs (Li et al. 2001, Park et al. 2008). MANS inhibits mucus release from goblet cells by inhibiting MARCKS binding to intracellular mucin granules (Li et al. 2001, Singer et al. 2004). However, recent studies suggest MANS inhibits the mucin binding assay rather than mucin secretion (Haddock et al. 2014).	Decreased mucus production	*In vitro* studies of HBECs and *in vivo* animal studies (Foster et al. 2010) indicate potential therapeutic use for this peptide, although this has been contradicted (Haddock et al. 2014). No clinical trial data published.
Azithromycin	The macrolide azithromycin inhibits TNF-α-induced MUC5AC secretion and mRNA expression in human nasal epithelial cells (Shimizu et al. 2012) and H292 cells (Poachanukoon et al. 2014)	Decreased mucus production	*In vitro* results suggest that azithromycin may be a useful therapy for mucus hypersecretion caused by allergic inflammation (Shimizu et al. 2012, Poachanukoon et al. 2014).
Heparin	Anticoagulant reduces TNF-α-induced MUC5AC secretion and mRNA expression from an airway epithelial cell line (Ogawa et al. 2011), and reduces mucus hypersecretion in the	Decreased mucus production	*In vitro* results from studies investigating MUC5AC secretion and expression suggest that heparin may be a useful therapy for

	nasal epithelium in rat models (Ogawa et al. 2012).		mucus hypersecretion.
N-acetylcysteine	Antioxidant thought to break down disulphide bridges that form between mucin monomers to form polymers (Fahy et al. 2010), and also to reduce oxidative stress which can contribute to excessive inflammation.	Promotion of mucus clearance, reduced mucus viscosity and reduced inflammation	No improvement in pulmonary function with high doses seen in clinical trials (Dauletbaev et al. 2009, Tirouvanziam et al. 2006).

Table 1-1: A summary of a selection of existing and potential therapeutic compounds for the treatment of airway mucus hypersecretion.

1.2   *The airway epithelium*

1.2.1   *The structure and function of airway epithelium*

The conducting airways form branches from the trachea to the alveoli that are covered with a continuous epithelial sheet. Inhaled air passes through the airways from the nasal and tracheal passages to the alveoli, where the large surface area facilitates gas exchange (Mercer et al. 1994). The airway epithelium is directly exposed to inhaled particles and is tightly regulated and renewed to maintain normal airway function (Crystal et al. 2008). The epithelium provides an essential protective barrier for the underlying airway structures and is capable of responding to different environmental and endogenous stimuli. As a result, the pathogenesis of lung disorders such as asthma can be significantly affected by airway epithelial cells.

1.2.1.1   *The basement membrane*

In large airways such as the bronchioles, epithelial cells form a pseudostratified structure resting on a basement membrane (**Figure 1-3**). The basement membrane consists of the upper lamina densa and lower lamina reticularis, formed by collagen, laminin, and fibronectin. Together, these structures facilitate adhesion of basal cells and polarisation of the epithelial cells (Paulsson 1992, Knight et al. 2003, Terranova et al. 1980). Basement membrane thickness is increased in asthma and is an indicator of steroid unresponsiveness and disease severity (Honkova et al. 2014, Bourdin et al. 2012). Consequently, reticular basement membrane measurements are a hallmark of airway remodelling in asthma (Bourdin et al. 2007, Shiba et al. 2002, Kasahara et al. 2002).

1.2.1.2   *Basal cells*

Above the basement membrane lie basal cells. These ubiquitous epithelial cells are non-ciliated and to attach to the basal lamina of the basement membrane via adhesion proteins (**Figure 1-3**) (Evans et al. 1989). Basal cells are ubiquitous within the airway epithelium, although smaller airways appear to contain lower numbers of these cells (Evans et al. 1988). Their multipotent differentiation

potential allows basal cells to behave like progenitor cells to facilitate repair mechanisms in response to epithelial injury (Knight et al. 2003, Hong et al. 2004). Characteristic features of the cells include expression of the cytokeratins 5 and 14. These proteins form the cytoskeleton of epithelial cells and are frequently used as a basal cell marker (Purkis et al. 1990). Basal cells express hemidesmosomes and desmosomes, which allow them to bind to both the basal lamina and the columnar epithelial cells (Evans et al. 1989). Thus, basal cells provide a point of attachment for columnar and other superficial epithelial cells with the basement membrane and are essential for the formation of the pseudostratified structure of the bronchial epithelium (Evans et al. 1989, Evans et al. 1990).

1.2.1.3   Tight junctions and epithelial damage

The bronchial epithelium forms a selectively impermeable obstacle. Regulated permeability permits the passage of certain ions and other molecules across the epithelium, and is facilitated by tight junctions. These junctions are constructed by adhesion proteins and receptors such as occludin, ZO-1, -2 and -3, claudins, E-cadherin, b-catenin and junctional adhesion molecule (JAM). Epithelial damage and disruption of the tight junctions allows inhaled pathogens and particles to infiltrate the airway tissue. Consequently, faulty permeability permits the capture of antigens by immune cells such as dendritic cells, leading to immune and inflammatory responses and tissue damage (Wan et al. 1999, Wan et al. 2001, Xiao et al. 2011).

In healthy airways, damaged epithelium can repair quickly. In contrast, chronic damage in asthmatic airways reduces the number of ciliated cells, exposes the basal cell layer, and promotes a mucus-secreting phenotype (Holgate et al. 2003, Holgate et al. 2000). Epithelial damage and abnormal repair mechanisms are implicated in adult and childhood asthma (Fedorov et al. 2005, Laitinen et al. 1985), and suggest that loss of the protective barrier and normal function of the epithelium contribute to asthma pathophysiology (Grainge et al. 2013).

1.2.1.4 Differentiated cells of the bronchial epithelium

The main differentiated cell types of the bronchial epithelium include ciliated, columnar, and secretory cells (**Figure 1-3**) (Fahy et al. 2010, Crystal et al. 2008). Ciliated cells express cilia that are approximately 7 μm long and aid clearance of mucus from the airways by beating, thus transporting mucus along the surface of the airways towards the throat (Widdicombe et al. 1995, Tarran et al. 2001, Rogers 1994). Ciliated epithelial cells originate from basal or secretory cells (Ayers et al. 1988) and are considered terminally differentiated cells; studies demonstrate that these cells do not proliferate or differentiate in response to epithelial injury (Rawlins et al. 2007). Ciliated epithelial cells express approximately 300 cilia per cell and are often identified by cellular markers such as β-Tubulin IV.

Secretory airway epithelial cells can be grouped into Clara, goblet, mucous and serous cells based on their microscopic appearances (Fahy et al. 2010). These cells play a role in the defence mechanisms of the lungs by constitutively releasing a variety of molecules to form a glycoprotein-rich mucus layer (Linden et al. 2008, Knowles et al. 2002). Mucus coats the airway lumen and acts as a protective barrier to guard the airways against foreign particles, damage and infection (Linden et al. 2008, Knowles et al. 2002).

The thickness of the mucus layer varies throughout the distal and proximal airways and is situated on top of an airway surface liquid (ASL) layer. Unlike the varying thicknesses of the mucus layer, ASL volume and height are tightly regulated by highly specific transport mechanisms, involving pulmonary epithelial ion transport processes (Widdicombe 2002, Hollenhorst et al. 2011). The ASL layer, also known as the periciliary layer, aids mucociliary clearance by hydrating the airway surfaces, preventing adherence of mucus to airway surfaces, and providing a low viscosity environment for cilia to beat in (Tarran et al. 2001, Mall et al. 2004, Matsui et al. 1998).
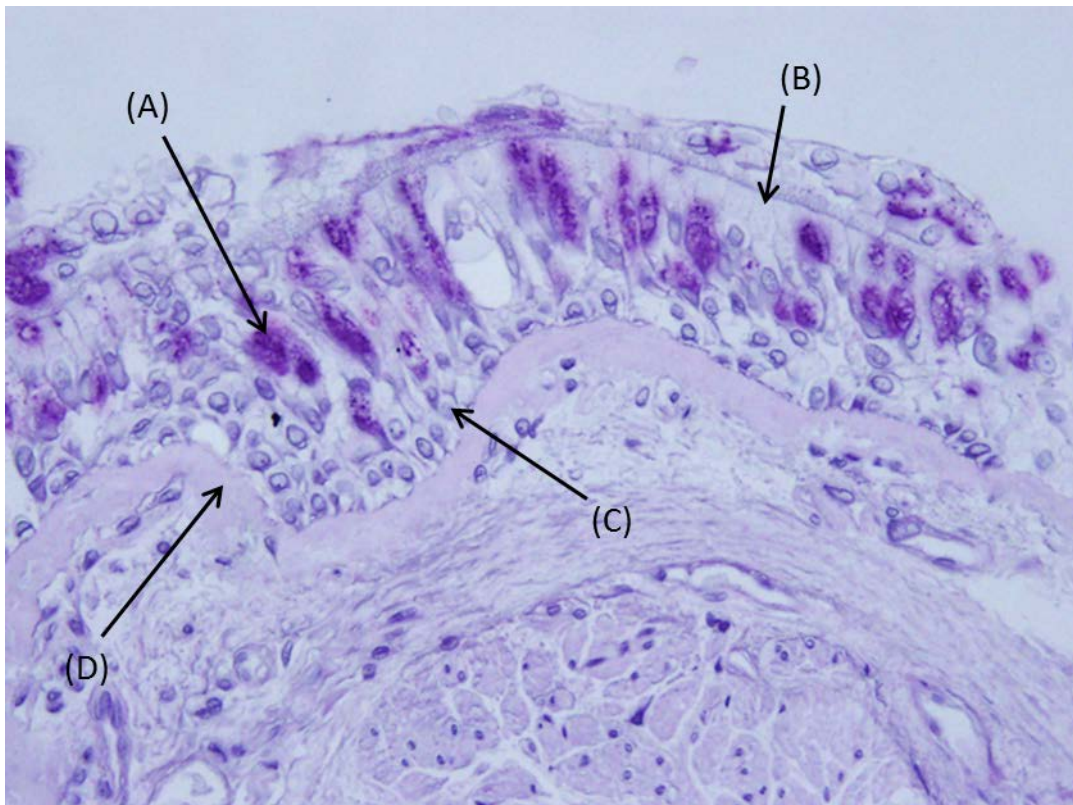
Figure 1-3: Structure of the human bronchial epithelium.

Human bronchial biopsy specimen stained with periodic acid-Schiff reagent (Sigma), counterstained with Mayer's haematoxylin (Sigma). (**A**) Mucin glycoproteins are visualised by their bright purple colour, and are representative markers of goblet cells. (**B**) Ciliated cells are present within the epithelial layer, and together with the goblet cells, are situated on top of a layer of basal cells. (**C**) Basal cells act as progenitor cells and adhere to the basal lamina of (**D**) the basement membrane.

1.2.2 Mucus-secreting cells of the airway epithelium

One of the major distinguishing features of the asthmatic airway epithelium in comparison to healthy epithelium is the large number of mucus-secreting cells. This is represented histologically by goblet cell hyperplasia and submucosal gland hypertrophy in asthmatic bronchial epithelium (Aikawa et al. 1992, Bullen 1952, Houston et al. 1953, Durward et al. 2000, Kuyper et al. 2003). Goblet cells and mucous cells of the glands maintain the mucus barrier by secreting mucins, high molecular weight glycoproteins which confer viscoelasticity to the mucus layer. This has been implicated by findings that intraluminal mucins and intracellular mucins of goblet cells within the airways of patients with asthma are comparable (Shimura et al. 1996, Hovenberg et al. 1996, Groneberg et al. 2002a). In this way, mucus-secreting cells provide protection for the airways.

1.2.2.1 Goblet cells

In the human lung, goblet cells are the principal secretory cell of the superficial epithelium that lines the upper airways, including the larger cartilaginous airways (Davis et al. 2008). Goblet cells represent a primary source of mucus, indicated by their intracellular mucin content (Jeffery 1983, Plopper et al. 1989). In contrast, submucosal glands in nasal, tracheal and bronchial epithelium consist of both mucous and serous cells. The mucous cells make up over 50% of the gland volume and are the main contributors to mucin secretion from the glands (Finkbeiner 1999), although store lower quantities of intracellular mucins than goblet cells (Fahy et al. 2010, Plopper et al. 1989). Serous cells make up approximately 40% of the gland volume but play a different role, thought to involve the release of antimicrobial proteins (Fahy et al. 2010, Joo et al. 2004), though there is limited data to directly support this.

Like other epithelial cells, goblet cells respond to a diverse range of stimuli, both exogenous (such as inhaled allergens and viruses) and endogenous (including proinflammatory mediators and cytokines) (Fallon et al. 2001, Mishra et al. 2008, Shim et al. 2001, Okamoto et al. 2007, Salmon et al. 1999, Dabbagh et al. 1999, Temann et al. 1997, Mukherjee et al. 2011). This enables the epithelium to react to different situations and maintain airway defence. However, inappropriate

responses can contribute to a pathological hypersecretory state of the airways in asthma.

Goblet cells respond to stimuli by secreting mucins, the principal solid components of healthy mucus. Excessive mucin production is associated with hypersecretory diseases, including asthma (Kirkham et al. 2002, Sheehan et al. 2000, Henke et al. 2007, Zuhdi Alimam et al. 2000, Young et al. 2007), and is driven by upregulated mucin secretion, gene expression, and synthesis, and goblet cell hyperplasia. Understanding these mechanisms will help identify novel methods for treating the hypersecretory phenotype. In particular, the molecular mechanism of mucin secretion is poorly understood.

A distinctive feature of goblet cell morphology is the presence of intracellular mucin granules (Perini et al. 1989, Raiford et al. 2011) which occupy more than 75% of the cell's cytoplasm (Evans et al. 2009). The granules are visualised histochemically using Alcian blue (AB) or Periodic Acid/Schiff (PAS) staining of airway tissue sections (**Figure 1-3**) (Fahy 2002, McGuckin et al. 2012). Mucins are detected by PAS when periodate oxidation of hydroxyl groups on the sugar residues of each mucin molecule yields aldehyde groups, which subsequently react with Schiff's reagent to produce a characteristic pink/purple colour (Dabbagh et al. 1999, Mantle et al. 1978, Grainge et al. 2011). Goblet cells are also identified by their positive-staining with antibodies targeting intracellular MUC5AC (Stewart et al. 2012).

Intracellular mucin stores are larger in goblet cells isolated from asthmatic subjects compared to normal subjects (Ordonez et al. 2001). Although this might be an indication of reduced mucin secretion by these cells, the hypersecretory nature of asthma and the continuity of luminal mucins with intracellular mucins of goblet cells suggest that goblet cell secretions contribute to mucus hypersecretion (Shimura et al. 1996). Mucin granules are thought to be regularly secreted by the cells to maintain the mucus layer. However, in hypersecretory disease mucin production exceeds the rate of baseline mucin secretion, resulting in greater intracellular accumulation and larger mucin granules (Zhu et al. 1999, Yasuo et al. 2006).

1.2.2.2 Goblet cell hyperplasia in asthma

Goblet cells are found throughout the surface epithelium in healthy airways. Due to their important role in mucin secretion, goblet cell numbers are maintained carefully. However, in asthma, the number of goblet cells increases beyond typically healthy levels, a feature known as goblet cell hyperplasia (Ordonez et al. 2001, Aikawa et al. 1992, Fahy 2002). This is a characteristic feature of the asthmatic airways and demonstrates that goblet cell dysregulation has important pathophysiological consequences (Ordonez et al. 2001, Aikawa et al. 1992, Fahy 2002). Goblet cell metaplasia is also a common occurrence in the asthmatic airway epithelium; metaplasia refers to the differentiation of epithelial cells into goblet cells in areas where goblet cells are normally present at low numbers or completely absent (Dabbagh et al. 1999, Rogers 2003). Larger numbers of goblet cells are thought to contribute to mucus hypersecretion by increasing the amount of secretory machinery, thereby enhancing the ability of the epithelium to synthesise and secrete mucus.

In vitro and animal models have provided insight into the mechanisms of goblet cell hyperplasia in asthma. Studies have shown intracellular Muc5ac expression, a marker of goblet cells, significantly increases in murine airways following challenge with aerosol antigens (Zuhdi Alimam et al. 2000, Yu et al. 2006). This suggests that Muc5ac upregulation in goblet cells is a central event in the development of airway mucus hypersecretion in asthma (Young et al. 2007).

Proinflammatory cytokines are strongly associated with goblet cell hyperplasia and subsequent mucus hypersecretion. For example, IL-13 is a potent inducer of goblet cell hyperplasia; IL-13 induces goblet cell hyperplasia within murine airways after 4 to 6 days (Kibe et al. 2003, Wills-Karp et al. 1998) and in *in vitro* models of the human airway epithelium after 9 to 14 days (Atherton et al. 2003, Booth et al. 2001). In addition, administering a neutralising anti-IL-13 protein prior to airway challenge with ovalbumin reduces the number of PAS-positive cells within murine airways (Wills-Karp et al. 1998). Studies have noted that treatment of *in vitro* cultures with 1 to 10 ng/ml IL-13, refreshed 3 times weekly, promoted goblet cell hyperplasia, whereas higher concentrations of IL-13 refreshed every 24 h induced a squamous phenotype (Atherton et al. 2003). IL-4 and IL-9 are also

capable of promoting goblet cell hyperplasia in mice following intratracheal inoculation (Wan et al. 2004, Perkins et al. 2006, Reader et al. 2003). Tumour necrosis factor (TNF)-α and IL-1β also contribute to goblet cell hyperplasia and mucus hypersecretion. Overexpression of IL-1β in the lung epithelium of transgenic mice resulted in elevated numbers of goblet cells (Lappalainen et al. 2005), and upregulation of epithelial growth factor receptor (EGFR) expression by TNF-α promotes goblet cell hyperplasia in *in vitro* cultures and in rats (Takeyama et al. 1999).

IL-4 and IL-13-driven goblet cell hyperplasia involves activation of IL-13 receptors and inhibition of the transcription factor forkhead box A2 (FOXA2), which suppresses MUC5AC gene transcription (Wan et al. 2004, Zhen et al. 2007). IL-13 and IL-4 pathways also require signal transducer and activator of transcription 6 (STAT6) and SPDEF activation, which subsequently facilitates the activation of gene transcription. SPDEF is a downstream signalling molecule of the Notch pathway, and modulates the transcriptional program for mucous cell metaplasia, goblet cell differentiation, and mucin gene expression (Chen et al. 2009, Maeda et al. 2011, Gras et al. 2013); *in vivo* expression of SPDEF induces goblet cell differentiation and is associated with increased expression of genes such as FOXA3, required for goblet cell differentiation (Chen et al. 2009). SPDEF expression is increased in murine goblet cells following allergen sensitisation. Furthermore, SPDEF is detected in the goblet cells of patients with chronic airways disease (Chen et al. 2009).

Histamine and EGFR ligands such as AREG and TGF-α also contribute to increased goblet cell numbers. For instance, treatment of *in vitro* airway epithelial ALI cultures or the airway epithelial cell line H292 with histamine or TGF-α induced goblet cell differentiation, identified by an increase in Alcian blue/PAS staining (Hirota et al. 2012, Takeyama et al. 2008). As well as increasing MUC5AC protein production in H292 cells, TGF-α is also thought to contribute to goblet cell hyperplasia by exerting an anti-apoptotic response via the EGFR signalling pathway (Takeyama et al. 2008). Chronic exposure to AREG induces goblet cell metaplasia in the absence of IL-13 in *in vitro* HBEC cultures (Enomoto et al. 2009). AR is elevated in the airways during acute asthma attacks (Enomoto et al. 2009).

Consequently, AREG may contribute to the pathophysiology of acute severe asthma and mucus hypersecretion, and also airway remodelling and mucous cell metaplasia in asthma.

The ability of cytokines and mediators such as IL-13, histamine and AREG to induce goblet cell hyperplasia implicate a role for mast cells in goblet cell hyperplasia. In mice, mast cells are essential for the development of goblet cell hyperplasia and elevated mucin gene expression (Yu et al. 2006); goblet cell hyperplasia is accompanied by increased numbers of airway mast cells, but goblet cell hyperplasia is absent in mast cell-deficient mice (Yu et al. 2006). This effect could be attributed to the ability of mast cells to release AREG and IL-13 (Bradding et al. 2006, Bradding et al. 1994, Wang et al. 2005), and also enhance lymphocyte recruitment and Th2 cytokine production in mice following ovalbumin challenge (Nakae et al. 2007).

1.2.3   *Models of the airway epithelium*

Mice are frequently used to investigate the functions and molecular mechanisms of the airway epithelium *in vivo* due to histological similarities between the tracheobronchial airways of adult mice with the conducting airways of the human lung (Rock et al. 2010). In both murine and human airways the epithelium is pseudostratified and contains similar proportions of differentiated cell types, such as ciliated, basal and goblet cells (Boers et al. 1998, Nakajima et al. 1998, Rock et al. 2009).

To study the dysfunction of airway epithelium in asthma, *in vitro* models of primary human bronchial epithelial cells (HBECs) and cell lines are also essential. Many studies utilise primary HBECs cultured as submerged, de-differentiated monolayers on plastic surfaces. However, air-liquid-interface (ALI) HBEC cultures provide a model with greater physiological relevance in terms of cell morphology and biological processes (Stewart et al. 2012, Fulcher et al. 2013, Kesimer et al. 2009).

1.2.3.1 Primary cell models of the airway epithelium

Primary HBECs are thought to provide a useful representation of the human airway epithelium by exhibiting similar properties to the epithelium *in vivo*. HBECs are isolated from fresh bronchial tissue and then grown in a phase of de-differentiation by submerging the cells with media. Once confluent, exposure to air in an ALI culture encourages cell differentiation and the development of a mucociliary phenotype. The cultures contain ciliated cells and goblet cells and develop a pseudostratified and polarised structure (Stewart et al. 2012, Fulcher et al. 2013, Kesimer et al. 2009). MUC5AC and MUC5B mucins secreted by ALI cultures are reportedly similar in density to mucins found in *in vivo* human respiratory mucus secretions (Kesimer et al. 2009, Thornton et al. 2000). In addition, ALI cultures exhibit higher expression of mucin mRNA in comparison to submerged, de-differentiated cultures (Bernacki et al. 1999). As a result, ALI cultures are preferable for studying mechanisms of mucin synthesis and secretion rather than de-differentiated cultures.

Studies have demonstrated that primary HBEC ALI cultures derived from HBECs isolated from endobronchial biopsy specimens retain inherent differences typical in different asthma severities (Bernacki et al. 1999). For instance, ALI cultures from severe asthma produced more mucin, released more IL-8 and less lipoxin A4 in comparison to cultures derived from mild asthma. This highlights the relevance of *in vitro* cultures in the study of the airway epithelium.

1.2.3.2 Cell line models of the airway epithelium

There are several limitations of primary HBEC ALI cultures. These include the limited life-span of the cell cultures and broad donor variability (Shen et al. 1994, Prytherch et al. 2011). In addition, primary HBEC culture is expensive and finite numbers of cells are isolated from primary tissue. Cell lines offer useful alternative models of airway epithelial cells.

The H292 cell line is a human mucoepidermoid pulmonary carcinoma cell line and is frequently used as a model of human bronchial epithelium (Okumura et al. 2005, Iwashita et al. 2010, Rose et al. 2000). Originally isolated from lung cancer, a

significant limitation of this cell line is that the cells demonstrate important phenotypic differences compared to normal and asthmatic HBECs. However, advantages of this cell line include a potentially unlimited supply of cells (Shen et al. 1994). In addition, submerged H292 cultures express and secrete mucins, and for this reason are a useful mucus-secreting epithelial cell model (Kai et al. 1996, Iwashita et al. 2010, Takeyama et al. 2000, Bautista et al. 2009).

1.3   *The mucus layer*

1.3.1   *Mucus acts as a physical barrier*

Mucus is a gel that lines the tracheobronchial tree and is a key component of innate defence against foreign particles (Rose et al. 2006, Jeffery et al. 1997). Mucus is secreted in response to environmental and endogenous triggers to provide a protective barrier for the underlying epithelium. In the airways, its protective functions include dilution of noxious gases and sequestering of inhaled particles and pathogens to aid their clearance from the lungs by ciliary transport and coughing (Knowles et al. 2002). This suggests that a key property of mucus is that it is not static, but can be moved along the airways towards the throat. In chronic airways diseases such as asthma, aberrant transport properties of mucus, particularly when combined with excessive production and secretion, can contribute to disease pathophysiology.

1.3.2   *Mucins are the principle solid components of mucus*

Normal mucus of healthy human airways consists of approximately 97% water with 3% solid components, which include mucins, non-mucin proteins, electrolytes, cell-derived mediators and cellular debris (Fahy et al. 2010, Rogers 2004). Among these components, water, electrolytes and mucins are secreted from specialised mucosal epithelial cells via tightly regulated mechanisms to form a complex gel-like structure. Mucins are the predominant solid components of healthy mucus and form large polymeric networks throughout the mucus gel (Davies et al. 2002). Together with their anionic water-binding properties, mucin

polymer networks provide structure to the gel (Sheehan et al. 2006, Thornton et al. 2008).

The secretion of water and electrolytes is tightly regulated to ensure optimum hydration of mucus and surface of the airways; for efficient mucus clearance, a layer of airway surface liquid (ASL) must be carefully maintained (Tarran et al. 2006, Gaillard et al. 2010b). This prevents mucus stasis within the lumen, allowing mucus to be removed via ciliary transport (Widdicombe 2002, Ballard et al. 2007, Boucher 2003). Ion transport systems of the airway epithelium maintain consistent ASL hydration is tightly regulated by ion transport systems of the airway epithelium and mucosal glands. Ion channels such as the cystic fibrosis transmembrane conductance regulator (CFTR) and the airway epithelial sodium channel (ENaC) modulate transepithelial Cl^- secretion and Na^+ absorption, thus facilitating the movement of water and maintaining airway hydration (Gaillard et al. 2010b, Boucher 2003, Gaillard et al. 2010a, Toczylowska-Maminska et al. 2011). The effect of airway surface dehydration is seen in CF and bronchiectasis (Daviskas et al. 2007, Daviskas et al. 1999, Robinson et al. 1999, Daviskas et al. 2008, Daviskas et al. 2010, Bilton et al. 2013). In these diseases, mucus clearance can be restored by treatments such as inhaled mannitol and hypertonic saline, which improve mucus hydration and stimulate mucociliary clearance (Donaldson et al. 2006, Elkins et al. 2006, Daviskas et al. 2007, Daviskas et al. 1999).

1.3.3 *Abnormal mucin production in asthma*

As the principal solid components of mucus, mucins affect the composition and biophysical properties of mucus within the airways. Consequently, abnormal mucin production could contribute to the pathophysiology of asthma. This is supported by findings of elevated mucins in asthmatic sputum compared to normal mucus samples (Kirkham et al. 2002, Jinnai et al. 2010). In addition, larger quantities of mucins are detected in the airway epithelium in asthma and following allergen challenge, compared with healthy epithelium (Ordonez et al. 2001, Aikawa et al. 1992, Shimura et al. 1996, Hays et al. 2001). Augmented mucin secretion and mucin gene expression are also associated with increased sputum production in asthma (Ordonez et al. 2001). Abnormal expression, storage and synthesis of

mucins, in addition to goblet cell hyperplasia, are likely to contribute to the hypersecretory phenotype of asthma (Ordonez et al. 2001, Jinnai et al. 2010). Improved understanding of mucin expression, synthesis and secretion will provide an opportunity to identify useful targets for novel therapeutics against mucus hypersecretion.

1.4   *Respiratory mucins*

1.4.1   *Structure and function of respiratory mucins*

Mucins are large, high molecular weight glycoproteins consisting of a mucin polypeptide backbone. This backbone is rich in serine, proline and threonine amino acid residues and is often referred to as the mucin domain (Shankar et al. 1994). A large number of tandem repeats are present in the mucin domain, which helps distinguish mucins from other glycoproteins (Shankar et al. 1994, Meezaman et al. 1994). Mucin domains can be detected by specific antibodies, allowing identification of mucin subtypes within unknown samples (Hovenberg et al. 1996, Thornton et al. 1996b, Podolsky et al. 1986).

A variety of oligosaccharides bind to the mucin domain via a linkage sugar, *N*-acetylgalactosamine (GalNAc; **Figure 1-4**) (Fahy et al. 2010, Thornton et al. 2008, Brockhausen et al. 1983, Brockhausen et al. 1985), creating a large heavily glycosylated macromolecular structure (Thornton et al. 2008, Podolsky 1985). The diversity of oligosaccharides, differing in structure, creates a heterogeneous molecule (**Figure 1-4**) (Podolsky 1985, Schulz et al. 2002), with structural variations of mucins found in different tissues and species (Thornton et al. 2008, Nordman et al. 1998, Thornton et al. 1999, Nordman et al. 2002). Many of the oligosaccharides are sialylated or sulfated. This makes the mucin highly anionic and capable of ion and water binding (Podolsky 1985, Schulz et al. 2002, Goso et al. 1990), and allows mucins to affect the hydration of the mucus layer (Fahy et al. 2010, Thornton et al. 2008). Adjacent anionic oligosaccharides are thought to repel each other, causing the glycosylated mucin to stiffen and remain as an expanded structure that fills space (Shogren et al. 1989, Gerken 1993). This is thought to

contribute to gel-like properties of mucus (Thornton et al. 2008, Shogren et al. 1989, Jentoft 1990, McMaster et al. 1999).

The glycosylation of mucins are important in bacterial sequestration, and thus contribute to the defence mechanisms of the mucus layer (Sajjan et al. 1992, Linden et al. 2002). For example, oligosaccharides of gastric epithelial mucins bind to bacteria such as *Helicobacter pylori* (Linden et al. 2002), and airway epithelial mucins are thought to adhere to *Pseudomonas aeruginosa*. However, this defence mechanism may facilitate disease process such as bacterial colonisation of airway mucus (Delmotte et al. 2002, Thomsson et al. 1998).
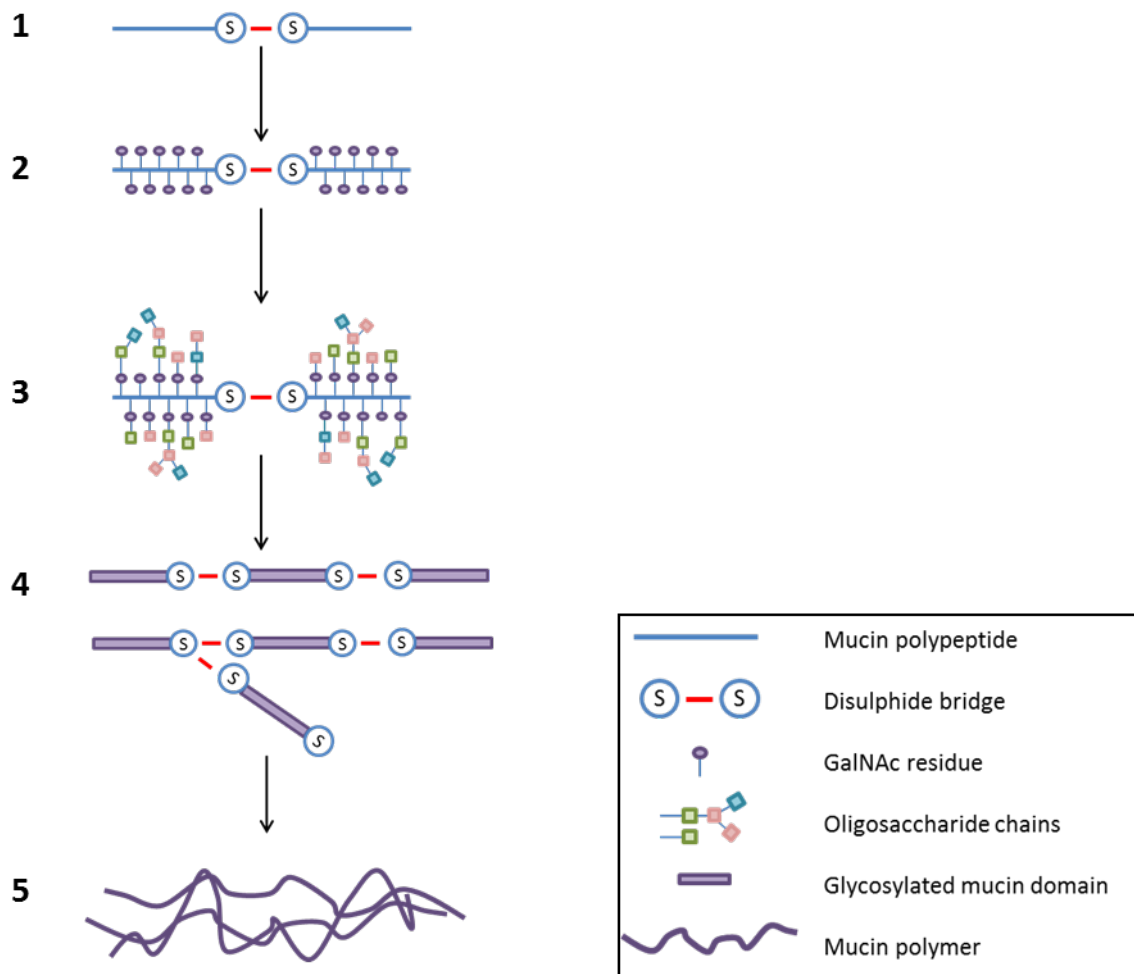
Figure 1-4: The assembly pathway of secreted airway mucin polymers.

(**1**) In the endoplasmic reticulum, mucin dimers are formed by creating disulphide bonds between mucin domain monomers. (**2**) Once transferred to the Golgi, GalNAc is added to amino acid residues of the mucin domain. (**3**) A variety of oligosaccharides are added to GalNAc, thus creating a heavily glycosylated molecule. (**4**) Mucin dimers form long polymers within the Golgi or specialised secretory granule via disulphide bonds. (**5**) Once secreted, large mucin polymers form networks throughout the mucus gel. Diagram based on (Thornton et al. 2008).

1.4.1.1 MUC5AC and MUC5B are the predominant secreted respiratory mucins and are elevated in asthma

Mature mucins can be secreted by the epithelium or remain tethered to the airway surface (Evans et al. 2009, Thornton et al. 2008, Gum et al. 1994, Hattrup et al. 2008). Cell surface membrane-tethered mucins, such as MUC1 and MUC4, play important roles in innate immunity and aid hydration of the airway surfaces due to their water-binding properties (Sheehan et al. 2006, Hattrup et al. 2008). These mucins have a large extracellular domain within the airway lumen and a membrane-spanning region and cytoplasmic tail (Sheehan et al. 2006, Hattrup et al. 2008).

In contrast, secreted mucins do not remain attached to the cells and are released into the mucus layer (Sheehan et al. 2006, Hattrup et al. 2008). The predominant secreted mucin glycoproteins in the airways are the products of MUC5AC and MUC5B genes, consistently detected in mucus and sputum (Thornton et al. 2000, Thornton et al. 1997, Thornton et al. 1996a). The quantities of mucin subtypes vary between healthy and diseased airways. For instance, MUC5AC and MUC5B mucins are present in larger quantities in asthmatic sputum and during exacerbations compared to normal healthy mucus (Kirkham et al. 2002, Henke et al. 2007). In addition, healthy mucus is predominated by the mucin MUC5AC, whereas mucus of CF and COPD contains higher quantities of MUC5B (Kirkham et al. 2002). Variations in mucin content influence the biophysical properties of the mucus gel, and subsequently contribute to the disease pathophysiology (Thornton et al. 2008). In mouse models of asthma, murine Muc5ac is a marker of goblet cell hyperplasia (Zuhdi Alimam et al. 2000, Yu et al. 2006). Challenging mice with aerosol antigens induces a significant increase in Muc5ac expression in the airways, and indicates that Muc5ac expression is a major event in the development of mucus hypersecretion in allergic mice (Young et al. 2007). Consequently, MUC5AC is an attractive target for investigating mechanisms of mucus hypersecretion in asthma.

1.4.2   *Mucin gene structure and expression*

The central mucin domain is encoded by genes named with the symbol MUC,
followed by a number (Voynow et al. 2009). Human mucin-encoding genes such as
MUC5AC, MUC5B and MUC2 are located in a gene complex on chromosome
11p15.5 (Pigny et al. 1996). The constant baseline expression of these genes
supports the innate role of both MUC5AC and MUC5B mucins in the human lung
(Pigny et al. 1996). Within the airways, MUC5AC is mainly expressed in surface
airway epithelial goblet cells, whereas MUC5B is primarily expressed by mucous
cells of submucosal glands (Hovenberg et al. 1996, Wickstrom et al. 1998, Buisine
et al. 1999, Groneberg et al. 2002b).

MUC5AC and MUC5B genes consist of a large exon encoding the main mucin
domain, the translated polypeptide of which is rich in serine, proline and threonine
residues (Escande et al. 2001, Desseyn et al. 1997). Scattered throughout this exon
are regions encoding cysteine residues comparable to the blood glycoprotein von
Willebrand Factor (vWF) (Gum et al. 1994, Desseyn et al. 1998). As shown by
studies of vWF, cysteine-rich domains are required for disulfide-mediated
polymerisation of the glycoproteins (Thornton et al. 2008, Gum et al. 1994,
Escande et al. 2001, Desseyn et al. 1998, Gum et al. 1992). Hence, cysteine residues
of MUC genes are considered important for the formation of the large mucin
polymers in mucus by constructing disulphide bonds between mucin monomers
(Escande et al. 2001, Desseyn et al. 1997, Desseyn et al. 1998, Desseyn et al. 1997).

1.4.2.1 *Mucin gene abnormalities in asthma*

The well-characterised role of mucus hypersecretion in asthma has led to
investigations into whether mucin gene expression is abnormal in asthma, and
whether this would provide a therapeutic target for asthma. Ordonez CL et al.
found that the most frequently expressed MUC gene in homogenates of bronchial
biopsy specimens obtained from patients with asthma and healthy controls was
MUC5AC, with a trend in elevated MUC5AC mRNA in asthmatic biopsies in
comparison to healthy biopsies (Ordonez et al. 2001). This finding was consistent
with increased immunohistochemical expression of MUC5AC in asthmatic

bronchial epithelium (Ordonez et al. 2001) and implicates a role for abnormal mucin gene expression in asthma pathogenesis.

1.4.2.1.1 Upregulation of mucin gene expression

Mucin gene expression in airway epithelial cells can be upregulated *in vitro* and *in vivo* by a variety of stimulants such as inflammatory cytokines and mediators (e.g. IL-13, IL-6, IL-9, TNF-α, IL-8, IL-4 and AREG), proteases (e.g. neutrophil elastase), bacterial products (e.g. lipopolysaccharide), irritant gases (e.g. cigarette smoke), and viral mediators (e.g. rhinovirus) (Okumura et al. 2005, Kim et al. 2007, Dabbagh et al. 1999, Bautista et al. 2009, Di et al. 2012, Takeyama et al. 2001, Tanabe et al. 2008, Lora et al. 2005). Inflammatory cytokines modulate mucin gene expression by activating specific cell surface receptors and transcription factors, and their role has been extensively studied because of their predominant expression in asthma.

One of the most extensively studied profiles of asthma is the Th2 phenotype. Th2 cytokines such as IL-4, IL-9 and IL-13 stimulate mucin production in *in vitro* and animal models of asthma (Kuperman et al. 2002, Webb et al. 2000, Grunig et al. 1998, Steenwinckel et al. 2007, Reader et al. 2003, Dabbagh et al. 1999, Temann et al. 1997, Zhen et al. 2007, Tanabe et al. 2008, Kuperman et al. 1998). In particular, IL-4 and IL-13 exert direct control over mucin gene expression. For instance, IL-4 upregulates MUC5 gene expression *in vitro* and *in vivo*, and is accompanied by increased Alcian blue/PAS mucin staining (Dabbagh et al. 1999). In addition, IL-13 increases expression of MUC5AC mRNA in primary HBEC cultures and leads to elevated MUC5AC protein synthesis (Kuperman et al. 2002, Zhen et al. 2007). A study examining the mechanism of IL-13 induced MUC5AC gene expression *in vitro* found that IL-13 stimulates MUC5AC promoter-driven luciferase reporter activity (Kuperman et al. 2002, Zhen et al. 2007), indicating that IL-13 directly activates MUC5AC gene transcription. However, IL-13-induced upregulation of MUC5AC expression is also thought to occur via indirect mechanisms such as activation of STAT6, which suppresses the transcription factor FOXA2 (Zhen et al. 2007). IL-4 is thought to act via a similar mechanism (Wan et al. 2004, Kuperman et al. 1998).

EGFR activation also regulates MUC5AC gene expression via inhibition of FOXA2 (Zhen et al. 2007), among other mechanisms. EGF, TGF-α and AREG upregulate mucin gene transcription by acting as ligands for EGFR (Okumura et al. 2005, Baginski et al. 2006, Casalino-Matsuda et al. 2006, Shao et al. 2003). For example, AREG released from FcεRI-activated human lung mast cells increases mucin gene expression in H292 cells (Okumura et al. 2005). Downstream steps of EGFR pathways are not fully understood, but involve activation of protein kinase C (Yuan-Chen Wu et al. 2007), extracellular signal-related kinases 1/2 (ERK1/2), phosphatidylinositol 3-kinase (PI3K) and Akt kinase (Yang et al. 2011, Li et al. 2012, Lee et al. 2010).

EGFR pathways ultimately lead to the activation of transcription factors including nuclear factor (NF)-κB, cyclic adenosine monophosphate response element binding protein (CREB), activator protein (AP)-1, and specificity protein (SP)-1 (Voynow et al. 2009). NF-κB plays a central role in the development of inflammation in asthma by increasing the expression of genes encoding proinflammatory cytokines such as IL-6, IL-8 and TNF-α, but also induces MUC5AC gene transcription (Yang et al. 2011, Lixuan et al. 2010). When inactive, NF-κB is stored in the cell cytoplasm with inhibitory proteins which prevent the binding of NF-κB to DNA. Activation of NF-κB involves phosphorylation of the inhibitory proteins, which permits the formation of a heterodimer between NF-κB and another transcription factor, p50. This allows NF-κB to translocate to the nucleus where it binds to DNA and initiates transcription (Kida et al. 2005, Nie et al. 2012).

1.4.3   *Synthesis and storage of respiratory mucins*

The synthesis of mucin macromolecules is a tightly regulated process involving multiple steps, from translation of the mucin domain to disulphide-linked polymerisation of mucin monomers. Initially, mucin mRNA is translated into the mucin domain on the endoplasmic reticulum (ER) (Rogers 2007, Davis et al. 2008, Sheehan et al. 2004). Once formed, the large mucin domains form flexible compact coils, making them easier to move through the synthesis process (Perez-Vilar 2007). Mucin domains form dimers via disulphide bonds (**Figure 1-4**) (Sheehan et

al. 2004, Perez-Vilar et al. 1999, Bell et al. 2001), and are then transferred from the ER to the Golgi for glycosylation.

Glycosylation involves the attachment of the linkage sugar GalNAc to the serine and threonine residues of the mucin domain, catalysed by *N*-acetylgalactosaminyl peptidyltransferase (Fahy et al. 2010, Rose et al. 2006, Thornton et al. 2008, Brockhausen et al. 1983, Brockhausen et al. 1985, Sheehan et al. 2004). Next, oligosaccharide chains are added to GalNAc residues by glycosyltransferases (Hang et al. 2005). Glycosylation is tightly controlled to create precise conformations of final glycosylated mucins (Gerken et al. 2006, de Graffenried et al. 2004). Once glycosylation is completed, mucin dimers undergo polymerisation (**Figure 1-4**) (Thornton et al. 2008, Perez-Vilar et al. 1999).

Final mucin polymers are characteristically large and approximately 40 MDa in size (Sheehan et al. 2000, Thornton et al. 1990). Each polymer is anionic due to the presence of sialic acid or sulfate groups at the terminus of their own oligosaccharides (Fahy et al. 2010, Rose et al. 2006, Thornton et al. 2008). The large size and anionic charge of mucins creates storage problems for the cell's cytoplasm. To solve this, mucins are packaged into specialised storage granules containing Ca^{2+} which "shield" the negative charges (Thornton et al. 2008, Verdugo et al. 1987, Baconnais et al. 2005, Paz et al. 2003).

Mucin granules occupy a significant quantity of the cytoplasm of goblet cells (Evans et al. 2009). These granules can be visualised either histochemically or by electron microscopy (Fahy 2002, Evans et al. 2009, Perini et al. 1989, Raiford et al. 2011, McGuckin et al. 2012). The storage of mucins within granules provides a stock of mucins ready for release. This allows secretory cells to respond to stimuli quickly rather than waiting for mucin synthesis to be completed first. As a result, mucus-secreting cells such as goblet cells can rapidly release mucins from their preformed stores once a trigger is received.

1.4.3.1 *Increased mucin synthesis in asthma*

Mechanisms of mucin synthesis are carefully regulated to ensure mucin production does not exceed mucin secretion, and a healthy mucus layer is

maintained. In pathological situations, goblet cell hypertrophy and hyperplasia contribute to the excessive secretion of mucins into the airway lumen by enhancing the secretory capacity of the airways. This has been reported by Ordonez CL, et al., who found that the airway epithelium in mild and moderate asthma contains larger quantities of stored mucin in comparison with healthy epithelium (Ordonez et al. 2001). MUC5AC and MUC5B mucins are present in lower quantities in normal healthy mucus, and are elevated in asthmatic sputum and during exacerbations (Kirkham et al. 2002). Accordingly, increased mucin synthesis may enhance the secretory capacity of the epithelium in asthma.

Increased mucin production is thought to be regulated by factors that are also capable of controlling mucin gene expression; mucin production is likely to be a downstream consequence of mucin gene transcription. Increased mucin production may also be a consequence of elevated goblet cell numbers. For instance, reduced Foxa2 expression in mice is associated with goblet cell hyperplasia and increased mucin staining within the epithelium (Wan et al. 2004). Similarly, Spdef transcription factor expression is associated with increased mucin content in the airway epithelium (Park et al. 2007) and goblet cell hyperplasia in mice, identified by immunohistochemistry (Chen et al. 2009, Park et al. 2007). EGFR agonists increase mucin production by activating mitogen-activated protein kinase (MAPK) (Tadaki et al. 2009), a downstream effect of EGFR stimulation, and pathways involving activation of protein kinase C (Yuan-Chen Wu et al. 2007, Semlali et al. 2008, Heijink et al. 2007), extracellular signal-related kinases 1/2 (ERK1/2), and phosphatidylinositol 3-kinase (PI3K) and Akt kinase (Yang et al. 2011, Li et al. 2012, Lee et al. 2010). Similarly to gene expression, allergen-induced mucus production is thought to involve IL-13; blockade of IL-13 by a soluble anti-IL-13 fusion protein prevented ovalbumin-induced in mucin expression in murine airways (Wills-Karp et al. 1998). IL-4 is also implicated in allergen-induced mucus expression (Temann et al. 1997, Rankin et al. 1996). However, Th2 cells isolated from IL-4-deficient mice still induce mucus over-expression (Cohn et al. 1997), so IL-4 may have more of a regulatory role than an effector role in mucin production.

1.4.3.2 *Notch pathways are involved in the development of a mucous phenotype*

Basal epithelial cells act as resident tissue stem cells capable of differentiating into specialised airway luminal cells, and in this way aid epithelial renewal and repair (Hong et al. 2004, Rock et al. 2009, Hackett et al. 2008, Hajj et al. 2007, Puchelle et al. 2006). The role of goblet cells in mucus secretion and goblet cell hyperplasia in asthma suggest that careful regulation of the number of mucus-secreting cells is important in healthy epithelium. Understanding mechanisms of basal cell differentiation may provide insight into the development of a hypersecretory phenotype in asthma.

Basal cell differentiation is affected by cytokines such as IL-13, which acts via Notch and SPDEF pathways (Chen et al. 2009, Gras et al. 2013). Notch pathways are a highly conserved signalling system and central to stem cell differentiation in different tissue types (Chiba 2006, Liu et al. 2010, Fre et al. 2005). The Notch gene encodes a transmembrane surface receptor containing an EGF-repeat region, which is activated by membrane-bound ligands expressed on the surface of neighbouring cells (Wharton et al. 1985). Consequently, direct cell interactions and communication are required for Notch signalling (Wharton et al. 1985, Guruharsha et al. 2012).

Down-regulation of Notch signalling, when combined with SPDEF activation and IL-13 signalling, promotes goblet cell differentiation (Gras et al. 2013). This is demonstrated in *in vivo* models. Transgenic mice carrying Notch transgenes lack goblet cells in intestinal tracts, in comparison with Notch transgene-negative mice (Fre et al. 2005). Targeting the Notch pathway with microRNAs affects Notch1 protein expression in epithelial cells and promotes differentiation of basal cells into ciliated cells (Marcet et al. 2011). Consequently, Notch signalling is thought to be capable of altering the balance between a mucus and ciliated cell phenotype of the epithelium.

1.4.4 *Mechanisms of mucin secretion*

Before secretion, MUC5AC and MUC5B mucins are stored within specialised intracellular secretory granules as preformed molecules (Evans et al. 2004). Once secreted, they form large polymers throughout the airway mucus layer (Thornton et al. 2008, Thornton et al. 1997, Escande et al. 2001, Gum et al. 1992, Perez-Vilar 2007, Verdugo et al. 1987). The polymers become entangled due to their large size, but also form non-covalent Ca^{2+}-dependent cross-links between the sialic and sulfate residues of oligosaccharides from different polymers (Perez-Vilar 2007, Verdugo et al. 1987, Raynal et al. 2003, Thornton et al. 2004).

In healthy airways, mucins are secreted from distinct sites. Immunohistochemistry and density-gradient centrifugation of mucins extracted from surface epithelium and submucosal epithelium have indicated that goblet cells of the surface epithelium are mainly responsible for MUC5AC synthesis and secretion, whereas MUC5B is present in greater quantities in submucosal glands (Hovenberg et al. 1996, Groneberg et al. 2002a, Wickstrom et al. 1998). Due to the different mucins types and sites of mucin synthesis, specific mechanisms might regulate the composition of mucus by altering the quantities of specific mucins secreted into airways (Thornton et al. 2008).

In healthy airways, steady continuous mucin production and secretion ensures maintenance of the mucus layer. In hypersecretory situations such as asthma, the airway epithelium exhibits a heightened secretory capacity, associated with goblet cell hyperplasia and submucosal gland hypertrophy (Ordonez et al. 2001, Aikawa et al. 1992, Fahy 2002). However, these features are not detected in all patients with asthma (Lozewicz et al. 1990, Jeffery et al. 2002). This implies that aberrant secretion may also contribute to the hypersecretory phenotype of asthma, rather than increased secretory apparatus alone. This is supported by the higher levels of proinflammatory cytokine and mediator expression seen in asthma (Robinson et al. 1993, Woodruff et al. 2009), and their ability to induce mucin gene expression and mucin synthesis (Kanoh et al. 2011, Kemp et al. 2004, Kreda et al. 2007, Alevy et al. 2012). Understanding mechanisms of mucin secretion may identify novel therapeutic targets for mucus hypersecretion in asthma.

1.4.4.1 *Mucin granules are released via exocytosis*

Mucins are secreted from goblet cells via exocytosis of the storage granules (**Figure 1-5**). The exocytic process and machinery of goblet cells are comparable to other secretory cells such as mast cells. This indicated by animal models exhibiting specific gene knockdowns. For instance, reduced expression of Munc18b, an exocytic protein, simultaneously decreased mucin secretion and mast cell degranulation in mice (Kim et al. 2012). This indicates that Munc18b is a central component of the exocytic machinery of both secretory epithelial cells and mast cells.

Mucin granules are released constitutively at a steady rate, or rapidly following stimulation. This has been indicated by observations of radiolabelled mature mucins, which show that mucins are either continuously secreted at low levels or stored within the cell in mucin granules (Sheehan et al. 2004, McCool et al. 1995). Constitutive exocytosis is controlled by the rate of mucin synthesis and provides a continual supply of mucins required for maintenance of the protective mucus layer. In contrast, stimulation of goblet cells leads to the exocytic release of mucin granules. For instance, treatment of airway epithelial cells with phorbol 12-myristate 13-acetate (PMA; a mimic of diacylglycerol), the serine protease thrombin, or human neutrophil elastase rapidly induces mucin secretion (Poachanukoon et al. 2014, Kreda et al. 2010, Park et al. 2005). This suggests that secretory epithelial cells provide a fast defensive secretory response to inhaled particles or stimuli without needing to wait for mucin synthesis to occur first.

Finding similarities between goblet cell and mast cell granule secretion has provided a greater understanding of the mechanisms driving mucin secretion. The initial transfer of the mucin granule towards the apical plasma membrane is aided by myristoylated alanine-rich C kinase substrate (MARCKS) protein, which acts as a chaperone. MARCKS requires phosphorylation by protein kinase C before it can facilitate trafficking of the mucin granule across the goblet cell cytoplasm (Rogers 2007, Park et al. 2008, Park et al. 2007). The mucin granule membrane fuses with the plasma membrane by forming a soluble *N*-ethyl-maleimide-sensitive factor attachment protein receptor (SNARE) complex (Jones et al. 2012, Rogers 2007, Jahn et al. 2006, Burgoyne et al. 2007). Membrane fusion involves the construction

of a 4 helix-bundle complex between 4 SNARE proteins; 1 SNARE protein is anchored in the granule membrane, and this binds to 3 SNARE proteins located in the target plasma membrane (Davis et al. 2008, Burgoyne et al. 2007, McNew 2008). The formation of SNARE complexes is regulated by proteins such as Sec1/Munc18. These proteins are thought to provide a scaffold for the assembly of the SNARE complex and ensure that fusion is specific (Davis et al. 2008, Burgoyne et al. 2007, McNew 2008, Sudhof et al. 2009). Once irreversibly bound to the apical membrane, actin filaments within the membranes are disassembled and the granule contents are released from the cell into the airway lumen (**Figure 1-5**) (Rogers 2007, Oliver et al. 1990, Ehre et al. 2005).
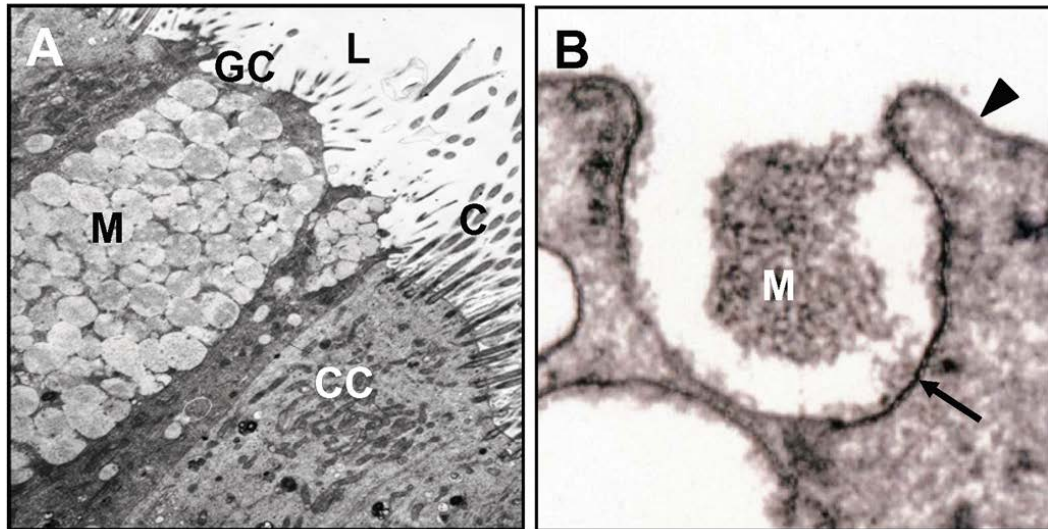
Figure 1-5: Mucins are released from goblet cells via exocytosis of specialised mucin granules.

(**A**) Mucins are stored within granules located in the goblet cell cytoplasm. GC = goblet cell; CC = ciliated cell; M = mucus; L = lumen; C = cilia. (**B**) Mucin granules are released via exocytosis from goblet cells. Images taken from (Rogers 2007).

1.4.4.2 *Increased mucin secretion in asthma*

In addition to goblet cell hyperplasia in asthma, indicating chronic over-production of mucus, goblet cells respond to stimuli by secreting mucins. Although this contributes to innate defence in healthy airways, in pathological situations excessive stimulation or hyperreactivity can lead to mucus hypersecretion.

Mucin secretion from *in vitro* cultures can be induced by different proinflammatory stimuli. For instance, the treatment of airway epithelial cultures with PMA, a diacylglycerol mimic, for 2 h induces mucin secretion, measured by enzyme-linked lectin assay (ELLA) and enzyme-linked immunosorbent assay (ELISA) (Poachanukoon et al. 2014, Kemp et al. 2004). The serine protease thrombin and human neutrophil elastase rapidly induce mucin secretion from cultures within 15 min, measured by immuno-slot blot and ELISA (Poachanukoon et al. 2014, Kreda et al. 2010, Park et al. 2005). ATP for 30 min (Jones et al. 2012, Kemp et al. 2004) and TNF-α for 24 h induces mucin secretion from human airway epithelial cells *in vitro*, as measured by ELISA (Shimizu et al. 2012). The role of AREG has also been implicated by *in vitro* studies; neutralising antibodies directed against AREG inhibit mucin secretion from H292 cells, detected by ELISA (Shimizu et al. 2014). Finally, *in vitro* co-cultures have demonstrated a role for eosinophilic inflammation in mucus overproduction in asthma (Burgel et al. 2001, Lee et al. 2004); co-culture of H292 cells with eosinophils upregulated MUC5AC secretion (quantified by ELISA) via EGFR activation (Burgel et al. 2001, Shimizu et al. 2014, Lundgren et al. 1991). The role of eosinophils in elevated airway mucus secretion has also been indicated by findings that eosinophil cationic protein increases the release of respiratory glycoconjugates from feline and human airway explants (Lundgren et al. 1991).

In vivo, intratracheal instillation of lipopolysaccharide induces mucus hypersecretion in rats, shown by findings of elevated mucin concentrations within bronchoalveolar lavage (BAL) samples (Ou et al. 2008). In addition, blocking PGD_2 activity reduces mucus hypersecretion induced by chronic inhalation of house dust mite in mice (Stebbins et al. 2010).

1.4.4.3   The role of intracellular Ca^{2+} signalling in mucin secretion

The precise mechanism of mucin secretion from goblet cells is not yet fully understood, but is thought to involve fluctuations in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$). For instance, Kreda et al. have shown that stimulation of purinergic $P2Y_2$ receptors induced Ca^{2+}-dependent mucin secretion from airway epithelial SPOC1 cells (Kreda et al. 2007). Fluorescence imaging of Fura-2-loaded cells has shown that increased $[Ca^{2+}]_i$ accompanies stimulated mucin secretion from primary human airway epithelial cells (Kemp et al. 2004, Rossi et al. 2004, Bahra et al. 2004). Furthermore, chelation of intracellular Ca^{2+} by BAPTA diminishes the mucin secretory response of SPOC1 cells following PMA stimulation (Rossi et al. 2004).

The role of intracellular Ca^{2+} signalling pathways in mucin secretion have also been indicated by manipulating the intracellular environment. For instance, intracellular treatment of SPOC1 cells with inositol triphosphate (IP3) induces mucin secretion (Rossi et al. 2004). IP3 modulates Ca^{2+} signalling by depleting intracellular stores (Hoth et al. 1992, Hoth et al. 1993). Consequently, intracellular store depletion is thought to contribute to the mucin secretory pathway. In other studies, ionomycin, an antibiotic capable of increasing $[Ca^{2+}]_i$ by permeabilising intracellular stores, and thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps, promoted mucin granule release (Kemp et al. 2004, Parekh et al. 2005, Parekh 2010, Abdullah et al. 1997). SERCA pumps are responsible for transporting Ca^{2+} from the cytoplasm into the ER and replenishing stores.

In other studies, high concentrations of extracellular $[Ca^{2+}]$ have been shown to promote the differentiation of *in vitro* tracheal epithelial cultures into a mucous cell-dominated phenotype (Martin et al. 1991). In contrast, low extracellular $[Ca^{2+}]$ resulted in squamous cell differentiation (Martin et al. 1991). In pancreatic duct epithelial cells and chromaffin cells, $[Ca^{2+}]_i$ is thought to have a role in secretory granule fusion and actin filament disassembly during exocytosis (Martin 2002, Trifaro et al. 2000, Gerber et al. 2002, Jung et al. 2009).

1.4.4.4 The role of ion channels in the regulation of Ca^{2+} signalling and mucin secretion

Intracellular Ca^{2+} signalling and store depletion are implicated in mucin secretion and production. Consequently, ion channels capable of modulating Ca^{2+} signalling may play a role in mucin secretion.

Since granule release from goblet cells is thought to be comparable to mechanisms of mast cell degranulation (Kim et al. 2012), intracellular Ca^{2+} signalling in goblet cells may be similar to Ca^{2+} signalling in activated mast cells. Mast cells can be activated by endothelin-1 or by cross-linking of IgE bound to cell surface FcεRI (Cruse et al. 2005, Yamamura et al. 1995). This leads to the generation of IP3 (Cruse et al. 2005, Wu 2011), and induces a biphasic increase in $[Ca^{2+}]_i$; Ca^{2+} is released from intracellular stores, and this stimulates store-operated Ca^{2+} influx from the extracellular environment (Hoth et al. 1992, Hoth et al. 1993, Kerst et al. 1995).

Studies have found that specific ion channels in mast cells modulate this Ca^{2+} entry, and thus exert control over Ca^{2+} signalling (Melendez et al. 2002, Ashmole et al. 2013, Ashmole et al. 2012). These ion channels include the Ca^{2+} release-activated Ca^{2+} channel Orai and the Ca^{2+}-activated K^+ channel $K_{Ca}3.1$.

The role of Orai channels in mast cell degranulation have been demonstrated by knockdown studies; knockdown of the Orai1 channel subunit in human lung mast cells resulted in a significant reduction in Ca^{2+} influx and β-hexosaminidase release, a marker of mast cell degranulation (Ashmole et al. 2013). Evidence for the role of $K_{Ca}3.1$ in mast cell degranulation was found in knockout animals. In $K_{Ca}3.1$ knockout mice, mast cell activation failed to induce Ca^{2+} release from intracellular stores, and mast cell degranulation was significantly attenuated, as identified by reduced histamine and β-hexosaminidase release (Shumilina et al. 2008). Additionally, $K_{Ca}3.1$ opening enhances IgE-dependent Ca^{2+} influx and subsequent degranulation in human lung mast cells (Duffy et al. 2004).

These ion channels may modulate similar mechanisms in goblet cells (Rossi et al. 2004, Nguyen et al. 1998). Activation of P2Y$_2$ receptors of primary HBEC cultures by ATP and UTP induces mucin secretion accompanied by Ca^{2+} entry (Bahra et al.

2004). This Ca^{2+} entry was found to be sensitive to lanthanum (La^{3+}) and gadolinium (Gd^{3+}) (Bahra et al. 2004). Ion channels such as transient receptor potential (TRP) and Orai are Ld^{3+}- and Gd^{3+}-sensitive and also Ca^{2+}-selective. TRP and Orai channels facilitate selective Ca^{2+} entry via the plasma membrane, and thus regulate fluctuations in $[Ca^{2+}]_i$ (Berridge et al. 2000, Berridge 1993). It could be possible that the receptor-stimulated Ca^{2+} entry of HBECs is mediated by either of these ion channels (Bahra et al. 2004, Hoth et al. 1993, Derler et al. 2013, Chang et al. 2008).

However, while ion channels such as Orai directly regulate $[Ca^{2+}]_i$ signalling by controlling Ca^{2+} entry, other ion channels are involved in $[Ca^{2+}]_i$ signalling pathways. For instance, Ca^{2+} entry into mast cells can be enhanced or attenuated by changes in the membrane potential (Di Capite et al. 2009b). This has been shown by findings that closure of K^+ channels within the plasma membrane of mast cells causes membrane depolarisation. The resulting positive-shift in membrane potential reduces the electrical driving force for Ca^{2+} entry, and subsequently reduces $[Ca^{2+}]_i$ signalling (Di Capite et al. 2009b). Thus, ion channels capable of indirectly regulating $[Ca^{2+}]_i$ signalling may affect Ca^{2+}-dependent pathways of the cell, such as mucin secretion.

Due to the proposed role of Ca^{2+} store release in mucin release from goblet cells, and the similarities between the exocytic machinery of both mast cells and goblet cells, $K_{Ca}3.1$ may be involved in mucin secretion from airway epithelial cells. Consequently, examining the expression and function of ion channels capable of controlling Ca^{2+} influx and $[Ca^{2+}]_i$ signalling in airway goblet cells might provide further insight into the mechanisms of mucin secretion in health and in asthma.

1.5   *Ion channels*

1.5.1   *Overview*

Ion channels have vital roles in the physiological activity of all living cells and are essential for the generation of electrical signals required for neuronal messaging in nerves and muscles. The channels form a group of proteins that are embedded

within the plasma membranes of cells and allow the passage of charged particles, such as Na^+, K^+ and Ca^{2+} ions, across the membrane (Aidley et al. 1996).

The plasma membrane is composed of two layers of lipid molecules and is impermeable to ions unless specific ion channels are open to permit their transport. Ion channels bypass the hydrophobicity of the plasma membrane by providing a hydrophilic aqueous pore which facilitates the movement of ions. Different channels selectively allow specific ionic species to pass through. This control of ion transport allows ions to be maintained at highly explicit concentrations within the cell. The intracellular condition is different to that of the extracellular fluid; in comparison to the extracellular environment, the intracellular condition usually contains high concentrations of K^+ and fluctuations in Ca^{2+}, with comparably lower concentrations of Na^+ and Cl^- ions (Aidley et al. 1996, Reuss 2011).

Ion channels selectively allow certain ionic species to pass through by discriminating between the size and charge of the molecule. Some ion channels only allow a single species to move through; others transport a range of cations. There are particular methods for studying different types of ion channels and these utilise the gating mechanisms of the channels (i.e. factors inducing the opening or closing of an ion channel, such as voltage, Ca^{2+}, or ligand-gated), as well as their specific or broad selectivity. Studying the properties of ion channels using patch clamp electrophysiology provides an insight into the activity of ion channels in different cells.

1.5.2 *Ion channels in non-excitable cells*

Excitable cells propagate all-or-nothing action potentials by allowing a rapid fluctuation in membrane potential. In contrast, non-excitable cells lack voltage-gated Na^+ and Ca^{2+} channels and therefore cannot respond to depolarising stimuli in the same way (Rink et al. 1989, Fewtrell 1993, Clapham 1995). Non-excitable cells, such as leukocytes or structural cells (e.g. epithelial cells) express a variety of ion channels which are used for specific physiological purposes, including

proliferation (Duffy et al. 2003), differentiation (Tharp et al. 2006), migration (Cruse et al. 2006, Cruse et al. 2011) and apoptosis (Lang et al. 2012).

Ion channels continuously regulate the internal ionic concentrations of the cell. Differences in ionic concentrations are determined by ion diffusion or active transport across the plasma membrane and generate an ionic gradient across the membrane for each ionic species. Each ion carries a specific charge, and differences in charge across the plasma membrane create an electrical potential difference between the cell cytoplasm and extracellular fluid (Aidley et al. 1996). This is known as the membrane potential and is quantified as a voltage (mV). The membrane potential reflects the separation of charges by the membrane and alongside the concentration gradient, also influences the movement of ions. As ions travel across the plasma membrane their carriage of charge is detected as a flow of current. Together with the membrane potential, this can be recorded during patch clamp experiments (Aidley et al. 1996).

The movement of ions through an open ion channel is rapid and occurs passively down the ionic concentration and electrical gradient. However, some ion channels act as pumps or transporters and move ions across the membrane against these gradients. The rate of passive ion movement is determined by the magnitude of the gradients, the selectivity of the ion channels, the number of ion channels present within the plasma membrane, and the proportion of open channels (Aidley et al. 1996).

The passive movement of ions continues until the channel closes. This often involves a conformational change in the channel's structure. The ability of the channel to open or close is controlled by different factors, and is known as its gating. If the channel remains open, ions will continue to move passively until equilibrium is reached; that is, the concentration gradient of ions is balanced with the electrical gradient and there is no net movement of ions or charge. At this point, the potential difference between the interior and exterior of the cell is known as the equilibrium potential (**Figure 1-6**). This value can be calculated for each ionic species using the Nernst equation. The equilibrium potential is not the same as the resting potential, which requires active ion transport (Aidley et al. 1996).
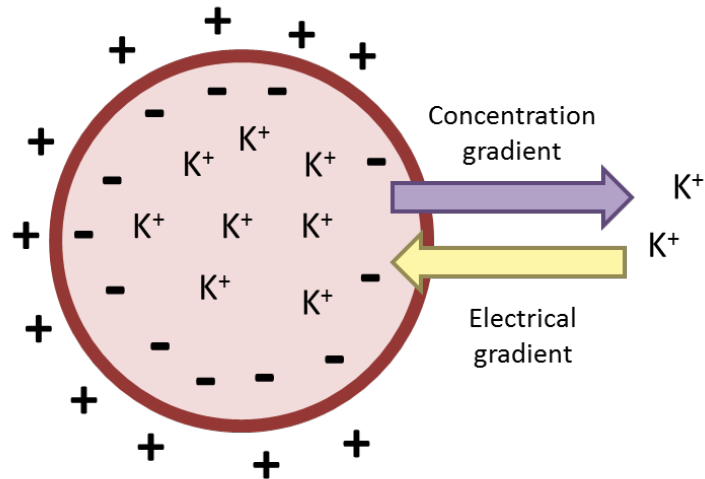
Figure 1-6: The equilibrium potential for an ionic species is determined by the concentration and electrical potential gradients.

The forces driving the passive movement of ions are the electrical potential, determined by the ion's charge, and the concentration, determined by the amount of each ion on either side of the membrane. When ions are driven by these forces in equal but opposite directions, this is known as the equilibrium potential. This can be calculated by the Nernst equation.

The current carried by ions will flow in or out of the cell depending on the gradients present. The direction of currents will reverse in response to a change in the electrical or concentration gradient; ions move from an area of high energy to low energy. This energy can be created by the concentration gradient, the electrical gradient, or a combination of both (electrochemical gradient; the sum of both gradients/forces). The point at which the current reverses is known as the reversal potential and depends upon the properties of the ionic species carrying the current and the surrounding ionic environment. If the current is conducted by a particular channel with high selectivity for a specific ion, the reversal potential has a similar value to the equilibrium potential (Aidley et al. 1996). Ion channels exhibit characteristic reversal potentials under experimental conditions, and this can be used to identify ion channels during experiments.

1.5.3   *Store-operated Ca²⁺ channels*

Store-operated Ca^{2+} channels (SOCs) are Ca^{2+} permeable ion channels expressed in the plasma membrane of a variety of eukaryotic cells (Parekh et al. 2005). SOCs facilitate Ca^{2+} entry into the cell to rapidly increase $[Ca^{2+}]_i$ and regulate a wide range of cellular processes (Parekh et al. 2005, Parekh 2010). Ca^{2+} can also enter cells via Ca^{2+} channels operated by voltage, receptor activation or secondary messengers, such as the transient receptor potential (TRP) channel. Together, Ca^{2+} channels of internal Ca^{2+} stores and the plasma membrane maintain tight regulation of $[Ca^{2+}]_i$ (Parekh et al. 2005). The best characterised SOC is the Ca^{2+} release-activated Ca^{2+} Orai channel (Parekh et al. 2005).

1.5.3.1   *Orai channels*

1.5.3.1.1 Cellular expression and biological role of Orai channels

The Orai channel was first characterised in rat peritoneal mast cells by Hoth M, et al. in 1992 using patch clamp electrophysiology and Fura-2 measurements to detect the emptying of intracellular Ca^{2+} stores by IP3, ionomycin, and the Ca^{2+} chelator EGTA (Hoth et al. 1992, Hoth et al. 1993). These methods caused

depletion of intracellular Ca^{2+} stores and induced a highly selective inward Ca^{2+} current known as the Orai current (Hoth et al. 1992).

In mast cells and T cells, Orai channels have central roles in various physiological processes including cytokine release, mitochondrial metabolism, cell growth, proliferation and gene transcription (Parekh et al. 2005, Parekh 2010, Berridge 1993, Lewis 2003). Orai channel activation and Ca^{2+} influx facilitates a rapid rise in $[Ca^{2+}]_i$, replenishes intracellular stores and helps sustain Ca^{2+} signals (Hoth et al. 1992, Parekh et al. 2005, Parekh 2010, Di Capite et al. 2009b, Di Capite et al. 2009a). As a ubiquitous signalling messenger in most cells, the kinetics and location of Ca^{2+} signals trigger specific downstream pathways (Berridge et al. 2000, Neher 1998, Rizzuto et al. 2006, Berridge 1990). Consequently, the Orai channel and Ca^{2+} influx play precise roles in cell processes. For example, gene expression in mast cells is triggered when $[Ca^{2+}]_i$ fluctuations are accompanied by Ca^{2+} entry via Orai channels (Di Capite et al. 2009a), and shRNA knockdown of Orai1 attenuates Ca^{2+} influx in human lung mast cells and reduces degranulation (Ashmole et al. 2013).

The expression and activity of Orai channels have also been found in vascular smooth muscle cells (Zhang et al. 2014), macrophages (Gao et al. 2010) polarised secretory cells, such as pancreatic and salivary gland acinar duct cells (Hong et al. 2011), and in primary airway epithelial cells obtained during lung transplantation from CF patients and from healthy controls (Balghi et al. 2011). CF epithelial cells exhibited larger and faster Ca^{2+} influx than healthy epithelial cells, an effect attributed to increased cell surface expression of Orai1 in CF cells (Balghi et al. 2011). In polarised cells, most Ca^{2+} entered through the apical membrane (Balghi et al. 2011). Under resting conditions, Orai1 is stored intracellularly and is inserted into the plasma membrane by SNAP-25-mediated exocytosis (Woodard et al. 2008, Yu et al. 2010). In CF epithelial cells, increased exocytic insertion of Orai1 was detected (Balghi et al. 2011).

Aberrant Orai channel activity and Orai1 gene mutations are strongly associated with primary immunodeficiencies and severe combined immunodeficiency (SCID) due to the role of Orai channels in T cell cytokine production; loss of T cell activation increased susceptibility to severe infections (Feske et al. 2006, Feske

2009, Partiseti et al. 1994, McCarl et al. 2009). This strong association with immune dysfunction is attributed to the inability of Orai2 and Orai3 to fully restore Ca^{2+} influx in the absence of Orai1 (Gwack et al. 2007). Inhibition of Orai channels by the pyrazole derivative BTP2 may also have therapeutic effects in asthma; BTP2 inhibits interleukin secretion from T cells, histamine and leukotriene release from mast cells, and reduces asthmatic bronchoconstriction in animal models (Ashmole et al. 2013, Ashmole et al. 2012, Ishikawa et al. 2003, Ohga et al. 2008, Yoshino et al. 2007). Although the role of Orai in mucus secretion has not been identified, these findings suggest Orai1 expression and activity is associated with airways disease.

1.5.3.1.2 Orai channel features

As SOCs, Orai channels facilitate rapid Ca^{2+} entry in response to transient decreases in Ca^{2+} within ER stores. Store depletion is initiated by inositol-1,4,5-trisphosohate (IP3) activation of Ca^{2+} channels of the stores (Hoth et al. 1992). Orai channel gating, conductance and ionic selectivity are highly conserved between rodents, humans, the nematode *Caenorhabditis elegans*, and the fly *Drosophila melanogaster*. Hence, the Orai channel is thought to represent an essential Ca^{2+} entry pathway.

While studying at the University of Oxford, Professor Parekh described characteristic electrophysiological features of the Orai channel. A typical time course for Ca^{2+} stores depletion is 5 min and Orai channel kinetics last for approximately 1 min (Fierro et al. 2000). Orai channel activity causes a positive shift in reversal potential and strong inward rectification, and is sensitive to 3 μM of the Orai channel blocker GSK-7975A (Ashmole et al. 2013, Ashmole et al. 2012, Derler et al. 2013). The SERCA pump inhibitor, thapsigargin, and IP3 in the presence of the intracellular Ca^{2+} chelator EGTA are capable of depleting Ca^{2+} stores and thus activating Orai channels (Fierro et al. 2000, Zweifach et al. 1993, Zweifach et al. 1995). Orai channels have high selectivity for Ca^{2+} and can discriminate between different divalent cations as well as between monovalent and divalent cations, such as between Ca^{2+} and Na^+ (Hoth et al. 1992). This high selectivity results in a low single channel conductance, as identified by fluctuation

analysis (a method that distinguishes between individual channel activity and the background noise of each current) (Parekh et al. 2005, Zweifach et al. 1993).

Orai channels are not the only SOCs present within the plasma membrane (Parekh et al. 2005). For example, mammalian homologs of the *Drosophila* TRP channel conduct Ca^{2+} entry and respond to IP3 receptor activation (Zhu et al. 1996, Boulay et al. 1999, Rosado et al. 2002). In addition, TRP homologs such as TRPM5 associate with stromal interaction molecule (STIM)-1 and Orai1 to form a complex that permits store-operated Ca^{2+} entry (Hong et al. 2011, Jardin et al. 2009, Jardin et al. 2008, Ong et al. 2007).

Orai1 is a predominant component of the Ca^{2+}-selective pore of the Orai channel (Feske et al. 2006, Prakriya et al. 2006, Vig et al. 2006). While overexpression of Orai1 does not upregulate Orai currents, knockdown of Orai1 attenuates activation of the channel (Feske et al. 2006, Vig et al. 2006). Orai2 and Orai3 are homologs of the Orai channel protein components displaying similarities in their sequences with Orai1, but different functional properties. Overexpression of Orai2 and Orai3 results in Orai currents with larger amplitudes and altered selectivity for Ca^{2+} and Na^+ (Lis et al. 2007). The 3 homologs form heteromultimeric complexes of the Orai channel (Lis et al. 2007). Knockdown experiments in human lung mast cells have demonstrated that Orai1 knockdown significantly reduces Ca^{2+} influx and mast cell degranulation, whereas only marginal reductions were seen with Orai2 knockdowns (Ashmole et al. 2013).

1.5.3.1.3 Orai channel activation and pharmacological study

STIM1 a 77 kDa protein that spans the ER membrane and is the Ca^{2+} sensor responsible for linking store depletion with Ca^{2+} entry (Roos et al. 2005, Liou et al. 2005, Dziadek et al. 2007). STIM1 activates SOCs such as Orai following transient decreases in store Ca^{2+} concentrations (Roos et al. 2005, Liou et al. 2005). The NH_3 terminus of STIM1 is located within the ER lumen and contains a Ca^{2+}-binding domain, allowing it to detect the Ca^{2+} content of stores (Liou et al. 2005, Cahalan 2009, Lewis 2007, Zhang et al. 2005). Store depletion induces STIM1 translocation to areas within close proximity to the plasma membrane (Zhang et al. 2005, Wu et

al. 2006). Under resting conditions, Orai subunits are thought to exist as dimers within the plasma membrane (Penna et al. 2008). Aggregation of STIM1 close to the plasma membrane facilitates clustering of Orai subunits at the cell surface (Zhang et al. 2005, Mignen et al. 2008, Luik et al. 2008, Yeromin et al. 2006). Together, STIM1 and Orai modulate the store-operated Ca^{2+} signalling pathway by recognising and transducing store-dependent signals to facilitate Ca^{2+} entry into the cell.

Orai subunits form the multimeric structure of the Orai channel with a Ca^{2+}-selective central pore, and this facilitates the entry of extracellular Ca^{2+} ions across the plasma membrane (Yeromin et al. 2006). Penna A, al. have proposed that STIM1 interacts with Orai dimers to induce dimerization, resulting in a tetrameric Orai channel with a Ca^{2+}-selective pore (Penna et al. 2008). In contrast, Hou X, et al. report a hexameric assembly of Orai subunits around a central membrane-spanning ion pore (**Figure 1-7**) (Hou et al. 2012). Glutamate residues around the extracellular portion of the channel are thought to facilitate selectivity for Ca^{2+}, and basic residues of the intracellular portion are hypothesised to bind anions when the channel is closed (Hou et al. 2012).

Activation of cell surface receptors such as G protein-coupled receptors initiates a cellular messenger cascade involving the activation of phospholipase C (von Kugelgen 2006, Rossi et al. 2007). This enzyme hydrolyses phosphatidylinositol 4,5-bisphosphate to generate of the intracellular secondary messenger IP3 (Berridge 1984). IP3 activates receptors on the ER membrane, releasing Ca^{2+} from the stores and subsequently inducing STIM1 activation of Orai channels (Redondo et al. 2008).

Orai channel activity can be triggered in patch clamp experiments by administering intracellular IP3 to activate IP3 receptors and Ca^{2+} ionophores such as ionomycin and A23187, which facilitate Ca^{2+} passage across the cell membrane (Parekh et al. 2005, Parekh 2010, Zweifach et al. 1995, Jardin et al. 2009). Inhibitors of the SERCA pump such as thapsigargin also induce store depletion (Parekh et al. 2005, Parekh 2010, Zweifach et al. 1995); inhibition of these pumps prevents replenishing of Ca^{2+} stores once depleted. Dialysing the cytoplasm with

high concentrations of Ca^{2+} chelators, such as EGTA or BAPTA, can prevent store refilling and activate Orai channels (Parekh et al. 2005).

Pharmacological blockade also helps identify Orai channels during experiments. For instance, 2-aminoethoxydiphenylborate (2-APB) and a bistrifluoromethyl-pyrazole derivative (BTP2, also known as YM-58483) block Orai channels, although these blockers exhibit non-specific inhibitory effects on other channels (Derler et al. 2013, Ishikawa et al. 2003, Zitt et al. 2004, Prakriya et al. 2001). 2-APB also has a complex pharmacology; Orai currents are enhanced by low concentrations of 2-APB (1-5 μM), but are blocked by high concentrations of 2-APB (above 10 μM) (Prakriya et al. 2001). Synta-66 and GSK-7975A are also capable of inhibiting Orai channel activity and reducing Ca^{2+} influx, mast cell degranulation and cytokine secretion (Ashmole et al. 2013, Ashmole et al. 2012, Derler et al. 2013). In addition, trivalent ions such as lanthanum (La^{3+}) and gadolinium (Gd^{3+}) non-specifically block Ca^{2+}-selective influx via TRPV5/6 and Orai channels (Hoth et al. 1993, Derler et al. 2013, Chang et al. 2008).

A   **B**
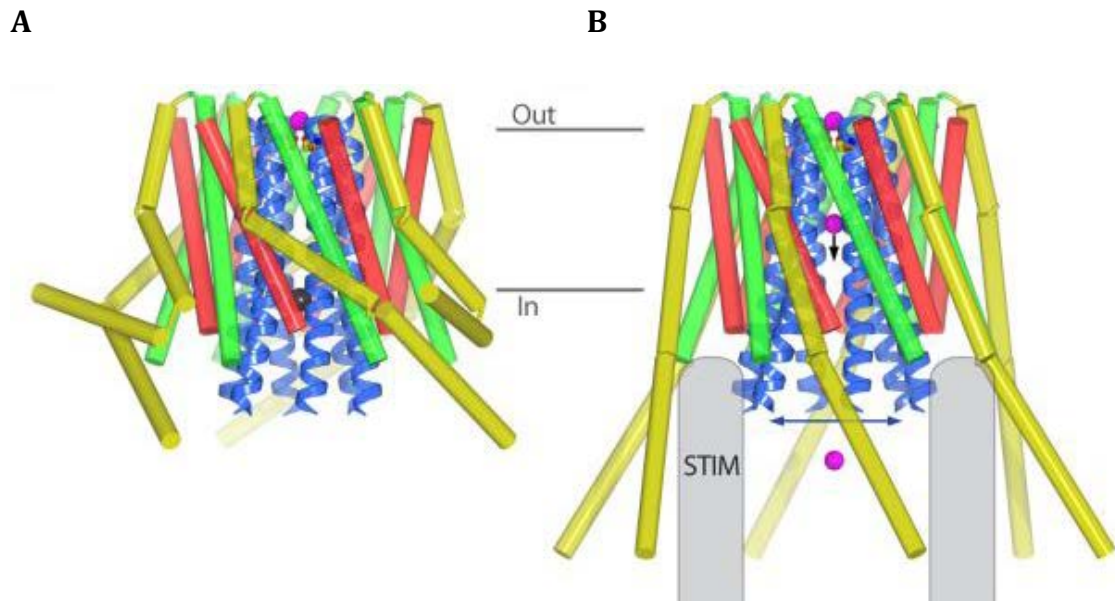
Figure 1-7: Structure of the Orai channel.

(**A**) Proposed structure of Orai when closed. (**B**) The blue central helices are thought dilate to allow the central pore to widen when the channel opens (blue arrow), allowing Ca^{2+} ions (pink sphere) to move through the channel. Lines labelled "In" and "Out" represent predicted boundaries of the lipid membrane. The blue helices are hypothesised to interact with STIM1. Images taken from (Hou et al. 2012).

1.5.3.1.4 Toxicity of Orai channel blockade and knockdown

Studies investigating the role of Orai channels in human lung mast cells reported that expression of dominant-negative Orai subunit mutants inhibited Orai currents but did not alter human lung mast cells survival (Ashmole et al. 2013). Similar effects were seen in activated mast cells isolated from mice lacking Orai1 or STIM1 (Vig et al. 2008, Baba et al. 2008). Orai1 or STIM1 knockdown also impaired mast cell degranulation and LTC$_4$ and TNF-α production and secretion (Vig et al. 2008, Baba et al. 2008). In addition, STIM1 and Orai1 deficiency attenuated passive cutaneous anaphylaxis reactions (Vig et al. 2008, Baba et al. 2008). These findings suggest that SOC is necessary for mast cell activation via a STIM1-dependent pathway, but is not essential for mast cell survival.

Knockout mice lacking Orai1 or STIM1 expression have provided important information about the physiological role of Orai channels and store-operated Ca^{2+} entry. STIM1$^{-/-}$ mice died perinatally despite a lack of abnormalities in STIM1$^{-/-}$ embryos and no obvious histological abnormality in whole body necroscopy (Vig et al. 2008, Baba et al. 2008). If STIM1$^{-/-}$ mice survived they appeared smaller in size than their wild-type littermates but died of respiratory failure within 1 day (Baba et al. 2008). Targeted disruption of the murine Orai1 gene, resulting in reduced Orai1 immunostaining, caused eyelid irritation, hair loss and perinatal death (Gwack et al. 2008). Perinatal death is thought to be caused by rapid muscle fatigue and skeletal muscle myopathy (Stiber et al. 2008).

1.5.3.1.5 Existing therapies targeting Orai channels

Evidence for Orai channel activity in human diseases implies that inhibition of Orai channel activity would yield clinical benefits. However, no Orai channel blockers exist as current therapies. Pharmacological blockers of the Orai channel successfully block Orai current, but more specifically selective drugs must be synthesised first.

The role of STIM1 in activating Orai channels complicates the process of identifying a specific Orai channel blocker; Ca^{2+} entry is modulated by the Orai channel and STIM1 (Parekh 2010). For instance, although 2-APB inhibits Ca^{2+}

entry, this effect is suppressed when Orai1 is over-expressed (DeHaven et al. 2008). STIM1 activity is not specific for Orai channel activation, and therefore inhibition of STIM1 could block more than just Orai channel-dependent SOCE. For example, depletion of intracellular stores and STIM1 activation leads to adenylyl cyclase activation in the absence of Ca^{2+} entry (Lefkimmiatis et al. 2009).

Orai channel blockers are not entirely specific. For instance, although BTP2 abolishes Ca^{2+} entry without affecting store depletion, it is capable of blocking more than one ion channel (Parekh 2010, Ishikawa et al. 2003, Djuric et al. 2000, Trevillyan et al. 2001). Similarly, the synthetic oestrogen agonist, diethylstilbestrol, inhibits Orai channels in mast cells and has greater specificity for the Orai channel, but exerts agonistic effects on oestrogen receptors. Consequently, it is unlikely to be suitable for clinical use (Parekh 2010, Zakharov et al. 2004).

However, one SOC blocker, carboxyamido-triazole, has reached Phase I and II clinical trials in epithelial ovarian cancer. Carboxyamido-triazole inhibits angiogenesis, tumour growth and metastasis (Kohn et al. 2001, Hussain et al. 2003), and inhibits store-operated Ca^{2+} entry in non-excitable cells by depolarising mitochondria; mitochondria buffer Ca^{2+} entry, and hence affect the activity of Orai channels (Mignen et al. 2005, Parekh 2008b, Hoth et al. 2000, Gilabert et al. 2000). Clinical trials reported that treatment with carboxyamido-triazole had minimal toxic effects of Grade 1 to 3 and was well tolerated by mice and trial patients (Kohn et al. 2001, Hussain et al. 2003). However, there are still concerns about the specificity of the blocker (Parekh 2010).

Synta-66 and GSK-7975A are capable of blocking Orai channels with good specificity. However, their use in a clinical setting may be restricted due its high value of 50% inhibitory concentration (IC_{50}; quantity required to inhibit 50% of the maximal response); Synta-66 and GSK-7975A block Orai channels with an IC_{50} value of 3 μM (Parekh 2010, Ng et al. 2008).

1.5.4 *Ca²⁺-activated K⁺ channels*

Ca^{2+}-activated K^+ channels (K_{Ca}) represent a large family of K^+ channels which become activated by elevated $[Ca^{2+}]_i$. These channels are ubiquitous throughout

the body and are classified into 3 groups according to the size of their single channel conductance: large, intermediate and small (Aidley et al. 1996). Similarly to Orai channels, K_{Ca} channels participate in Ca^{2+} signalling, and thus contribute to the regulation of various cellular processes (Berridge et al. 2000).

The ubiquity of Ca^{2+} signalling is due to the ability of Ca^{2+} to act as an allosteric activator or an inhibitor of a variety of enzymatic intracellular pathways (Berridge et al. 2000, Priddy et al. 2007, Greber et al. 1995). The specificity of Ca^{2+} signalling is conferred by the speed, amplitude and spatial locations of changes in $[Ca^{2+}]_i$ (Martin et al. 1991). However, Ca^{2+} signalling is also manipulated by changes in the membrane potential, which can affect the driving force for Ca^{2+} currents (Di Capite et al. 2009b). Here, K_{Ca} channels have been implicated as modulators of Ca^{2+} signalling. For example, $K_{Ca}3.1$ activation causes efflux of K^+, which produces a negative membrane potential (Duffy et al. 2004, Wulff et al. 2010). This increases the size of the electrical driving force for Ca^{2+} entry and thus enhances Ca^{2+} influx via SOCs. To support this, Orai channels have been shown to conduct larger currents at more negative membrane potentials (Hoth et al. 1992).

K_{Ca} channels affect Ca^{2+} signalling in mast cells and T cells. For instance, $K_{Ca}3.1$ enhances IgE-dependent Ca^{2+} influx in human lung mast cells (Duffy et al. 2004); suppression of K_{Ca} channels in T cells decreases Ca^{2+} influx (Fanger et al. 2001); introduction of $K_{Ca}3.1$ channels into T cells facilitates Ca^{2+} entry (Fanger et al. 2001); and $K_{Ca}3.1$ blockade inhibits cytokine release and T cell activation by suppressing signals that elicit a rise in $[Ca^{2+}]_i$ (Jensen et al. 1999).

These findings implicate a role for K_{Ca} channels in mediating Ca^{2+} signalling pathways and downstream pathways. Accordingly, $K_{Ca}3.1$ may contribute to the regulation of mucin granule exocytosis due to the known role of Ca^{2+} signalling mucin granule exocytosis (Rossi et al. 2007, Tuvim et al. 2009, Jacquot et al. 1995, Maizieres et al. 1998, Takahashi et al. 1998).

1.5.4.1 $K_{Ca}3.1$

1.5.4.1.1 $K_{Ca}3.1$ structure and Ca^{2+} sensitivity

$K_{Ca}3.1$ is encoded by the gene KCNN4 located on chromosome 19q13.2, and has a molecular weight of 48 kDa. $K_{Ca}3.1$ was originally described by Gardos G. in erythrocytes (Gardos 1958). As a member of the K_{Ca} channel family, $K_{Ca}3.1$ is gated by intracellular free Ca^{2+} (Vergara et al. 1998). $K_{Ca}3.1$ (also known as SK4, IK, IK_1, $IK_{Ca}1$) is an intermediate conductance channels with a single channel conductance of 18-50 pS, but shares similarities in the pore region with small conductance channels (Joiner et al. 1997). Unlike large conductance channels, $K_{Ca}3.1$ is not modulated by membrane voltage (Wei et al. 2005).

$K_{Ca}3.1$ is a tetrameric protein expressed within the plasma membrane with six transmembrane segments per subunit, within which a K^+-selective pore is formed (**Figure 1-8**) (Morales et al. 2013). The Ca^{2+}-sensitivity of $K_{Ca}3.1$ is conferred by calmodulin, which acts as a sensor of $[Ca^{2+}]_i$ and is constitutively bound to the channel's intracellular C-terminus (**Figure 1-8**) (Morales et al. 2013, Joiner et al. 2001, Maylie et al. 2004). $K_{Ca}3.1$ is activated in response to binding of Ca^{2+} to calmodulin, and this is thought to involve a conformational change of the channel in the presence of Ca^{2+} (Fanger et al. 1999, Xia et al. 1998, Schumacher et al. 2001). $K_{Ca}3.1$ activation also occurs by phosphorylation of a histidine residue in the C-terminus of the channel by nucleoside diphosphate kinase B (NDPK-B); NDPK-B knockout mice exhibit impaired $K_{Ca}3.1$ activation (Srivastava et al. 2006, Di et al. 2010).
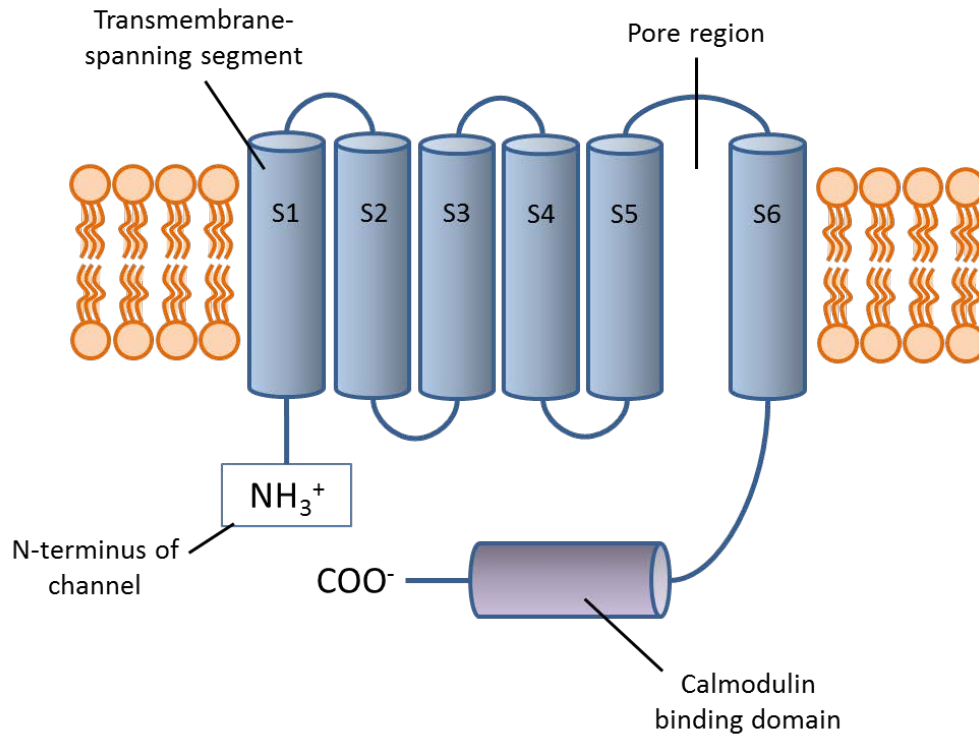
Figure 1-8: Structure of the $K_{Ca}3.1$ channel.

Each channel consists of 6 transmembrane segments (S1-6), within which a pore region permitting K^+ selectivity is formed (Morales et al. 2013). The pore contains highly specific amino acid sequences which allow it to be selective for K^+ ions (Neylon et al. 1999). Calmodulin is bound to channel's C-terminus and contains a Ca^{2+}-dependent binding region (Morales et al. 2013). The N-terminus of the monomer is required for efficient packaging and trafficking of the channel to the plasma membrane from the ER. Diagram based on (Tharp et al. 2009, Neylon et al. 1999).

1.5.4.1.2 K_{Ca}3.1 gating and pharmacological study

The sensitivity of calmodulin to Ca^{2+} allows K_{Ca}3.1 channels to be activated by increased $[Ca^{2+}]_i$. Thus, Ca^{2+} influx via SOCs such as Orai and depletion of intracellular stores by IP3 induce K_{Ca}3.1 activity (Ishii et al. 1997). K_{Ca}3.1 is sensitive to submicromolar $[Ca^{2+}]_i$ (approximately 100-300 nM), with half-maximal activation achieved with approximately 95 nM $[Ca^{2+}]_i$ (Joiner et al. 1997).

K_{Ca}3.1 activation facilitates rapid K^+ efflux, causing hyperpolarisation of the membrane potential. K_{Ca}3.1 channels are not voltage-gated and thereby play a vital role in producing negative membrane potential values close to -80 mV (K^+ equilibrium potential) without becoming inactivated (Duffy et al. 2004, Wulff et al. 2010, Grgic et al. 2009a). This effect is useful for several cellular processes, including the control of proliferation in mast cells, T lymphocytes and smooth muscle cells (Duffy et al. 2003, Khanna et al. 1999, Shepherd et al. 2007). K_{Ca}3.1 also contributes to reduction of HCO_3^{-} secretion and increase in Cl^- secretion from epithelial cells, required for ASL hydration (Devor et al. 1999).

K_{Ca}3.1 channels can be pharmacologically distinguished from other K_{Ca} channels by their sensitivity to charybdotoxin, clotrimazole, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34), ICA-17043 (bis(4-fluorophenyl)phenyl acetamide; also known as Senicapoc), and insensitivity to apamin (Grissmer et al. 1993, Logsdon et al. 1997).

Clotrimazole is an azole antimycotic agent and inhibits cytochrome P450 enzymes, oxidases required for xenobiotic metabolism (Zhang et al. 2002, Ortiz de Montellano 2005). TRAM-34, modified from clotrimazole by substitution of imidazole with pyrazole, is a useful pharmacological tool for the study of K_{Ca}3.1 activity (Wulff et al. 2010, Wulff et al. 2001) and blocks K_{Ca}3.1 currents expressed by human lung mast cells (Duffy et al. 2004). TRAM-34 has an IC_{50} of 20 nM, making it more potent than clotrimazole (IC_{50} of 25 to 387 nM) (Stocker et al. 2003, Wulff et al. 2000, Triggle 1999). Unlike clotrimazole, which has a variety of side effects and is poorly absorbed with a short half-life, TRAM-34 has improved metabolic stability and is consequently considered to have greater clinical benefits (Wulff et al. 2000, Suzuki et al. 2000). Similarly to clotrimazole, TRAM-34 inhibits K_{Ca}3.1 by blocking the inner pore of the channel; TRAM-34 interacts with

threonine residues within the pore, and with valine residues in segment 6 of the channel (Wulff et al. 2001). However, TRAM-34 has a half-life of approximately 2 hours, and is not currently available as an oral therapeutic (Al-Ghananeem et al. 2010). In contrast, ICA-17043 (Senicapoc), also an analog of clotrimazole, has a half-life of 12.8 days and is suitable for oral administration. In addition, ICA-17043 has an even greater potency, with an IC_{50} of 11 nM (Triggle 1999, Ataga et al. 2006).

$K_{Ca}3.1$ can be distinguished from $K_{Ca}2$ channels by its sensitivity to charybdotoxin, a neurotoxin isolated from the venom of the scorpion *Leiurus quinquestriatus* var. *hebraeu* (Grissmer et al. 1993, Leonard et al. 1992). Blockade by this toxin is reversible and occurs by binding of the toxin to the external component of $K_{Ca}3.1$ (Lucchesi et al. 1989, Gimenez-Gallego et al. 1988). However, charybdotoxin for pharmacological study of $K_{Ca}3.1$ is not always favoured over TRAM-34 and ICA-17043 because the toxin also blocks voltage-gated K^+ channels (Sands et al. 1989, Deutsch et al. 1991).

Other compounds frequently used for the study of $K_{Ca}3.1$ channel activity are pharmacological channel openers with high selectivity for $K_{Ca}3.1$. These openers include 1-ethyl-2-benzimidazolinone (1-EBIO) and the more potent 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazole-2-one (DC-EBIO) (Devor et al. 1996, Singh et al. 2001). The compound NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) also activates $K_{Ca}3.1$ with 1000 times greater potency than 1-EBIO and 30 times greater than DC-EBIO (Strobaek et al. 2004). The maximal effect of 1-EBIO is achieved at approximately 300 μM, and acts by enhancing the sensitivity of $K_{Ca}3.1$ to $[Ca^{2+}]_i$ (Pedersen et al. 1999); maximal K^+ currents are achieved with 100 μM 1-EBIO in the presence of 100 nM free Ca^{2+}, which is lower than the resting $[Ca^{2+}]_i$ level of human lung mast cells (Duffy et al. 2004, Duffy et al. 2007). Despite its selectivity for $K_{Ca}3.1$, 1-EBIO also activates $K_{Ca}2.3$ and $K_{Ca}2.3$ channels (Jensen et al. 2001). In addition, 1-EBIO is ineffective in the absence of Ca^{2+}, so the presence of $K_{Ca}3.1$ currents should be confirmed by inhibition of 1-EBIO-induced currents with a selective blocker such as TRAM-34. 1-EBIO and DC-EBIO have been proposed as possible therapies for diseases such as CF; activating Cl^- secretion from airway epithelial cells by augmenting $K_{Ca}3.1$ activity could promote hydration of the

mucus layer and improved mucus clearance (Devor et al. 1996, Singh et al. 2001).
Activated $K_{Ca}3.1$ channels cause hyperpolarisation of the membrane potential, thus
increasing the driving force for Cl^- efflux (Wang et al. 2013). A $K_{Ca}3.1$ activator used
in vivo in rats is known as SKA-31, although the use of $K_{Ca}3.1$ activators for
treatment of conditions such as hypertension requires further validation; $K_{Ca}3.1$ is
expressed in cancer cell lines, so activation could have neoplastic effects (Chou et
al. 2008, Sankaranarayanan et al. 2009).

1.5.4.1.3 Cellular expression and biological function of $K_{Ca}3.1$

$K_{Ca}3.1$ is found in cells of peripheral tissues such as smooth muscle cells (Wang et
al. 2013) and in immune cells such as mast cells and T cells (Shepherd et al. 2007,
Chachi et al. 2013). The channel is also expressed by several epithelia, including
primary human renal and colonic epithelial cells and human airway epithelial cell
lines (Calu-3, Nu-Li and Cu-Fi) (Devor et al. 1999, Rufo et al. 1996, Albaqumi et al.
2008, Trinh et al. 2008). In epithelial cells, $K_{Ca}3.1$ channels are often found on the
basolateral membrane, where their ability to modulate K^+ currents facilitates Cl^-
secretion and simultaneous water transport across the epithelial layer (Devor et al.
1999, Rufo et al. 1996). Pharmacological studies have suggested that $K_{Ca}3.1$ is
expressed in primary HBECs, but these were not definitive (Singh et al. 2000).

$K_{Ca}3.1$ has various roles in different cell types, including T cell proliferation and
cytokine secretion (Ghanshani et al. 2000), proliferation of endothelial cells (Grgic
et al. 2009a, Grgic et al. 2005), and regulation of vasodilator release (part of a
pathway known as "endothelium-derived hyperpolarising factor") and blood
pressure control by skeletal muscle arterial endothelium (Grgic et al. 2009a).
$K_{Ca}3.1$ also regulates proliferation of cell types such as human airway smooth
muscle cells (ASMCs), vascular smooth muscle cells (VSMCs) and myofibroblasts
(Shepherd et al. 2007, Roach et al. 2013, Bi et al. 2013). In VSMCs, this is associated
with Ca^{2+}-dependent signalling pathways. For instance, reducing $[Ca^{2+}]_i$ using the
Ca^{2+} chelator BAPTA decreases VSMC proliferation, whereas elevating $[Ca^{2+}]_i$ with
Ca^{2+} ionophore A23187 enhances proliferation. In ASMCs, elevated $[Ca^{2+}]_i$
promoted $K_{Ca}3.1$ activity even in the presence of TRAM-34 (Bi et al. 2013).
Pharmacological blockade of $K_{Ca}3.1$ also inhibits Ca^{2+}-dependent signalling

pathways required for TGF-β-induced collagen secretion and contraction in myofibroblasts (Roach et al. 2013).

$K_{Ca}3.1$ has been implicated in specific disease processes. For instance, $K_{Ca}3.1$ regulates glucocorticoid-resistant inflammatory pathways in asthma (Chachi et al. 2013); $K_{Ca}3.1$ blockade reduced steroid-resistant proinflammatory gene expression and restored phosphorylation of the glucocorticoid receptor in ASMCs. This suggests that $K_{Ca}3.1$ may facilitate corticosteroid insensitivity in severe asthma (Chachi et al. 2013). The role of $K_{Ca}3.1$ in asthma is implicated further by the sheep model of chronic asthma; administration of ICA-17043 attenuated allergen challenge-induced airway resistance, carbachol-induced AHR, and allergen-induced eosinophilia in BAL fluid (Van Der Velden et al. 2013). Additionally, $K_{Ca}3.1$ blockade by TRAM-34 reduced ovalbumin-induced AHR, basement membrane collagen deposition, and airway eosinophilia in mice (Girodet et al. 2013).

The role of $K_{Ca}3.1$ in mast cell and eosinophil degranulation also implicates $K_{Ca}3.1$ in asthma; $K_{Ca}3.1$ mediates granular exocytosis. K_{Ca} channel activity is associated with granule secretion from human eosinophils isolated from mildly atopic donors (Saito et al. 1997); platelet-activating factor (PAF), which induces eosinophil degranulation, activated ion channels that were selectively permeable to K^+ and activated by ionomycin, thapsigargin, and A23187 (Saito et al. 1997). However, no publications have since indicated which channel is responsible for mediating human eosinophil degranulation. Expression of $K_{Ca}3.1$ in human lung mast cells has been reported in several studies (Duffy et al. 2004, Duffy et al. 2001), and plays a central role in human lung mast cells migration and degranulation (Duffy et al. 2004, Cruse et al. 2006, Duffy et al. 2007). The mechanism of human lung mast cell degranulation is Ca^{2+}-dependent, involving activation of SOCs by IP3 and store depletion. IgE-dependent activation of human lung mast cells activates $K_{Ca}3.1$, which enhances Ca^{2+} influx and leads to histamine release (Duffy et al. 2004, Wulff et al. 2010). Furthermore, $K_{Ca}3.1$-deficient mice exhibit attenuated degranulation, reduced Ca^{2+} influx and decreased anaphylactic responses to allergen challenge (Shumilina et al. 2008).

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In a sheep allergen-challenge model of asthma ICA-17043 reduced AHR and airway resistance (Robinette et al. 2008). In a phase II clinical trial of asthma, ICA-17043 also reduced the inflammatory marker exhaled nitric oxide and airway resistance after 2 weeks (Lam et al. 2011). However, in a subsequent proof-of-concept Phase II trial, no improvement in lung function in exercise-induced asthma was seen after 4 weeks of treatment (Lam et al. 2011). *In vitro*, *in vivo* and clinical trial data implicate a role for $K_{Ca}3.1$ in asthma, and suggest that $K_{Ca}3.1$ blockade might have greater clinical significance in airway remodelling in asthma.

1.5.4.1.4 Toxicity of $K_{Ca}3.1$ channel blockade and knockdown

Homozygous $K_{Ca}3.1$ knockout mice are viable and of normal appearance, and also exhibit normal fertility (Shumilina et al. 2008, Begenisich et al. 2004). There are no reports of visible signs of organ damage; only mild phenotypes have been described (Begenisich et al. 2004, Grgic et al. 2009c, Kohler et al. 2003). The role of $K_{Ca}3.1$ in cell volume regulation is thought to explain small changes in blood pressure, erythrocyte macrocytosis and splenomegaly with age see in $K_{Ca}3.1^{-/-}$ mice (Grgic et al. 2009b, Brahler et al. 2009).

So far, $K_{Ca}3.1$ blockers have not shown efficacy in clinical trials, but have demonstrated that $K_{Ca}3.1$ represents a potential therapeutic target due to the lack of toxicity seen following $K_{Ca}3.1$ blockade. Administration of high doses of TRAM-34 to rodents over several weeks is well-tolerated (Grgic et al. 2009c). High-dose treatment with oral ICA-17043 in phase 2 and 3 human trials of sickle cell disease were safe and well-tolerated, with only minor side effects (such as nausea and urinary tract infections) detected (Ataga et al. 2008, Ataga et al. 2011). In addition, a phase 2 clinical trial examining the effect of ICA-17043 on exercise-induced asthma (Icagen Inc. www.icagen.com) reported no major toxicity (Lam et al. 2011).

1.6   *Summary*

Mucus hypersecretion and goblet cell hyperplasia are characteristic of the asthmatic condition. Mucins are the predominant solid components of mucus and are released from goblet cells via exocytosis. *In vitro* studies suggest that mucin granule exocytosis is comparable to mast cell degranulation and requires Ca^{2+} signalling. The ion channels $K_{Ca}3.1$ and Orai modulate Ca^{2+} signalling in a variety of cell types, and have been shown to directly regulate mast cell degranulation. Consequently, investigating whether $K_{Ca}3.1$ and Orai channels play a role in mucin granule release could provide insight into mechanisms of mucin hypersecretion in asthma.

1.7   *Hypothesis*

$K_{Ca}3.1$ and Orai channels play key roles in the mucin expression, production and secretion, and thereby contribute to the hypersecretory phenotype of asthma. The proposed role of $K_{Ca}3.1$ and Orai channels is summarised in **Figure 1-9**.

1.8   *Aims*

The aims of this research are to examine:

- The expression and activity of $K_{Ca}3.1$ and Orai channels in human bronchial epithelial cells derived from patients with asthma and healthy controls
- The expression of MUC5AC mucin in the bronchial epithelium in patients with asthma and healthy controls
- Methods of quantifying mucin expression, production and secretion from *in vitro* cultures
- The effect of Orai and $K_{Ca}3.1$ blockade on mucin expression, production and secretion from *in vitro* cultures
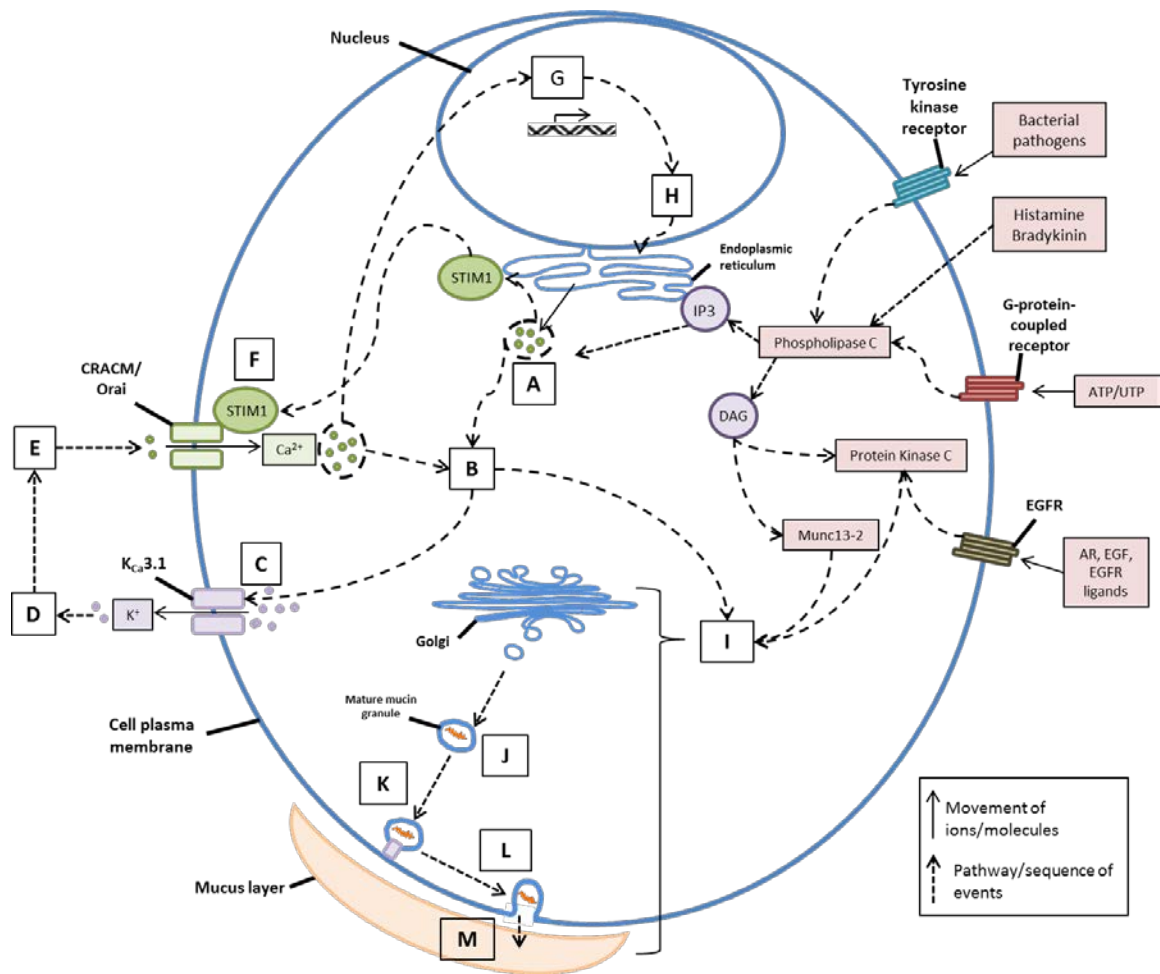
Figure 1-9: Diagram representing the hypothetical role of $K_{Ca}3.1$ and Orai channels in mucin secretion from goblet cells.

The letters (**A**) – (**M**) identify a process rather than a sequence of events. (**A**) Intracellular Ca^{2+} store depletion. (**B**) Spatial and temporal fluctuations in $[Ca_{2+}]_i$. (**C**) Activation of $K_{Ca}3.1$. (**D**) Membrane potential hyperpolarisation. (**E**) Increased driving force for Ca_{2+} influx. (**F**) Activation of Orai channels by STIM1. (**G**) Mucin gene expression. (**H**) Mucin protein synthesis. (**I**) Mucin granule exocytosis. (**J**) Chaperoning and translocation of mucin granule by MARCKS. (**K**) Fusion with core exocytic machinery. (**L**) Actin cytoskeleton disassembly. (**M**) Release of mucin glycoproteins into mucus layer. Apical and basolateral membranes of the cell are not indicated. Full pathways leading to mucin gene expression are not shown. Tyrosine kinase receptor, G protein-coupled receptor and EGFR are shown in diagram, but do not represent the only receptors responsible for triggering mucin secretion.

Chapter 2:

Methods

2.1   Subjects

For the study of gene and protein expression, patch clamp electrophysiology and immunohistochemistry, patients with asthma and healthy volunteers were recruited from respiratory clinics, from staff at Glenfield Hospital, and from the general population through advertisements. Patients with asthma exhibited objective evidence for variable airflow obstruction, indicated by one or more of the following: methacholine airway hyperresponsiveness ($PC_{20}FEV_1$, <8 mg/ml); greater than 15% improvement in FEV_1 10 min following 200 μg inhaled salbutamol; peak expiratory flow (PEF; >20% maximum within-day amplitude from twice-daily peak expiratory flow measurements over 14 days). Asthmatic patients also provided a suggestive history indicative of asthma. All subjects underwent spirometry tests to assess lung function and airway obstruction, and allergen skin prick tests (against *Dermatophagoides pteronyssinus*, dog, cat, grass pollen, and *Aspergillus fumigatus*). Subjects also took a methacholine inhalation test (tidal breathing method) to assess reductions in lung function, an indicator of asthma; methacholine induces bronchial spasms in hyperresponsive airways. Sputum was induced by incremental concentrations of nebulised hypertonic saline (Brightling et al. 2000). Asthmatic patients recorded daytime and night-time symptoms, daily SABA use, and twice-daily PEF, for 2 weeks prior to bronchoscopy. Asthma severity was defined according to the "British guideline on the management of asthma" treatment steps: Step 1, mild, β_2-agonist as required; Step 2 and 3, moderate, with inhaled corticosteroid dose ≤800 mg of beclomethasone equivalent per day with or without LABA; Step 4 and 5, severe, with Step 5 patients receiving courses of oral corticosteroids (British Thoracic Society 2014).

2.2   In vitro cell culture

2.2.1   Primary HBECs

2.2.1.1   Isolation from bronchial brushings

Primary HBECs were isolated from healthy control donors and patients with severe asthma by bronchial brushings obtained during fiberoptic bronchoscopy

(Brightling et al. 2000). Brushings were incubated overnight at 4°C in Bronchial Epithelial Basal Medium (BEBM) media (Lonza), supplemented with a SingleQuot kit (Lonza). The SingleQuot kits contained bovine pituitary extract, recombinant human insulin, hydrocortisone, gentamicin and amphotericin-B (GA-1000), retinoic acid, transferrin, triiodothyronine, epinephrine, and recombinant human epithelial growth factor. 0.3% fungizone (Gibco; amphotericin-B with sodium deoxycholate) and 1% antibiotic-antimycotic solution (AA; Invitrogen; 100 units/ml penicillin, 100 mg/ml streptomycin sulphate, and 0.25 µg/ml amphotericin-B) was also added to the media, herein referred to as Bronchial Epithelial Growth Media (BEGM). Brushings were shaken vigorously to dislodge cells from brushes. After removing brushes, the cell suspension was centrifuged (1300 rpm, 8 min) and the supernatant was discarded. Cells were resuspended in BEGM and then split between 4 wells of a 12-well plate pre-coated with 30 µg/ml bovine collagen type I PureCol solution (Advanced BioMatrix) diluted in Dulbecco's phosphate-buffered saline (PBS; Sigma, containing $MgCl_2$ and $CaCl_2$). These cultures will herein be referred to as freshly brushed cultures.

2.2.1.2 Culturing undifferentiated, submerged HBECs

Freshly brushed cultures were fed 3 times weekly until confluence was reached. Cells were washed with PBS, treated with trypsin-EDTA solution (Sigma) and then centrifuged (1300 rpm, 8 min). Viable cells were counted on a haemocytometer using trypan blue before being plated out into pre-coated T75 cm^2 flasks or 6-well plates, as required. These cultures will herein be referred to as submerged cultures.

2.2.1.3 Culturing differentiated HBEC ALI cultures

Submerged HBECs were seeded into the apical chamber of 12-well Transwell plates with 0.4 µm polyester membranes (Costar, Invitrogen) pre-coated with 30 µg/ml PureCol. Cells were seeded at a density of 2×10^5 cells per well and then incubated at 37°C with 95% air, 5% CO_2. Each Transwell was fed with 800 µl BEGM in the basolateral chamber and 200 µl BEGM in the apical chamber.

Upon reaching confluence, media in the basolateral chamber was replaced with ALI media consisting of BEGM and Dulbecco's Modified Eagles Medium (DMEM; Invitrogen; containing 400 mM glucose) at a ratio of 1:1. All media was supplemented with a SingleQuot kit (Lonza), 0.3% fungizone (Invitrogen), 1% AA, and 100 nM retinoic acid (Sigma). Media in the apical chamber was removed to expose the apical surface of the cell culture to air, thus creating an air-liquid-interface (ALI). On approximately Day 10 after emersion to ALI, apical surfaces of cultures were gently washed with 200 µl PBS to promote mucus secretion and cilia expression. These cultures will herein be referred to as ALI cultures or differentiated cells, and were only used for experiments if they expressed cilia, as visualised using a light microscope.

2.2.2   *H292 cells*

The human lung cancer cell line NCI-H292 (herein referred to as H292 cells) and the virus-transformed normal HBEC cell line BEAS-2B were used as alternatives to primary HBECs. Both cell lines were purchased from American Type Culture Collection.

H292 cells were cultured in RPMI-1640 (LGC Standards) supplemented with 10% FBS (Invitrogen) and 1% AA in a 5% CO_2 incubator. Culture containers did not require coating before plating cells. For establishment of ALI cultures, H292 cells were plated at a density of 3 x 10^5 cells per well on polyester Transwell membranes (12 mm diameter, 0.4 µm-pore size, 12-well plate, Transwell Clear; Costar) and maintained in RPMI-1640 until confluent. Once confluent, media from the apical chamber of the Transwell was removed expose the culture to air. Cells were maintained at ALI with RPMI-1640 supplemented with 10% FBS and 1% AA.

2.2.3   *Human lung mast cells*

Human lung mast cells (HLMCs) were purified from macroscopically normal human lung tissue obtained within one hour of resection for lung cancer (British Thoracic Society et al. 2014). Tissue was cut into small pieces and incubated

overnight at 4°C in DMEM with Glutamax™ and HEPES (Invitrogen) and 2% heat-inactivated FBS (Invitrogen), and then treated with collagenase type 1A (Sigma) and hyaluronidase (Sigma) for 75 min. Digested tissue was filtered sequentially through 100 and 50 μm gauze, and then centrifuged and washed repeatedly to remove unwanted debris. Non-specific antibody binding was inhibited by incubating filtrate with Hank's Balanced Salt Solution (HBSS) and 2% FBS, 1% horse serum, and 0.5 g bovine serum albumin (BSA) for 30 min. HLMCs were purified using immunomagnetic affinity selection with anti-mouse IgG1 magnetic Dynal beads (Fisher Scientific) coated with anti-c-kit CD117 monoclonal antibody (BD Biosciences). The final purity of HLMC cultures was >99%, as visualised using Kimura stain (0.05% toluidine blue solution, 0.03% light green, with saponin and phosphate buffer, pH 6.4). HLMCs were maintained at 37°C with 5% CO_2/95% air in DMEM containing Glutamax™ and HEPES (Invitrogen), supplemented with 10% heat-inactivated FBS, 1% AA, 100 ng/ml recombinant human stem cell factor (rh-SCF), 50 ng/ml rh-IL-6, and 10 ng/ml rh-IL-10 (R&D Systems).

2.2.3.1 Activation of mast cells

HLMCs at 5 x 10^5 cells per ml were activated for 1 or 16 h with anti-FcεRI antibodies (Millipore; 1/300 dilution). Secreted mediators and cytokines were harvested by centrifuging (1300 rpm, 8 min) and collecting the supernatants. As a control, supernatants were harvested from non-activated HLMCs at 5 x 10^5 cells per ml. For experiments, H292 ALI cultures were incubated for 24 h with activated HLMC supernatants, 1 ml supernatant per culture.

2.2.4 *Human lung myofibroblasts*

Human lung myofibroblasts (HLFs) were grown from explanted lung tissue obtained from macroscopically healthy human lung specimens collected within one of resection for lung cancer. HLF cultures were maintained at 37°C with 5% CO_2/95% air in DMEM containing Glutamax™ and 4.5 g/L glucose (Invitrogen), supplemented with 10% FBS, 1% AA, and 100 μM non-essential amino acids (Invitrogen) (Shikotra et al. 2012).

2.3   qPCR

The quantity of messenger RNA (mRNA) encoding either Orai channel subunits or MUC5AC was measured using quantitative real time polymerase chain reaction (qPCR) with TaqMan probes. The probes are customised sequences of oligonucleotides complementary to the target DNA sequence, and have fluorophore and quencher components at the 5' and 3' ends of the oligonucleotide, respectively. During qPCR reactions the probe binds to its complementary sequence downstream from a sequence-specific PCR primer. DNA synthesis is carried out by Taq DNA polymerase to extend the primer. Upon reaching the probe, Taq polymerase cleaves the probe from the 5' end, allowing the fluorophore to be released and preventing the quenching activity of the 3' quencher. The resulting increase in fluorescence is detected by a real-time PCR thermocycler, and is assumed to be directly proportional to the amount of double-stranded DNA (dsDNA) in the PCR reaction. PCR instruments drive DNA amplification with a thermal cycler and simultaneously use an optical system to excite fluorophores and detect the fluorescence emitted from each reaction. The software collects the quantitative data.

The quantity of $K_{Ca}3.1$ mRNA was measured using SYBR Green-based qPCR. SYBR Green is a fluorescent dye that binds to dsDNA generated during amplification by DNA polymerases. SYBR Green is capable of producing low fluorescence when unbound, but when it intercalates between dsDNA bases it fluoresces more brightly. Exposure of the dye to a specific wavelength of light results in brighter fluorescence, which is detected by a real-time PCR thermocycler, and is assumed to be directly proportional to the amount of dsDNA in the reaction. SYBR Green binds to non-specific dsDNA so will not distinguish between target sequences and unwanted products such as primer-dimers. Consequently, PCR products were sequenced to ensure that the correct target sequence had been amplified.

2.3.1   Total RNA isolation

Total RNA was isolated from *in vitro* cell cultures using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

Cells were lysed by Buffer RLT, which contains a guanidine salt and inactivates RNases to prevent RNA degradation. Lysates were collected using a sterile plastic cell scraper, before undergoing homogenisation in the QIAshredder column (Qiagen) to shear genomic DNA and reduce sample viscosity. RNA within homogenised lysates was harvested by centrifuging samples with an RNeasy Spin column, which contains a silica gel-based membrane to which RNA adheres. RNA adherence was promoted by the presence of ethanol; the alcohol provides a lower dielectric constant in the presence of salt and encourages precipitation of RNA to improve its binding to the membrane. Contaminants were removed by washing the column with the stringent wash Buffer RW1, which contained a guanidine salt and ethanol, and efficiently removed non-specifically bound contaminants. Genomic DNA was destroyed by treating the spin columns with DNase I (Qiagen) for 15 min at room temperature. DNase I, contaminants and traces of salts were removed by washing again with Buffer RW1, and twice with Buffer RPE containing ethanol. Residual ethanol was removed from the columns by centrifuging for 1 min at 17,000 x g; contamination of RNA by ethanol will prevent optimal DNA polymerase activity in qPCR. RNA was eluted from the columns with RNase-free water and stored at -80°C until needed. RNA concentrations were determined by the Thermo Scientific NanoDrop 8000 Spectrophotometer.

2.3.2   *First strand cDNA synthesis*

First strand cDNA synthesis was used to provide a suitable template for use with TaqMan and SYBR Green qPCR kits. cDNA was synthesised from total RNA using the RevertAid cDNA synthesis kit (Fisher Scientific). Including a separate step for cDNA synthesis also reduced variability between the housekeeping gene and gene of interest in qPCR reactions.

RNA was incubated for 5 min at 65°C with random hexamer primers to form sequences of dsRNA/DNA to which reverse transcriptase could bind. A reaction buffer containing 500 mM Tris-HCl, 750 mM KCl, 30 mM $MgCl_2$, and 50 mM dithiothreitol was added to each sample, as well as RiboLock RNase Inhibitor, deoxyribonucleotide triphosphates (dNTPs) and Reverse Transcriptase. cDNA

synthesis was conducted by incubating reactions for 5 min at 25°C, and then 60 min at 42°C for synthesis of cDNA strands from the RNA sample. The reaction was terminated by inactivating the enzyme at 70°C for 5 min.

2.3.3 *TaqMan qPCR*

Target sequences were amplified with the Gene Expression Master Mix (Applied Biosystems) and first strand cDNA samples, with 100 ng/µl cDNA per reaction. The master mix contained ultra-pure AmpliTaq Gold DNA polymerase, dNTPs, Uracil-DNA Glycosylase (UDG), and ROX reference dye. UDG reduced contamination of qPCR reactions by excising dUTP and thus eliminating uracil-containing templates (Longo et al. 1990). Orai1 (Hs00385627_m1), Orai2 (Hs00259863_m1), Orai3 (Hs00752190_s1), and MUC5AC (Hs00873651_mH) TaqMan probes (Applied Biosystems) were used alongside probes targeting ribosomal 18S mRNA (Hs99999901_s1), here used as a housekeeping gene.

Initially, reactions were heated to 50°C to allow optimal UDG activity, followed by 95°C for 10 min to activate the AmpliTaq Gold DNA polymerase and ensure simultaneous denaturing of DNA. A thermal profile of denaturing at 95°C for 15 sec, and annealing and extension at 60°C for 1 min, was repeated for 50 cycles to allow amplification of the target sequence. Reactions were run on a Stratagene Mx3000P real-time thermocycler (Agilent Technologies).

2.3.3.1 *TaqMan PCR efficiency*

To compare mRNA expression levels of each Orai subunit homologue, TaqMan probes were assessed for their amplification efficiency. When comparing the expression of different PCR products, the efficiency of a qPCR reaction conducted with each probe must be similar to ensure the comparison is relative to the quantity of target RNA and not affected by discrepancies between probe amplification efficiencies. Ideally, the quantity of PCR product should double with every cycle. To assess qPCR efficiency, a series of RNA dilutions covering 7 concentrations were generated, and the C_T value for each dilution was determined.

Each RNA concentration was run in triplicate. The C_T value was plotted against $\log_{10}$ cDNA concentration and the gradient of the plot was recorded for each TaqMan probe. An example of this is shown in **Figure 2-1**.

To calculate the efficiency of each probe, the following equation was used:

$$\% \text{ efficiency} = (10^{(-1/\text{gradient})}-1) \times 100$$

The R^2 value, the coefficient of determination, was also noted to show the how close the PCR replicates are to the fitted regression line. Ideally, the R^2 value should be as close to 1 as possible; variations in triplicates reduce R^2.

The efficiencies calculated for each probe are:

Probe	Gradient	Efficiency (%)
Orai1 (Hs00385627_m1)	-3.3322	99.6
Orai2 (Hs00259863_m1)	-3.3381	99.3
Orai3 (Hs00752190_s1)	-3.4531	94.8

The similar efficiencies for Orai1 and Orai2 TaqMan probes allow a direct comparison between these two reactions. However, the slight difference in efficiency for Orai3 TaqMan probes prevented a direct comparison between all three. Consequently, the mRNA expression of each Orai subunit was assessed individually.
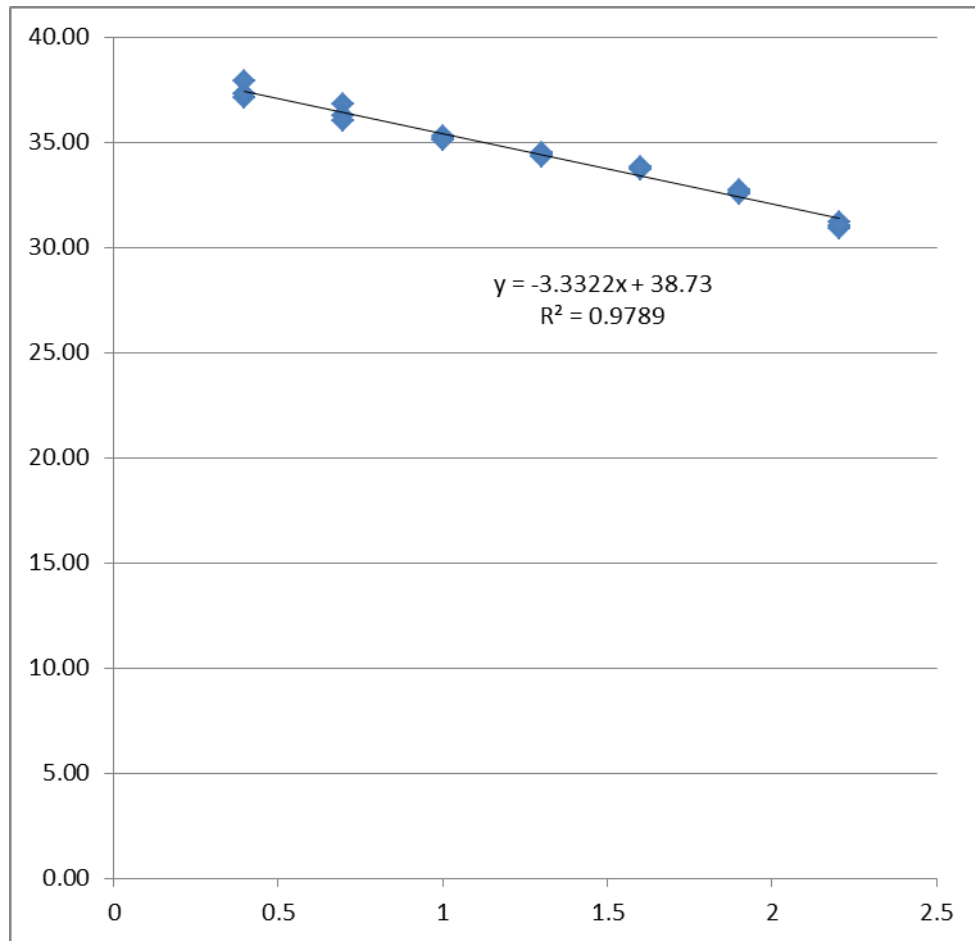
Figure 2-1: Determination of efficiency of Orai1 TaqMan probe assay using the C_T slope method. A series of 7 RNA concentrations were used, generated by serial dilution. The calculated efficiency for this assay was 99.6%. Each concentration of RNA was run in triplicate, represented by individual points on the graph.

2.3.4 *SYBR Green qPCR*

Target sequences were amplified using the Fast SYBR Green Master Mix (Applied Biosystems) and first strand cDNA samples, with 100 ng/µl cDNA per reaction. The master mix contained SYBR Green I dye, ultra-pure AmpliTaq Fast DNA polymerase, UDG, dNTPs, and ROX reference dye. Commercially available $K_{Ca}3.1$ primers (Qiagen) were used alongside primers targeting the housekeeping gene β-actin (Qiagen).

The reactions were heated to 95°C for 20 sec to activate the AmpliTaq Fast DNA polymerase. Denaturation of the double-stranded template was then achieved by holding the reaction at 95°C for 3 sec, before the temperature was lowered to 60°C for 30 sec to allow primer annealing and extension of a new DNA strand. The denaturation, annealing and extension stages were repeated for 40 cycles. Reactions were run on the Stratagene Mx3000P real-time thermocycler.

2.3.5 *Calculating the expression of target mRNA*

2.3.5.1 The ΔC_T method

The ΔC_T method was used to calculate the expression of Orai, $K_{Ca}3.1$ and MUC5AC mRNA in *in vitro* cultures, normalised to the expression of a housekeeping gene. All expression data were corrected using the reference dye ROX. The fluorescence emission was expressed as dRn (magnitude of fluorescence signal at each time point; determined by subtracting no template control signal from the normalised reporter signal), and was plotted against the cycle threshold (C_T; cycle number at which the fluorescence for the reaction crosses the threshold level). For TaqMan PCR, all data were normalised to 18S mRNA. For SYBR Green PCR, all data were normalised to β-actin mRNA. ΔC_T values were calculated as the difference in C_T between the gene of interest and the housekeeping gene, used as an endogenous control, for each sample: CT(gene of interest) – CT(18S/β-actin). C_T values were of a log scale, base 2, and calculated per 10^6 18S or 10^3 β-actin mRNA, as appropriate. Accordingly, to find the linear fold change in gene expression between the gene of interest and control samples, the following formula was used: $(1 \times 10^x) \times (2^{\Delta CT})$.

2.3.5.2 The $\Delta\Delta C_T$ method

The $\Delta\Delta C_T$ method was used to calculate the expression of MUC5AC mRNA in *in vitro* cultures treated with different experimental conditions, normalised to the expression of the housekeeping gene 18S. All expression data were corrected using the reference dye ROX and normalised to 18S mRNA using the ΔC_T. For relative quantification to assess the fold change caused by different treatment conditions, the $\Delta\Delta C_T$ method was used. $\Delta\Delta C_T$ values were calculated as the difference between the ΔC_T of the target gene in an experimental sample and the ΔC_T of the same gene in a control sample. C_T values were of a log scale, base 2. Consequently, to find the linear fold change in gene expression between the experimental and control samples, the following formula was used: 2^-[(C_T(gene of interest)-C_T(internal control)], or $2^{-\Delta\Delta C_t}$. Expression of the gene of interest (MUC5AC) is expressed as relative expression (i.e. fold change above control).

2.3.6 *Analysis of qPCR products by gel electrophoresis*

2% agarose gels were made using UltraPure Agarose (Sigma) with 50X Tris acetate-EDTA (TAE) buffer (Fisher Scientific UK) diluted to 1X with pure water. TAE allowed conductance of a current through the gel, and provided denaturing conditions to ensure linearity of DNA fragments. TAE also provided a consistent neutral pH during electrophoresis to allow the phosphate backbone of DNA, which has a net negative charge, to migrate towards the anode. Gels were treated with 0.5 µg/ml ethidium bromide (Sigma) to allow visualisation of the PCR products when exposed to UV light (ethidium bromide fluoresces when exposed to UV light after becoming intercalated within the major groove of the dsDNA). Samples were mixed with Blue/Orange loading dye (Promega) to aid loading into the gel's wells and to visualise their migration during electrophoresis. Samples were run alongside a 100 bp DNA ladder (Promega) to allow calculation of the product size. Gels were submerged in 1X TAE and run at 80 V for 1 h to separate DNA fragments according to their length. DNA fragments were visualised by exposing the gel to UV light using a UV Transilluminator (Gene Genius) and GeneSnap image acquisition software.

2.4   Western blotting

Expression of Orai and $K_{Ca}3.1$ protein were detected by western blotting, utilising the specificity of primary antibodies for the protein of interest, and the detection of successful antibody-binding by chemiluminescence.

2.4.1   Protein extraction

Cells were lysed by applying 500 µl radio-immunoprecipitation assay (RIPA) buffer to confluent cultures in T75 cm² flasks and using a sterile plastic scraper to harvest the lysates. RIPA buffer contained 50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS), as well as phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Santa Cruz) and a protease inhibitor cocktail (Sigma) were added to the buffer. Sodium orthovanadate is an inhibitor of protein tyrosine phosphatases, alkaline phosphatases and several ATPases; PMSF inhibits serine and cysteine proteases and acetylcholinesterase. Lysates were incubated on ice for 30 min to limit protease activity and promote cell lysis, and then centrifuged for 15 min (10,000 rpm at 4°C) to remove unwanted cell debris. The supernatant, here on in referred to as the lysate, was stored at -80°C.

The protein concentration of lysates was assessed using the colorimetric Bio-Rad DC Protein Assay. This assay is similar to the Lowry assay, in which total protein concentration is determined by measuring a colour change of the sample solution in proportion to protein concentration, but achieves faster colour development. Protein samples, diluted by 1/10 with RIPA buffer, were incubated with Reagent A/S, an alkaline copper tartrate solution, to allow copper treatment of the protein. Reagent B, a dilute Folin reagent, was also added to each sample. Copper-treated proteins reduce the Folin reagent, resulting in a blue colour. Colour was developed at room temperature for 15 min. The absorbance of samples was detected at a wavelength of 750 nm. The concentration of protein in lysates was determined by extrapolation from a standard curve produced using known concentrations of bovine serum albumin, ranging from 0 to 1.45 mg/ml. Final concentrations were calculated by multiplying by 10 to account for the 1/10 dilution in RIPA buffer.

2.4.2   *Protein electrophoresis and transfer*

Lysates were mixed with Laemmli buffer (Sigma) and denatured for 5 min at 100°C. Laemmli buffer contains 2-mercaptoethanol (reduces intra and inter-molecular disulphide bonds), SDS (to denature proteins and provide an overall negative charge), and also bromophenol blue (a glycerol-based dye to assist layering of samples into wells and visualisation of electrophoresis). Denatured samples were loaded onto precast polyacrylamide SDS Mini-PROTEAN TGX gels (Bio-Rad) alongside the MagicMark XP Western Protein Standard (Life Technologies). Mini-PROTEAN Tetra cell tanks (Bio-Rad) were filled with Tris-glycine running buffer (25 mM Tris base, 190 mM glycine, 0.1% SDS, pH 8.3). Gels were run at 200 V for 35 min. Proteins were separated according to their molecular weight, aided by the presence of the anionic detergent SDS; coating of proteins with a uniform charge facilitates their separation according to their size.

Proteins were transferred via a semi-dry method from the gel onto 0.45 μm nitrocellulose membrane (Whatman) using the Trans-Blot Turbo system (Bio-Rad). First, gels were submerged in Tris-glycine transfer buffer containing 48 mM Tris, 39 mM glycine, 0.04% SDS and 20% methanol (pH 8.3) and then placed on top of the membrane in between blotting paper (Bio-Rad). The gel and membrane were placed within a cassette with anode and cathode electrode plates. Protein transfer was achieved by running the Trans-Blot Turbo 2.5 A for 30 min; the current induced the transfer of SDS-coated proteins towards the anode and onto the membrane.

2.4.3   *Protein detection*

The membrane was washed with Wash Buffer (1X TBS + 0.1% Tween20). Non-specific antibody binding was inhibited by incubating the membrane with Blocking Buffer (5% milk in 1X TBS + 0.1% Tween20) for 1 h at room temperature. Membranes were probed with primary polyclonal antibodies targeting the protein of interest (**Table 2-1**) diluted in Blocking Buffer overnight at 4°C. Unbound antibody was removed by washing 3 times for 10 min. The membrane was incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated

secondary antibody (Santa Cruz; 1/2000 from 100 μg/ml stock) for 1 h at room temperature, and then washed 3 times to remove excess unbound antibody. Immunoreactive protein bands were visualised by applying Pierce ECL Western Blotting Substrate (Fisher Scientific) to the membrane for 1 min, and then detecting chemiluminescence (ECL acts as a substrate for the peroxidase enzyme) in a pre-cooled ImageQuant LA S 4000 biomolecular imager (GE Healthcare Life Sciences).

B-actin was used as a positive control for the western blot after the target protein had been identified on the blot. To re-stain blots, bound primary and secondary antibodies were removed by incubating the membrane in 0.2 M NaOH for 5 min. Membranes were washed, blocked with Blocking Buffer and then probed with primary monoclonal β-actin antibody HRP conjugate (Santa Cruz; 1/30,000 from 200 μg/ml stock) for 2 h at room temperature. Bands were visualised with Pierce ECL substrate and the ImageQuant LA S 4000.

For $K_{Ca}3.1$ detection, a rabbit polyclonal anti-$K_{Ca}3.1$ antibody from Biorbyt was selected due to its recommendation for western blotting by the manufacturer and prior optimisation by colleagues (Chachi et al. 2013). In contrast, for immunostaining a rabbit polyclonal anti-$K_{Ca}3.1$ antibody (AV35098) from Sigma was used due to its suitability for GMA staining and prior optimisation by colleagues (Roach et al. 2013). Both antibodies targeted the C-terminal of human $K_{Ca}3.1$. The Biorbyt antibody produced immunoreactive bands at the predicted target protein size in western blots. The Sigma antibody was used alongside rabbit IgG as a negative control in immunohistochemistry and this produced no staining.

Primary Antibody Target	Manufacturer	Animal species	Stock concentration	Dilution
Orai1	Alomone Labs	Rabbit polyclonal	0.65 mg/ml	1/1000
Orai2	Alomone Labs	Rabbit polyclonal	0.80 mg/ml	1/1000
Orai3	AbD Serotec	Rabbit polyclonal	1.00 mg/ml	1/100
$K_{Ca}3.1$	Biorbyt	Rabbit polyclonal	0.50 mg/ml	1/500
β-actin	Santa Cruz	Mouse monoclonal HRP conjugate	0.20 mg/ml	1/30,000

Table 2-1: Primary and secondary antibodies used for western blotting and detection of Orai1, Orai2 and Orai3, $K_{Ca}3.1$, and β-actin proteins.

All antibodies were diluted in Blocking Buffer (5% milk in 1X TBS 0.1% Tween20).

2.5   *Patch clamp electrophysiology*

The activity of Orai and $K_{Ca}3.1$ channels were investigated using the electrophysiological technique of patch clamping. This method involves using an electrode within a glass pipette to complete a circuit connected to an amplifier, which is held at a specific voltage. The cell membrane creates resistance by only permitting the carriage of ionic charge through ion channels expressed within the cell membrane. The difference in ionic charge across the membrane creates an electrical potential, known as the membrane potential. The presence and activity of specific ion channels determines the permeability of the cell to certain ions, and thus affects the membrane potential of the cell. Similarly, the size of the current is affected by the passage of ions across the membrane. By altering the ionic concentrations of the experimental solutions, and by adding particular ion channel openers and blockers, the activity of specific types of channels can be isolated and studied.

2.5.1   *Whole-cell patch clamp technique*

Membrane currents were recorded from single cells using the whole-cell variant of the patch clamp technique. Patch pipettes were produced from borosilicate fibre-containing glass (Clark Electromedical Instruments), and tips of the pipettes were heat-polished to create resistances of 6-10 MΩ. For recording, cells were placed in 35 mm dishes containing the standard external bath solution (**Table 2-2**). Pipettes were backfilled with an internal solution, and then inserted into the pipette holder containing a chloride-coated silver wire (the binding of chloride ions to the wire prevented electrical drift during experiments). A seal of at least 1 GΩ was formed between the pipette and cell membrane. Suction was applied to rupture the patch of membrane spanning the pipette and produce continuity between the membrane and pipette. At this point, the cell's contents dialyse and the intracellular conditions are controlled by the internal solution.

Membrane currents were recorded using an Axoclamp 200 A amplifier (Axon Instruments), and currents were evoked by applying voltage commands to a range of potentials (-120 to 100 mV) in 10 mV steps from a holding potential of 0 mV.

Each sequence of steps was conducted during a 100 ms pulse (**Figure 2-2**). Currents were digitized, stored on a computer, and analysed using Clampfit software version 10.3 (Molecular Devices). Cell capacitance was minimised using the capacitance neutralisation circuits on the amplifier.

Prior to patch clamp experiments, primary HBECs were prepared by treating with trypsin-EDTA solution and centrifuging to obtain a cell pellet, before resuspending in fresh media. Cells were incubated at 37°C for 20 min before beginning experiments.

External bath solution (mM)
NaCl, 140
KCl, 5
CaCl$_2$, 2
MgCl$_2$, 1
HEPES, 10

Table 2-2: Recipe for the standard external bath solution used for recording Orai and K$_{Ca}$3.1 channel activity.

The solution was buffered to pH 7.3 with NaOH.
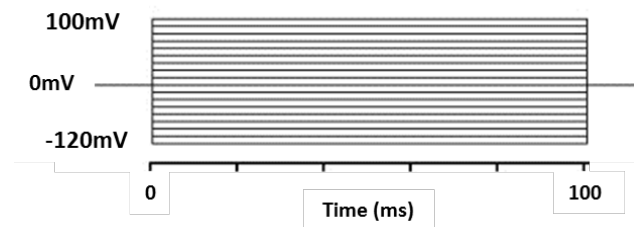
Figure 2-2: Voltage commands at a range of potentials (-120 to 100 mV) in 10mV steps were applied to cells during whole-cell patch clamp experiments.

2.6   *Immunohistochemistry* — *Flow cytometry*

The cell surface expression of the Orai channel was analysed by flow cytometry.

Primary HBECs were grown to confluence in T75 cm² flasks and then harvested using trypsin-EDTA solution. The cell suspension was centrifuged (1500 rpm, 8 min) and the supernatant was discarded. The cells were not fixed to avoid alteration of the antigen by the fixative, which could potentially prevent detection of the antigen by the antibody. Cells at a density of 2×10^5 cells per tube were incubated with PBS-2% FBS and 47 μg/ml anti-human IgG antibody on ice for 30 min to block Fc receptors and reduce the likelihood of false positives.

Cells were incubated on ice for 30 min with a range of concentrations of polyclonal rabbit anti-Orai1 antibody (Alomone Labs) targeting the extracellular portion of the Orai1 subunit. Unbound antibody was removed by washing twice with 1 ml PBS-2% FBS and centrifuging (1500 rpm, 8 min at 4°C). Cells were resuspended in PBS-2% FBS and incubated with 2 μg/ml secondary goat anti-rabbit allophycocyanin (APC)-conjugated antibody (Invitrogen) on ice in the dark for 30 min. Cells were washed twice to remove unbound antibody. Non-viable cells were identified by staining with 100 μg/ml propidium iodide; viable cells with intact membranes exclude this dye, whereas the membranes of dead cells are permeable. Before detection, cells were resuspended in 400 μl Fluorofix (BioLegend) to preserve the antigen and fluorophore. Orai1 cell surface expression was acquired by flow cytometry using the BD FACSCanto running BD FACSDiva software (BD Biosciences), analysed by FlowJo software and quantified as fold difference in the geometric mean fluorescence intensity (GMFI) in comparison with the rabbit isotype control.

2.7   *Immunohistochemistry*

Immunohistochemistry was used for the immunoenzymatic identification of $K_{Ca}3.1$ and MUC5AC visualised by a coloured end-product. Primary antibodies targeting either antigen were applied to the specimen mounted on a glass slide. The Fc portion of the primary antibody then acted as an antigen for the secondary antibody conjugated to the vitamin biotin, with specificity for the animal species of

the primary antibody. The chemical affinity of streptavidin for biotin permitted labelling of the primary-secondary antibody complex with streptavidin-horseradish peroxidase (Av-HRP). A coloured product was visualised by applying 3-amino-9-ethylcarbazole (AEC) to the specimen; AEC is a chromogenic substrate that produces a red end-product when catalysed by horseradish peroxidase activity. The resulting red colour is visible using a light microscope.

2.7.1 *Preparation of biopsy specimens*

Healthy control and asthmatic donors underwent fiberoptic bronchoscopy (Chachi et al. 2013). Biopsy specimens were collected from a subsegmental bronchus of the left lower lobe. Biopsies were fixed in phenylmethylsulphonyl fluoride (PMSF), iodoacetamide and acetone at -20°C overnight to stabilise tissue proteins and cellular components, and prevent tissue autolysis and putrefaction.

Biopsy specimens were embedded in glycol methacrylate acrylic (GMA) (Shikotra et al. 2012). This method of tissue fixation and embedding provides good antigen presentation and morphology preservation, and allows very thin sections of 1-2 μm to be cut. This is especially useful for small biopsies and when staining sequential sections. Fixed biopsies were incubated in acetone for 15 min at room temperature, and then in methyl benzoate for another 15 min at room temperature. This facilitated the infiltration of the tissue with GMA. Next, the biopsy was incubated at 4°C for 6 h in 5% methyl benzoate in GMA, with replacements of fresh methyl benzoate-GMA solution every 2 h. Biopsies were added to labelled capsules and incubated for 48 h at 4°C in GMA embedding solution containing benzoyl peroxide and N,N-dimethylaniline. This controlled the polymerisation of GMA monomers within the solution and facilitated the formation of a hard, brittle resin. GMA-embedded tissue blocks were stored at -20°C.

2.7.2 *Immunostaining of GMA-embedded bronchial biopsy specimens*

Biopsy specimens were cut into sequential sections of 2 μm thickness using a microtome (Leica) and floated onto a 0.2% ammonia water bath. Sections were

collected on charged Colorfrost Plus glass slides (Fisher Scientific) and left to air-dry for 1-4 h at room temperature, and then stained.

Endogenous peroxidase activity of the tissue was inhibited by applying 0.3% hydrogen peroxide in 0.1% sodium azide and incubating at room temperature for 30 min. This reduced the risk of non-specific background staining with AEC by hydrogen peroxidase activity later in the method. Slides were washed with 1X TBS 3 times for 5 min (**Table 2-3**). Non-specific antibody-antigen interactions were prevented by applying blocking medium for 30 min at room temperature (**Table 2-3**). Blocking medium was discarded, and primary antibodies (**Table 2-4**) diluted in 1X TBS were added to the specimens and incubated with coverslips overnight at room temperature.

Unbound antibody was removed by washing (3 x 5 min) and then incubated with secondary biotinylated antibodies (**Table 2-4**) for 2 h at room temperature. Unbound secondary antibody was removed by washing (3 x 5 min). Av-HRP solution (Dako) was applied for 2 h at room temperature. Slides were washed (3 x 5min) to remove excess Av-HRP, and then AEC (A Menarini Diagnostics Ltd) was applied for 2-10 min (**Figure 2-3**). Reactions were stopped by rinsing 1X TBS. Slides were washed under running tap water for 5 min. Specimen sections were counterstained with Mayer's haematoxylin (Sigma) for 10 min, which selectively stains nuclear chromatin to aid visualisation of the tissue and cellular structure (**Figure 2-3**), and then placed under running tap water for 30 sec (this provides a slightly alkaline solution to induce the characteristic blue colour of the haematoxylin stain). Slides were drained and coated with SuperMount (BioGenex), a mounting media that aids preservation of specimen sections for long-term storage. Slides were dried overnight in the dark at room temperature before being treated with DPX mountant (Sigma) and covered with a glass coverslip.

10X Tris-buffered saline (TBS)	Blocking medium
40 g NaCl	80 ml DMEM (Invitrogen)
3 g Tris	20 ml FBS
8 ml 2 M HCl	1 g BSA
5 L distilled water	

Table 2-3: Recipes for 10X TBS and blocking medium

pH of 10X TBS adjusted to 7.6 with NaOH or HCl as required. 10X TBS diluted to 1X TBS with distilled water.

Primary Antibody	Manufacturer	Polyclonal / Monoclonal	Concentration	Secondary Antibody	Manufacturer
KCNN4 PAb	Sigma	Rabbit polyclonal	5 µg/ml	Swine anti-rabbit IgG biotinylated	Dako
IgG isotype	BD Pharmingen	Rabbit polyclonal	5 µg/ml	Swine anti-rabbit IgG biotinylated	Dako
MUC5AC MAb	Millipore	Mouse monoclonal	20 µg/ml	Rabbit anti-mouse IgG biotinylated	Dako
IgG1 isotype	Dako	Mouse monoclonal	20 µg/ml	Rabbit anti-mouse IgG biotinylated	Dako

Table 2-4: List of primary and secondary antibodies used for immunohistochemistry.

All antibodies were diluted using 1X TBS. MUC5AC Ab = primary monoclonal anti-MUC5AC antibody; KCNN4 PAb; primary polyclonal anti-KCNN4 ($K_{Ca}3.1$) antibody.
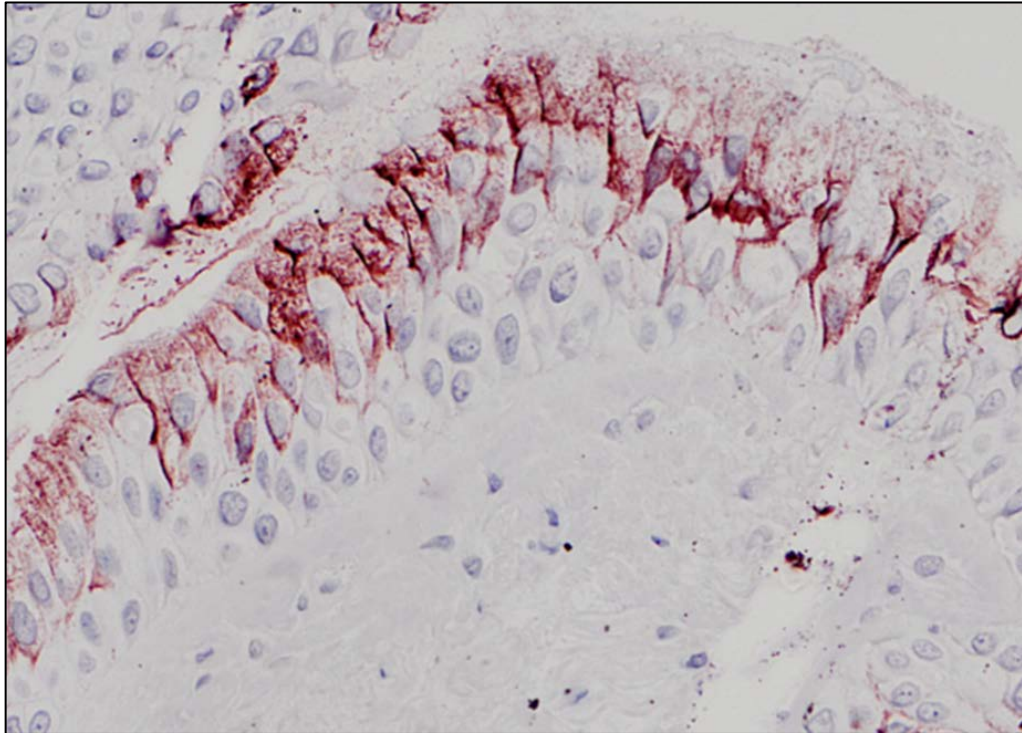
Figure 2-3: Example of immunostaining of human bronchial tissue with MUC5AC MAb.

Visualisation of the target antigen within the tissue section is aided by the AEC substrate system. This involves catalysis of a chromogenic substrate (AEC) by hydrogen peroxidase to produce a red colour. The surrounding tissue structure is visualised by counterstaining with haematoxylin.

2.7.3   Co-localising K$_{Ca}$3.1 and MUC5AC immunostaining

Areas of epithelium within each tissue section were identified using computer-assisted image analysis (Cell^F software, Olympus), and numbers of positively-stained nucleated cells in each area were counted blind. A minimum of 6 areas of epithelium per donor were counted. Cells staining positive for both MUC5AC and K$_{Ca}$3.1 were co-localised by ascertaining a reference point in each tissue section and then identifying cells present in sequential sections stained with MUC5AC MAb or KCNN4 PAb antibodies. The values obtained for each individual donor represent the total average of cells positive for both target antigens within all areas of epithelium per donor biopsy. Results of each donor group are expressed as a percentage of MUC5AC-positive epithelial cells displaying K$_{Ca}$3.1 immunostaining. Each percentage is presented as median ± interquartile range of the values obtained for each individual donor of the group.

2.7.4   Semi-quantitative analysis of K$_{Ca}$3.1 and MUC5AC immunostaining

The amount of positive staining in the epithelium of each biopsy was assessed using a thresholding technique (Shikotra et al. 2012). This was based on the hue, saturation and intensity of immunostaining.

The total area of all intact epithelium was identified and measured using Cell^F software. Damaged areas of epithelium were excluded. For thresholding, detection limits for the hue, saturation and intensity (HSI) of immunostaining were optimised using Cell^F software. Initially, the HSI colour system was visualised within a scale of 0 to 255.

Images of sections were acquired at x20 magnification and white balance was corrected for each individual section. Pixels representative of positive staining within all epithelium of each biopsy were measured. 2 sections were measured for each donor per primary antibody. The median of the lower and upper limit of the HSI values were calculated from 10 biopsies from severe asthmatic patients and healthy controls. Final HSI values were selected as the final threshold, and ensured that saturated red light was captured, representative of positive staining. All

epithelial sections within each bronchial biopsy were blinded and subsequently measured using this final threshold.

Thresholding values were representative of positive MUC5AC or $K_{Ca}3.1$ immunostaining and expressed as an average of the percentage of the total area of bronchial epithelium per biopsy (MUC5AC or $K_{Ca}3.1$ area fraction %). Prism 5 software (GraphPad Software) was used for statistical analysis. Immunohistochemistry data were expressed as median ± interquartile range.

2.8   *Mucin quantification*

2.8.1   *Enzyme-linked lectin assay*

The quantity of mucins within apical washes and lysates of H292 ALI cultures was determined by enzyme-linked lectin assay (ELLA) (**Chapter 5, 5.3.2.5**). Apical washes were collected by incubating the surface of H292 ALI cultures with warm PBS for 5 min, and gently washing the PBS over the culture to maximise collection of mucins. Lysates were harvested by removing the ALI culture from the Transwell and storing in pure water at -80°C.

2.8.2   *Staining intracellular mucins in H292 and HBEC ALI cultures*

Intracellular mucins were identified by immunohistochemistry and staining with MUC5AC MAb (**Chapter 5, 5.3.2.5**). MUC5AC immunostaining was used as an indicator of goblet cells in HBEC cultures, and as a marker of intracellular mucin expression.

Positive immunostaining in H292 cultures was identified by the semi-quantitative thresholding method (**Chapter 5, 5.3.2.4**). Results are expressed as MUC5AC area fraction %.

Positive immunostaining in primary HBEC ALI cultures was identified by counting the number of MUC5AC-positive cells in each section of each culture. Results are expressed as the number of MUC5AC-positive cells per μm^2, calculated by dividing the total number of stained cells per culture by the total area of the culture section.

Each experimental condition was stained in duplicate, and each value represents an average of the two counts.

2.9 *Experimental design*

To examine the role of $K_{Ca}3.1$ in rh-AREG-induced mucin production, MUC5AC mRNA expression in H292 cells was assessed by qPCR, and the mucin content of apical washes and lysates of H292 ALI cultures was analysed by ELLA and immunohistochemistry. For qPCR, H292 cells were grown to confluence in 6-well plates and treated with 10 ng/ml rh-AREG (R&D Systems) for 24 h. For ELLA or immunohistochemistry, H292 cells were grown into ALI cultures and maintained for 48 h before treating with rh-AREG for 24 h. RNA, apical washes and lysates were harvested after 24 h.

To examine the role of $K_{Ca}3.1$ in rh-IL-13-induced mucin production, primary HBEC ALI cultures were treated with 10 ng/ml rh-IL-13 (R&D Systems) for 12 days. Fresh media, supplemented with cytokines and blockers as applicable, was added 3 times weekly. Apical washes were collected on Day 0, 3, 5, 7, 9 and 12. Lysates and RNA were harvested on Day 12.

For $K_{Ca}3.1$ blockade, H292 and HBEC cultures were treated with 200 nM TRAM-34 or 100 nM ICA-17043, with a final DMSO concentration of 0.1%, for 30 min prior to stimulation with rh-AREG or rh-IL-13. $K_{Ca}3.1$ blockers were used alongside 0.1% DMSO and 200 nM TRAM-7 as negative controls. TRAM-7 has a similar structure to TRAM-34 but does not block $K_{Ca}3.1$ activity.

Chapter 3:

Orai channel expression in human bronchial epithelial cells

3.1   *Introduction*

Ca^{2+}-release activated Ca^{2+} entry via Orai channels is a predominant mechanism of Ca^{2+} influx, contributing to intracellular Ca^{2+} signalling and thus initiating downstream pathways. In mast cells, these channels modulate cellular processes such as degranulation (Parekh et al. 2005, Parekh 2010, Ashmole et al. 2013, Ashmole et al. 2012, Di Capite et al. 2009b). The mechanism of granule release is thought to be similar to mucin granule exocytosis from goblet cells, but the expression of the Orai channel in primary HBECs has not been examined before.

Mammalian cells express 3 different homologues of the channel subunits, herein referred to as Orai1, Orai2 and Orai3. Orai1 has been identified as the Ca^{2+}-selective pore-forming protein expressed within the plasma membrane of HLMCs and T cells (Prakriya et al. 2006, Yeromin et al. 2006). Orai2 and Orai3 homologues exhibit a significant degree of sequence homology with Orai1 but also reportedly have different functional properties, as shown in the HEK293 cell line (Lis et al. 2007, DeHaven et al. 2007). Consequently, the expressions of all 3 Orai channel subunit homologues were examined in primary HBECs.

3.2   *Methods*

3.2.1   *Subjects*

Patients with asthma and healthy volunteers were recruited (**Chapter 2, 2.1**) for the study of gene and protein expression, patch clamp electrophysiology and flow cytometry.

3.2.2   *In vitro cell culture*

Primary HBECs were isolated from healthy control and asthmatic donors and grown as submerged or differentiated ALI cultures (**Chapter 2, 2.2.1**). Primary HLMCs were purified from macroscopically normal human lung specimens (**Chapter 2, 2.2.3**).

3.2.3   *Quantification of Orai mRNA expression using TaqMan qPCR*

Total RNA was isolated from *in vitro* cultures and used for first strand cDNA synthesis (**Chapter 2, 2.3**). Orai1, Orai2 and Orai3 mRNA expression was quantified by qPCR with TaqMan probes alongside probes targeting the housekeeping gene, ribosomal 18S (**Chapter 2, 2.3.3**). Only experiments resulting in a clear, single PCR product band of the appropriate fragment size were used; the expected product size with Orai1, Orai2, Orai3, and 18S TaqMan probes were 110, 111, 153, and 187 base pairs (bp), respectively. PCR products were analysed by gel electrophoresis (**Chapter 2, 2.3.6**).

All expression data were corrected using the reference dye ROX and normalised to 18S mRNA using the ΔC_T method (**Chapter 2, 2.3.5.1**). C_T values were of a log scale, base 2, and calculated per 10^6 18S mRNA. All data are expressed as median (interquartile range; IQR).

3.2.4   *Detecting Orai protein expression by western blotting*

Expression of Orai proteins were detected by western blotting (**Chapter 2, 2.4**). Target proteins were identified with primary polyclonal anti-Orai1 (0.65 μg/ml), anti-Orai2 (0.80 μg/ml), and anti-Orai3 (10 μg/ml) antibodies, labelled with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (0.05 μg/ml). Immunoreactive protein bands were visualised by applying Pierce ECL Western Blotting Substrate and detecting chemiluminescence. B-actin was used as a positive control for the western blot and was identified by primary monoclonal β-actin antibody HRP conjugate (7 ng/ml).

3.2.5   *Patch clamp electrophysiology*

Orai channel activity in primary cells was examined using the whole cell patch clamp technique with specific experimental solutions (**Table 3-1**; **Chapter 2, 2.5**). Prior to experiments, primary HBECs were prepared by treating with trypsin-EDTA solution and centrifuging to obtain a cell pellet, before resuspending in fresh media. Cells were incubated at 37°C for 20 min before beginning experiments. Primary HLMCs did not require treatment with trypsin-EDTA.

Membrane currents were evoked by applying voltage commands to a range of potentials (-120 to 100 mV) in 10 mV steps from a holding potential of 0 mV during a 100 ms pulse. Upon reaching whole-cell status, cell contents became dialysed with the internal solution of the pipette (**Table 3-1**). Membrane currents were recorded from cells bathed in the standard external solution (this condition is herein referred to as "baseline") according to the different experimental designs. Characteristic electrophysiological features of the Orai channel include the appearance of a current over 5 min as Ca^{2+} stores are depleted, a positive shift in reversal potential, and strong inward rectification. To confirm the presence of Orai channel currents, 3 μM of the Orai channel blocker GSK-7975A, with a final DMSO concentration of 0.1%, was added to specifically block Orai channel activity (Prakriya et al. 2006, Yeromin et al. 2006). All data are expressed as mean ± SEM.

External bath solution (mM)	Internal solution (mM)
NaCl, 140	CsCl, 140
KCl, 5	$MgCl_2$, 2
$CaCl_2$, 2	HEPES, 10
$MgCl_2$, 1	EGTA, 5
HEPES, 10	IP3, 0.03
	Na^+-ATP, 2
	GTP, 0.1

Table 3-1: Recipes for the standard external bath solution and the internal solution used for recording Orai channel activity.

The solutions were buffered to pH 7.3 with NaOH.

3.2.6 *Flow cytometry*

The cell surface expression of the Orai channel in primary HBECs was analysed by flow cytometry (**Chapter 2, 2.6**). Before staining for target proteins, cells were incubated with anti-human IgG antibody on ice for 30 min to block Fc receptors and reduce the likelihood of false positives. Cells were stained with a range of concentrations (0.1, 0.5, 1.0, 5.0, and 10.0 μg/ml) of polyclonal rabbit anti-Orai1 antibody targeting the extracellular portion of the Orai1 subunit. Cells were labelled with 2 μg/ml secondary goat anti-rabbit APC-conjugated antibody. Non-viable cells were identified by staining with 100 μg/ml propidium iodide. Orai1 cell surface expression was quantified as fold difference in the geometric mean fluorescence intensity (GMFI) in comparison with the rabbit isotype control.

3.3 *Results*

3.3.1 *Expression of the Orai channel by primary HBECs*

3.3.1.1 *Patient characteristics*

In total, HBECs from 9 patients with asthma and 5 healthy controls were used for the following experiments. The patient clinical characteristics are displayed in **Table 3-2**.

3.3.1.2 *Primary HBEC cultures express Orai channel mRNA*

Orai1, Orai2 and Orai3 mRNA were detected in asthmatic ($n = 5$) and healthy ($n = 5$) HBECs cultured in submerged conditions (**Figure 3-1A**). All RNA was isolated from HBECs at either passage 2 or 3. Analysis of the PCR products of reactions with Orai1 (110 bp), Orai2 (111 bp), and Orai3 (153 bp) TaqMan probes using a 2% agarose gel confirmed the presence of a single PCR product of the appropriate size. All reactions were conducted alongside probes targeting the internal normaliser gene, ribosomal 18S, and produced a single product of approximately 187 bp. Similar levels of 18S mRNA expression were seen in all donors HBEC cultures, making it suitable for use as an internal normaliser gene (**Figure 3-1B**).

	Asthmatic *n* = 9	Healthy *n* = 5	P value
Disease severity	Severe	N/A	-
Age (y), mean ± SEM	48.0 ± 5.3	34.6 ± 7.4	0.2075
Sex (M/F)	5/4	3/2	0.9282
Asthma duration (y) , mean ± SEM	24.3 ± 7.3	N/A	-
Inhaled corticosteroid dose (µg), mean ± SEM (beclomethasone equivalents ‡)	1700 ± 181	0	**0.0008**
No. at BTS step 5	3	0	-
No. taking long-acting β-agonist	9	0	-
Exacerbations in last year, median (IQR)	2.0 (1.0 - 2.8)	N/A	-
Sputum eosinophil count (%), median (IQR)	5.3 (2.0 - 17.8)	0	0.0500
FEV1 (% predicted), mean ± SEM	70.8 ± 9.8	104.0 ± 3.3	**0.0186**
FEV1/FVC ratio (%), mean ± SEM	70.1 ± 6.2	84.6 ± 3.5	0.1368
Atopy (yes/no)	7/2	0/5	**0.0072**
Serum IgE (kU/L), median (IQR)	287.0 (66.6 – 372.1)	4.6 (3.1 - 16.9)	**0.0121**

Table 3-2: Clinical characteristics of donors recruited for collection of HBECs by bronchoscopy.

BTS, British Thoracic Society; *N/A*, not applicable; U/L, units per litre; IQR, interquartile range. ‡ Ratio for budesonide Turbuhaler calculated as 1.5; **bold** text highlights statistically significant P values. Data are presented as mean ± SEM or median (IQR).

A
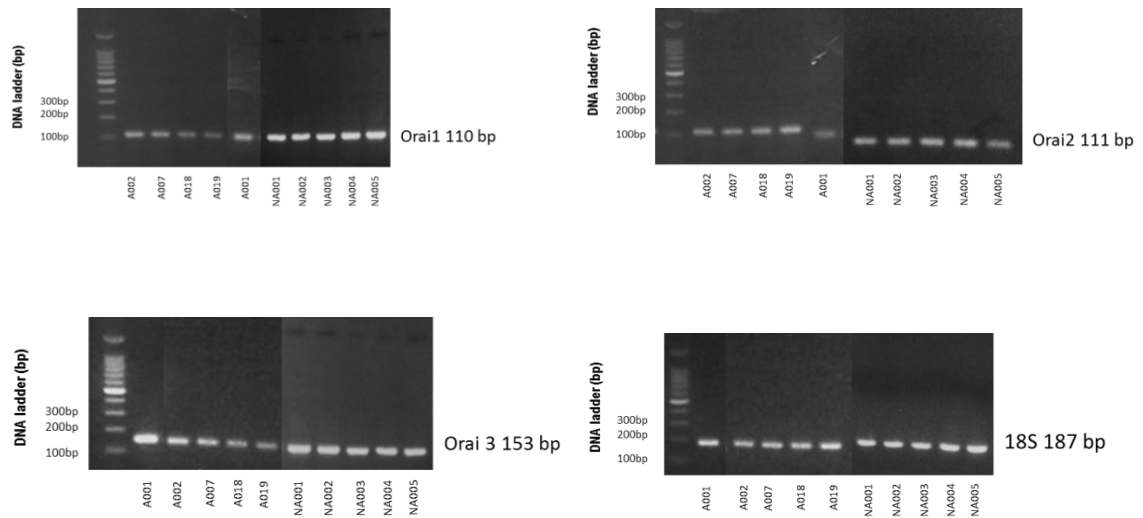
B
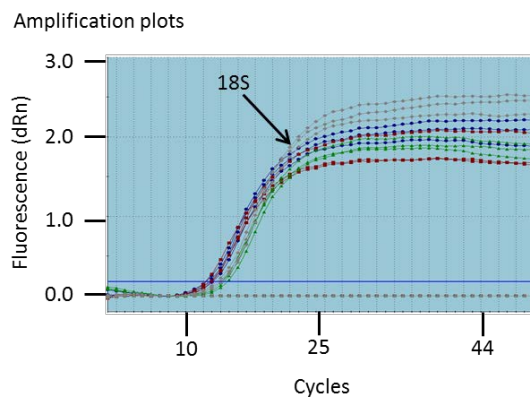
Figure 3-1: Submerged asthmatic (n = 5) and healthy (n = 5) HBEC cultures express Orai1, 2 and 3 mRNA.

(**A**) Products of qPCR for Orai1 (110 bp), Orai2 (111 bp) and Orai3 (153 bp) were visualised on a 2% agarose gel to confirm amplification of a single product of the correct size. Ribosomal 18S (187 bp) was used as the normalising control. (**B**) Similar amplification plots were seen in qPCR with asthmatic and healthy HBECs and 18S TaqMan probes.

The amount of mRNA encoding Orai1 (**Figure 3-2A**), Orai2 (**Figure 3-2B**) and Orai3 (**Figure 3-2C**) subunits were similar in asthmatic and healthy HBECs (Mann Whitney *t*-test).

When cultured at ALI, primary asthmatic HBECs continued to express all 3 Orai homologues at similar levels (n = 5; **Figure 3-3A**). 18S mRNA expression was consistent in all ALI cultures (**Figure 3-3B**). However, levels of 18S mRNA expression varied between submerged and ALI cultures. Consequently, no comparison of the amount of Orai mRNA in submerged and differentiated ALI HBEC cultures could be made (**Figure 3-3C**).

3.3.1.3 *Primary HBECs express Orai channel protein*

Western blots performed with the lysates of submerged primary HBEC cultures (n = 3) revealed the presence of bands close to the predicted molecular weights of Orai1 (32.7 kDa) and Orai2 (28.6 kDa). The anti-Orai3 antibody did not reveal any staining (**Figure 3-4**). These findings are similar to those reported in HLMCs (Ashmole et al. 2012).

A   **B**   **C**
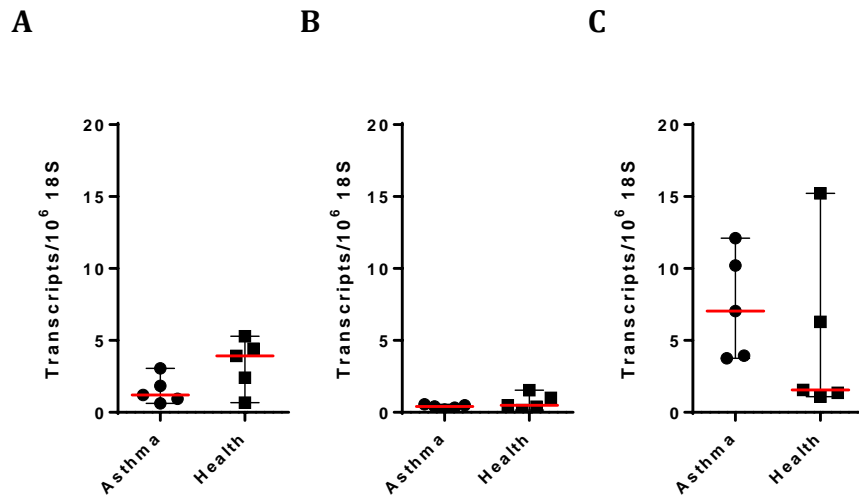
Figure 3-2: Asthmatic and healthy HBECs display similar patterns of Orai mRNA expression.

Comparison of Orai1, Orai2 and Orai3 channel mRNA expression in submerged HBECs isolated from asthmatic (n = 5) healthy (n = 5) donors. No significant differences in the level of expression of (**A**) Orai1, (**B**) Orai2 and (**C**) Orai3 mRNA were seen between asthmatic and healthy HBECs. Data are expressed as median (range).

A
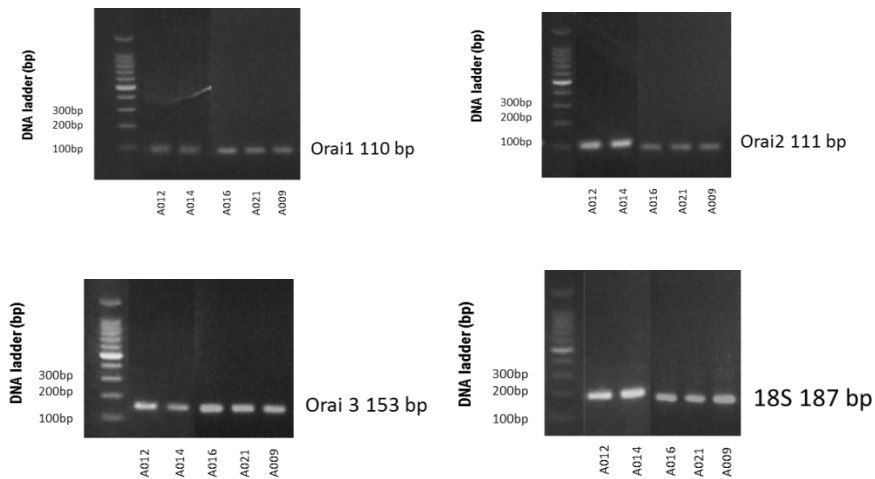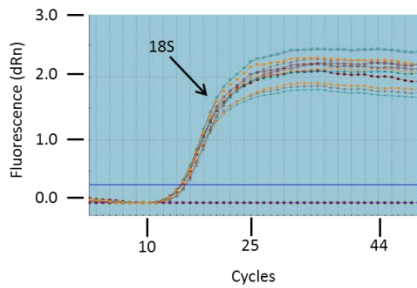
B   **C**
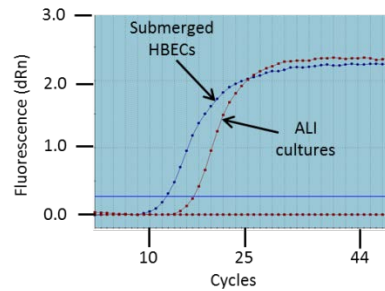
Figure 3-3: Asthmatic HBEC ALI cultures (*n* = 5) express Orai channel mRNA.

(**A**) qPCR products with Orai1 (110 bp), Orai2 (111 bp), Orai3 (153 bp) and r18S (187 bp) TaqMan probes were visualised on a 2% agarose gel. (**B**) Similar levels of 18S mRNA was detected in asthmatic HBEC ALI cultures. (**C**) The amount of 18S mRNA varied between submerged (blue) and ALI (red) cultures.
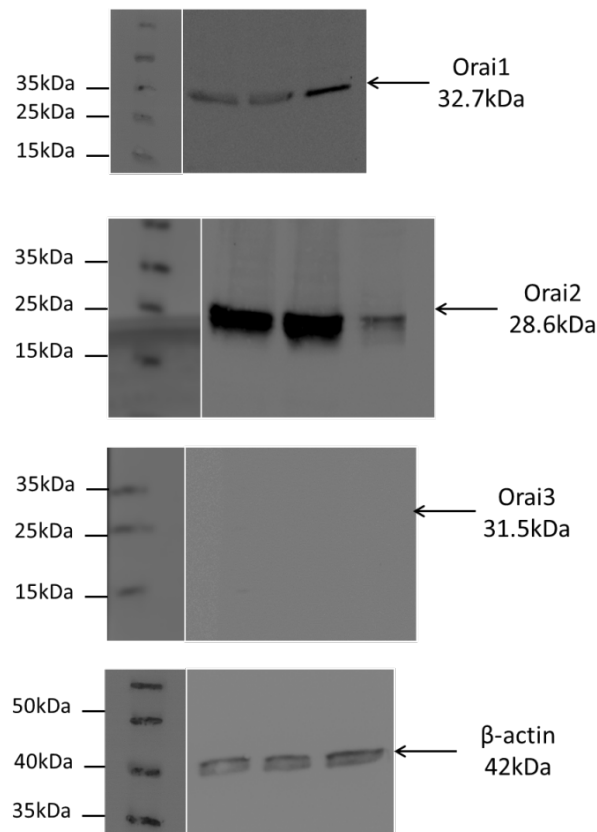
Figure 3-4: Orai1, Orai2 and Orai3 proteins are expressed by primary HBECs.

Representative western blots with antibodies targeting Orai1 and Orai2 and lysates of submerged primary HBECs isolated from 3 donors revealed the presence of bands close to the predicted molecular weight of Orai1 (32.7 kDa) and Orai2 (28.6 kDa). Protein bands in the protein ladder were marked on blots separately using a pen. No staining was achieved with antibodies targeting Orai3 (31.5 kDa). B-actin was used as a positive control (42 kDa).

3.3.1.4 Orai channel activity cannot be recorded from primary HBECs

3.3.1.4.1 Characteristic Orai channel activity is not detectable in submerged HBECs

To record Orai channel electrophysiological activity, membrane currents were recorded from primary HBECs following the application of 3 sequential conditions to the cells.

Initially, HBECs were dialysed with intracellular solution containing 30 µM IP3 and 500 µM EGTA (Baseline). The normal time course for Ca^{2+} store depletion is 5 min (Fierro et al. 1999). Accordingly, once whole-cell status was achieved cells were dialysed with the baseline internal solution for 5 min. During this time, 30 µM IP3 was used to induce depletion of intracellular stores, a prerequisite for Orai channel activation. Concomitant administration of EGTA was used to promote the passive depletion of intracellular stores by chelating $[Ca^{2+}]_i$ and buffering $[Ca^{2+}]_i$ to 0 nM (Hoth et al. 1992, Hoth et al. 1993, Fierro et al. 1999). Extracellular $[Ca^{2+}]$ was raised from 2 mM to 10 mM (herein referred to as 10 mM Ca^{2+}) to increase the driving force for Ca^{2+} entry via activated Orai channels, and thus increase the size of the Orai current (Fierro et al. 1999). Finally, 3 µM of the Orai channel blocker GSK-7975A, with a final DMSO concentration of 0.1%, was added to specifically block Orai channel activity (Ashmole et al. 2012, Ashmole et al. 2013, Derler et al. 2013).

Characteristic Orai channel activity was not detected in HBECs (**Figure 3-5**). No significant differences were found between the reversal potential values in the experimental conditions (Friedman one-way ANOVA). The size of the inward current at -100 mV differed significantly during the experiment (P = 0.0001), with significant differences between baseline (-23.1 ± 3.9 pA) and 10 mM Ca^{2+} (-39.1 ± 3.8 pA), and between 10 mM Ca^{2+} and GSK-7975A (-39.1 ± 3.8 pA; *P = 0.0005; Dunn's multiple comparisons test). This appears to show that higher extracellular $[Ca^{2+}]$ causes an increase in the size of the inward current, and that this is sensitive to GSK-7975A. Although this could represent Orai channel activity, the currents do not exhibit the characteristic electrophysiological properties of Orai channels (Ashmole et al. 2013). The reversal potential approaching 0 mV of the subtracted

current could suggest the presence of a non-selective cation current, such as that mediated by transient receptor potential (TRP) channels (McGarvey et al. 2014).

3.3.1.4.2 Characteristic Orai channel activity cannot be recorded from differentiated HBECs

To investigate whether the culture state of primary HBECs affected Orai channel electrophysiological activity, membrane currents were recorded from primary HBECs (n = 11) isolated from differentiated ALI cultures grown from HBECs of 2 donors (**Figure 3-6**). At baseline, membrane currents of differentiated HBECs exhibited a reversal potential of -15.8 ± 2.8 mV. 10 mM Ca^{2+} induced slight depolarisation of the reversal potential to -2.4 ± 3.7 mV, and caused a small increase in the size of the inward current at -100 mV from -20.5 ± 4.5 pA to -32.0 ± 5.4 pA. GSK-7975A reduced the size of membrane currents at -100 mV to -17.4 ± 4.0 pA, and the membrane potential became hyperpolarised to near-baseline values of -16.5 ± 4.1 mV.

The reversal potential values of the 3 conditions differed significantly (P = 0.0129; Friedman one-way ANOVA); 10 mM Ca^{2+} induced a positive shift in reversal potential, and GSK-7975A appeared to reduce this (*P = 0.0211; Tukey's multiple comparisons test). Although this suggests that higher extracellular $[Ca^{2+}]$ induces membrane potential depolarisation that is sensitive to GSK-7975A, the reversal potential values were not comparable to the equilibrium potential of Ca^{2+}. In addition, no significant differences in the size of the inward current between each experimental condition were found. The currents recorded from differentiated HBECs did not display characteristic Orai channel electrophysiological features.

A

B

C

D
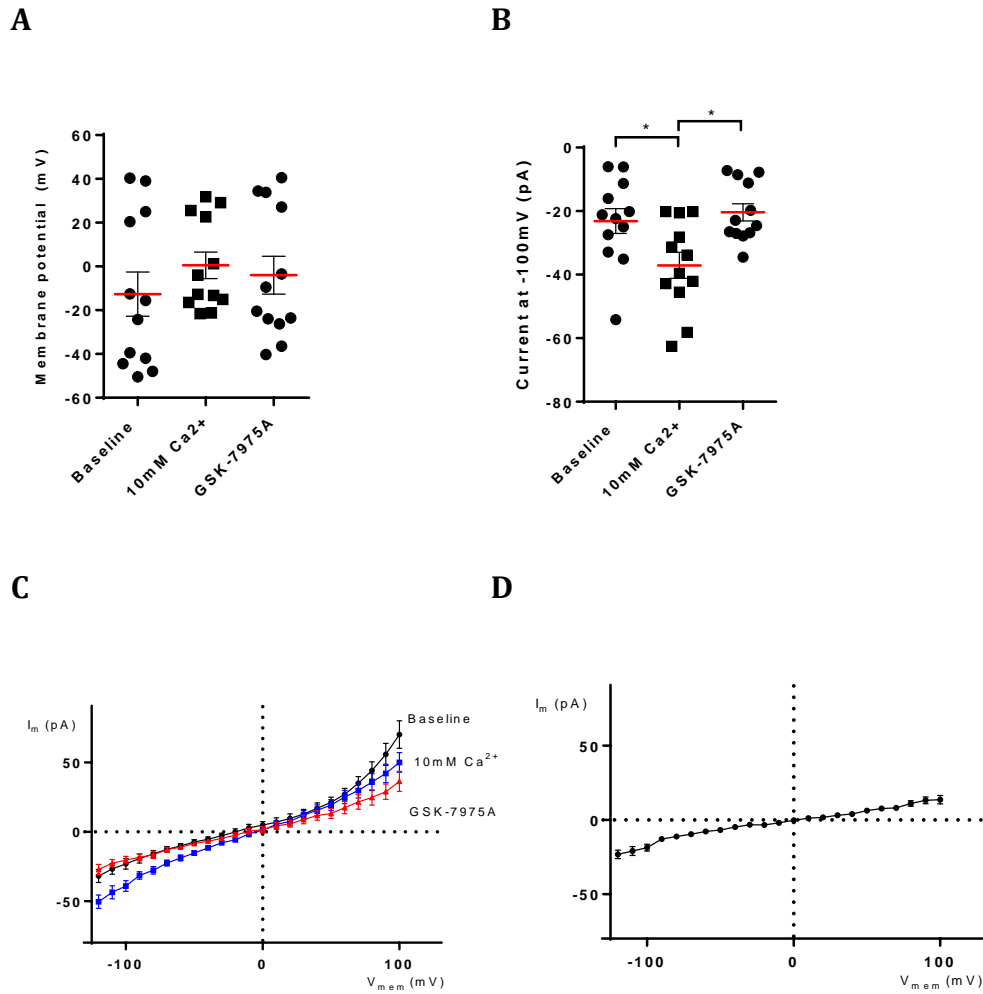
Figure 3-5: Characteristic Orai channel activity was not detected in submerged HBECs (n = 12 cells from 4 donors).

Comparison of (**A**) reversal potential values and (**B**) the size of the inward current at -100 mV (*P = 0.0005) generated in each experimental condition. (**C**) I_m-V_{mem} relationships for baseline (black graph), 10 mM Ca^{2+} (blue graph), and GSK-7975A (red graph). (**D**) Subtracted current (10 mM Ca^{2+} - GSK-7975A). Membrane current (I_m) is displayed in picoamps (pA), and membrane potential (V_{mem}) is displayed in millivolts (mV). All values are mean ± SEM.

A

B

C

D
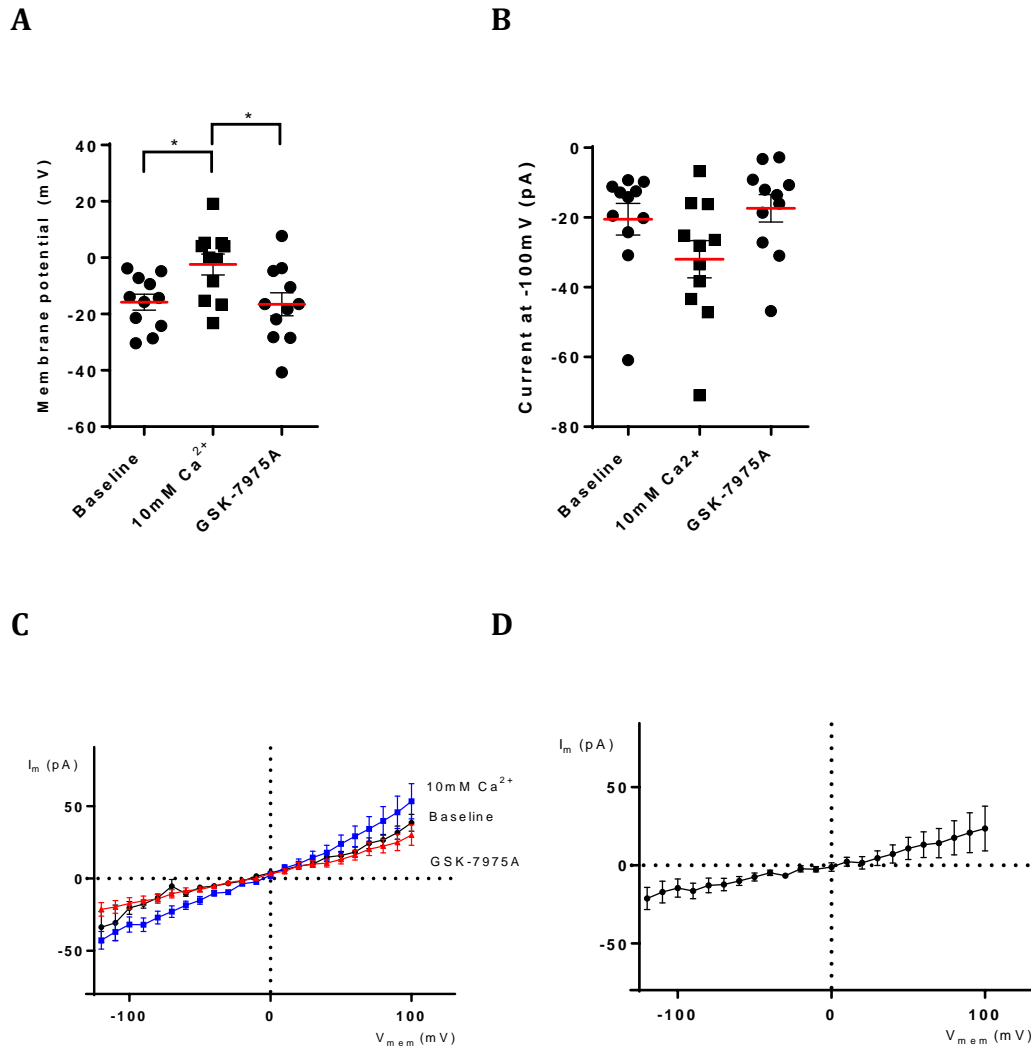
Figure 3-6: Characteristic Orai channel activity was not detected in differentiated HBECs (n = 11) from 2 donors.

Comparisons between (**A**) the reversal potentials and (**B**) the sizes of the inward currents at -100 mV generated by each experimental condition (*P = 0.0211). (**C**) I_m-V_{mem} relationships for baseline (black graph), 10 mM Ca^{2+} (blue graph) and GSK-7975A (red graph). (**D**) Subtracted current (10 mM Ca^{2+} - GSK-7975A).

To minimise the effect of Cl- currents on the recorded membrane currents, which may be masking smaller Orai currents, membrane currents were recorded in the presence of Cs glutamate instead of 140 mM CsCl. As demonstrated in previous studies, glutamate cannot pass through Cl- channels such as ClC-1 (Ashmole et al. 2013). Replacing Cl- with glutamate also provided less Cl- movement while maintaining a suitable osmotic concentration.

In the presence of glutamate, at baseline membrane currents exhibited a reversal potential of -16.4 ± 10.4 mV. 10 mM Ca^{2+} induced significant depolarisation of the reversal potential to -0.8 ± 6.4 mV, and caused a significant increase in the size of the inward current at -100 mV from -29.3 ± 9.4 pA to -62.6 ± 23.5 pA (*P = 0.0401; **Figure 3-7**; Friedman one-way ANOVA, Dunn's multiple comparisons test). However, GSK-7975A did not significantly reduce the size of membrane currents at -100 mV (-10.0 ± 7.3 pA) or hyperpolarise the membrane potential (-35.7 ± 11.0 mV). In addition, the subtracted current did not display characteristic Orai channel features (**Figure 3-7D**).

A

B

C

D
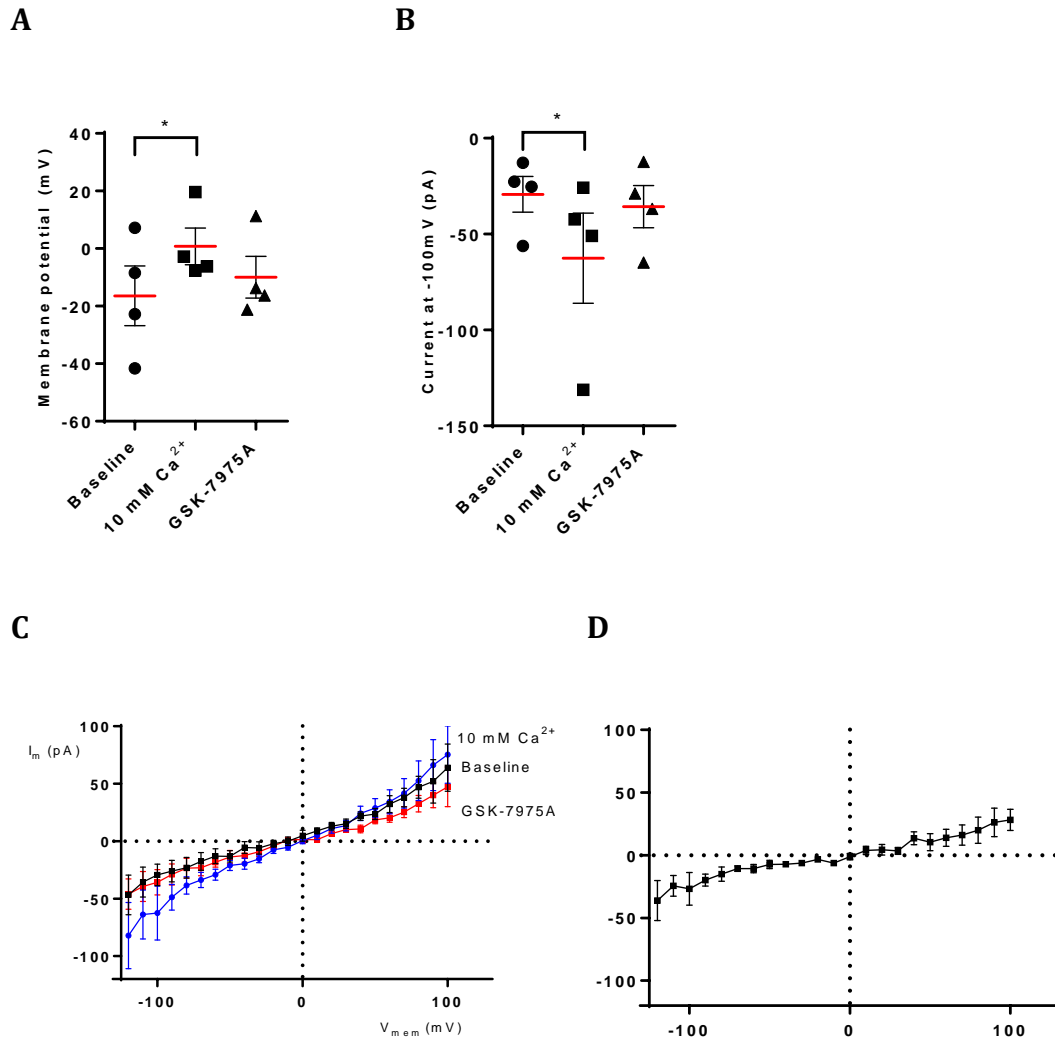
Figure 3-7: Characteristic Orai channel activity was not detected in HBECs (*n* = 4) in the absence of Cl⁻ currents.

Comparisons between (**A**) the reversal potentials and (**B**) the sizes of the inward currents at -100 mV generated by each experimental condition (*P = 0.0401). (**C**) I_m-V_{mem} relationships for baseline (black graph), 10 mM Ca^{2+} (blue graph) and GSK-7975A (red graph). (**D**) Subtracted current (10 mM Ca^{2+} - GSK-7975A).

3.3.1.4.3 Characteristic Orai channel activity in HBECs is not induced by IP3 and EGTA

Previous studies in cells such as mast cells and T cells have demonstrated that intracellular IP3 induces Orai channel activity. This occurs by depletion of intracellular Ca^{2+} stores and subsequent translocation of STIM1 to the plasma membrane, followed by assembly and activation of Orai channels within the plasma membrane (Ashmole et al. 2012, Ashmole et al. 2013, Di Capite et al. 2009b, Zhang et al. 2005, Wu et al. 2006, Luik et al. 2008, Redondo et al. 2008, Huang et al. 1998). Consequently, to investigate the lack of Orai activity in HBECs, patch clamp experiments assessed the response of submerged and differentiated HBECs to IP3 and EGTA.

To do this, membrane currents were recorded from HBECs bathed in the standard external bath solution as soon as whole-cell status was achieved (herein referred to as "baseline"). Cells were dialysed for 5 min with intracellular solution infused with 30 µM IP3 and 500 µM EGTA for (herein referred to as "IP3 + EGTA"). Baseline currents and IP3 + EGTA currents were compared to establish whether IP3 and intracellular Ca^{2+} chelation induced Orai channel activity. Experiments were conducted on submerged and differentiated ALI culture HBECs (**Figure 3-8**). All data was analysed by the Student's paired *t*-test.

At baseline, membrane currents of submerged HBECs (*n* = 22 cells from 5 donors) exhibited a reversal potential of -35.6 ± 3.9 mV. After 5 min, IP3 + EGTA caused significant depolarisation of the reversal potential to -24.4 ± 3.3 mV (*P < 0.0001; **Figure 3-8A**). However, the reversal potential after IP3 + EGTA was not similar to the equilibrium potential of Ca^{2+}. The size of the current recorded at -100 mV increased significantly from -24.5 ± 4.1 pA at baseline to -33.66 ± 3.7 pA with IP3 + EGTA (**P = 0.0008; **Figure 3-8A**), although this change was very small.

Similar results were found in differentiated HBECs (*n* = 18 cells from 2 donors). No significant difference was seen in the reversal potential of currents recorded at baseline (-18.8 ± 2.9 mV) or after 5 min with IP3 + EGTA (-11.3 ± 3.4 mV; **Figure 3-8B**). Furthermore, no significant differences were found in the size of the inward current at -100 mV (baseline: -13.6 ± 2.7 pA; IP3 + EGTA: -19.4 ± 3.6 pA; **Figure**

3-8B). I_m-V_{mem} graphs found no change with IP3 + EGTA in submerged or differentiated HBECs (**Figure 3-8C** & **D**).

These findings indicate that IP3 and EGTA do not evoke characteristic Orai channel activity in submerged or differentiated HBECs. To explore this further, primary HBECs were transported to the University of Oxford, and patch clamp studies were conducted with Professor Anant Parekh. No characteristic Orai activity was recorded from HBECs in these experiments. Consequently, the expression and activity of the Orai channel in primary HBECs requires more investigation.

A

Submerged HBECs

B

Differentiated HBECs

C

D
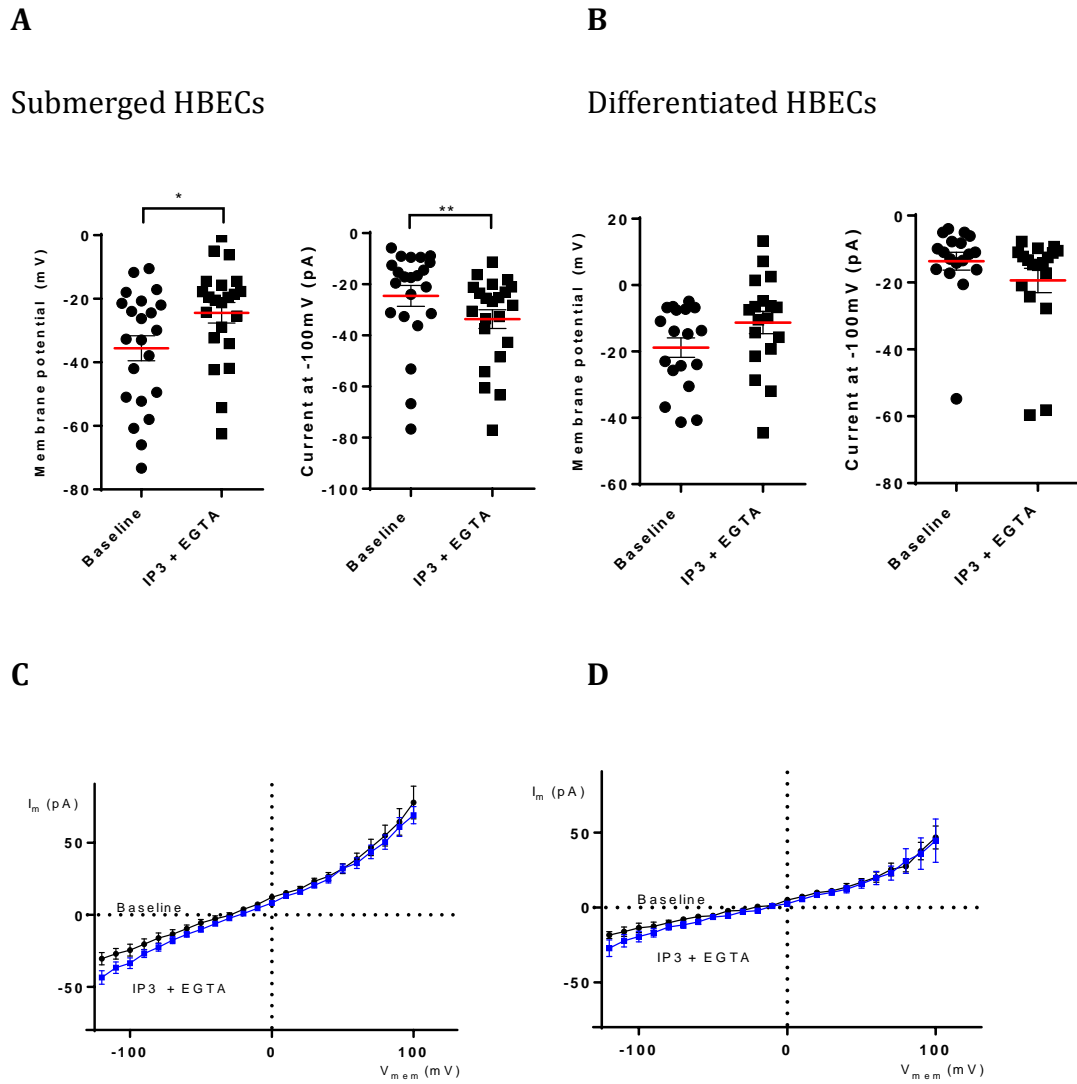
Figure 3-8: Membrane currents of HBECs recorded at baseline and after 5 min with IP3 + EGTA.

Comparison of the reversal potential values and size of the currents at -100 mV in (**A**) submerged HBECs (n = 22; *P < 0.0001; **P = 0.0008) and (**B**) differentiated HBECs (n = 18). I_m-V_{mem} relationships for baseline (black graph) and IP3 + EGTA (blue graph) in (**C**) submerged and (**D**) differentiated HBECs.

3.3.1.5 Characteristic Orai channel activity can be recorded from HLMCs

HLMCs were used as a positive control for Orai channel electrophysiological activity in patch clamp experiments (Ashmole et al. 2012, Ashmole et al. 2013, Di Capite et al. 2009b). Membrane currents were recorded at baseline as soon as whole-cell status was achieved, and again following incubation of cells for 5 min with intracellular 30 μM IP3 and 500 μM EGTA. Extracellular [Ca^{2+}] was raised to 10 mM to increase the size of the Orai current, and Orai channel activity was inhibited by 3 μM GSK-7975A.

Orai channel activity was detected in HLMCs (n = 10 cells from 3 donors; **Figure 3-9**). Unlike primary HBECs, HLMCs were cultured as a cell suspension so did not require treatment with trypsin for detachment from culture containers. All data were analysed by the Friedman one-way ANOVA and Dunn's multiple comparisons test.

At baseline, membrane currents of HLMCs exhibited a reversal potential of -12.2 ± 4.4 mV. IP3 + EGTA caused depolarisation of the membrane potential to induce a value of -6.8 ± 2.7 mV. 10 mM Ca^{2+} caused further depolarisation to 15.6 ± 5.7 mV. The size of the inward current at -100 mV increased from -14.3 ± 3.8 pA to -44.2 ± 11.4 pA with IP3 + EGTA. 10 mM Ca^{2+} did not significantly increase the size of the inward current at 100 mV (-40.9 ± 9.4 pA), but this might be because the maximal inward current via open Orai channels had already been achieved with IP3 + EGTA. GSK-7975A hyperpolarised the reversal potential to -19.7 ± 4.6 mV and reduced the size of the inward current at -100mV to -13.5 ± 3.8 pA.

The reversal potentials of each condition differed significantly (P = 0.0012), and the significant differences were found between baseline and 10mM Ca^{2+} (#P = 0.0224), and between GSK-7975A and 10 mM Ca^{2+} (##P = 0.0006). Significant differences were also found between the size of the inward current at -100 mV between baseline and IP3 + EGTA (*P = 0.0130), baseline and 10 mM Ca^{2+} (**P = 0.0021), and between 10 mM Ca^{2+} and GSK-7975A (***P = 0.0011).

IP3 + EGTA produced a large inward current in HLMCs similar to those reported previously (Ashmole et al. 2013, Ashmole et al. 2012). Although the reversal potential of 10 mM Ca^{2+} was not close to the equilibrium potential of Ca^{2+}

(Ashmole et al. 2012), the large inward current induced by IP3 + EGTA, the positive reversal potential of the subtracted current, and the sensitivity of this current to GSK-7975A indicate that characteristic Orai channel activity was recorded from HLMCs.

A

B

C

D

E
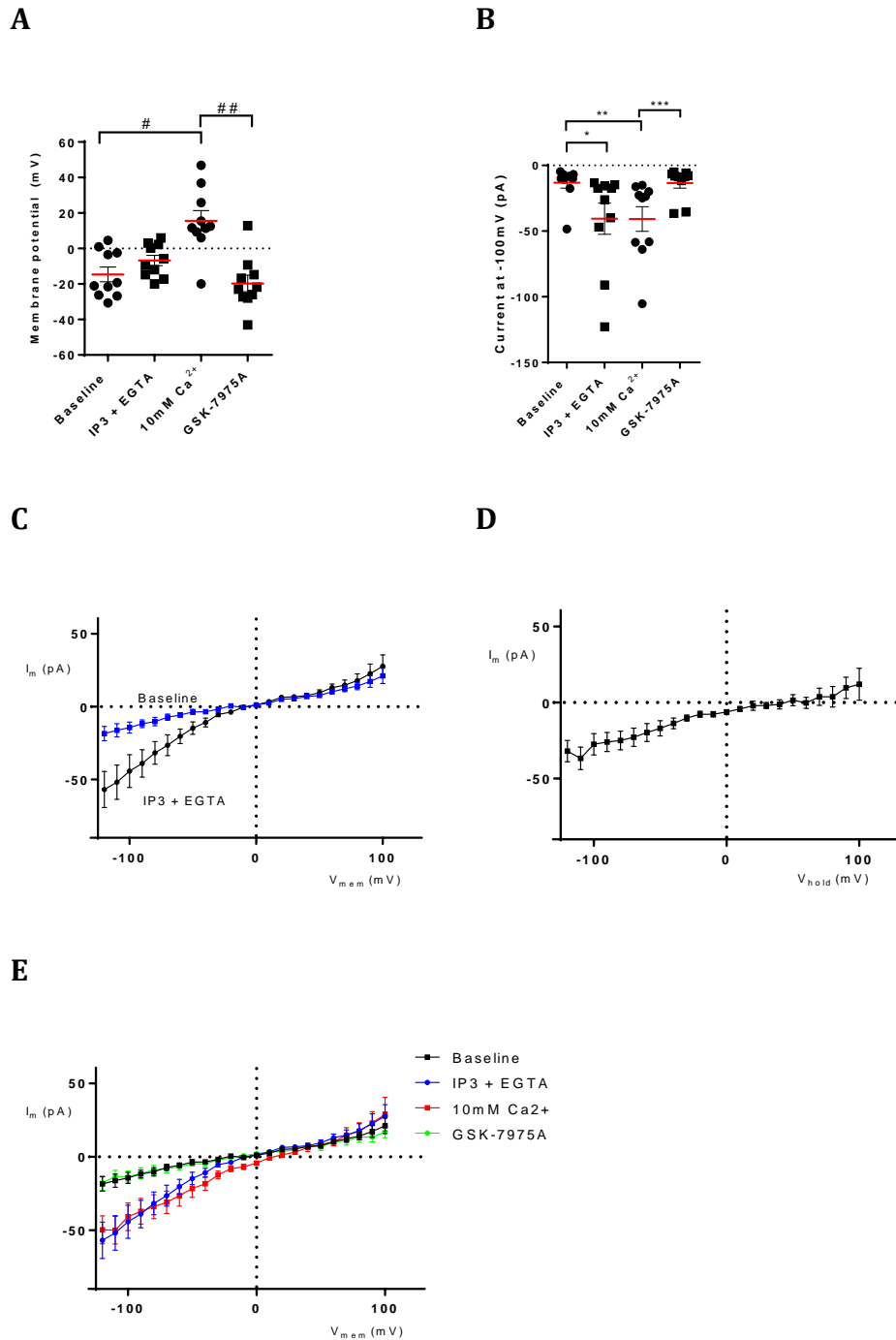
Figure 3-9: Primary HLMCs (*n* = 10) express Orai currents.

Comparison between (**A**) reversal potential values (# P = 0.0224; ## P = 0.0006) and (**B**) the size of the inward current recorded at -100 mV (*P = 0.0130; **P = 0.0021; ***P = 0.0011) in each experimental condition. (**C**) I_m-V_{mem} at baseline (blue graph) and IP3 + EGTA (black graph). (**D**) Subtracted current (10 mM Ca^{2+} - GSK-7975A). (**E**) I_m-V_{mem} relationships for baseline (black graph), IP3 + EGTA (blue graph), 10 mM Ca^{2+} (red graph), GSK-7975A (green graph).

3.3.1.6   Primary HBECs express cell surface Orai1

To investigate the lack of Orai channel activity detected in HBECs, extracellular cell surface expression of Orai1 was assessed by flow cytometry. HBECs were stained with propridium iodide to identify non-viable cells (**Figure 3-10A**) and single viable cells were selected for analysis (**Figure 3-10B**). Cells stained with primary polyclonal anti-Orai1 antibody (Orai1 PAb) were detected by goat anti-rabbit APC-conjugated secondary antibody. The fluorescence emission of cells labelled with the APC fluorochrome was detected using a fluorescence channel with wavelengths suitable for excitation of APC.

Cells stained with Orai1 PAb exhibited a difference in geometric mean fluorescence (ΔGMFI) compared with cells stained with rabbit IgG control antibody at a range of concentrations (**Figure 3-11**). ΔGMFI increased with the concentration of antibody (**Figure 3-11A**) and the ratio between IgG and Orai1 PAb was greatest at antibody concentrations of 5.0 µg/ml (**Figure 3-11B**). Due to a limited supply of cells, HBECs from one donor were analysed for Orai1 surface expression. Consequently, no statistical analysis was performed. However, these findings suggest that HBECs express extracellular cell surface Orai1.

A
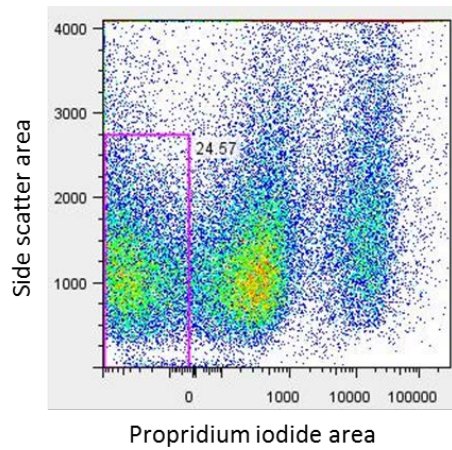
B
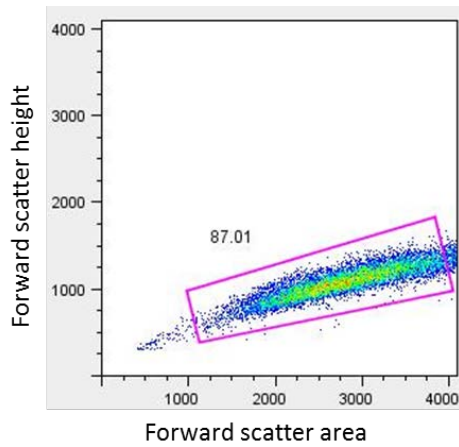
Figure 3-10: Primary HBECs were analysed by flow cytometry for Orai1 cell surface expression.

(**A**) HBECs were stained with propridium iodide to identify dead cells. Viable cells were selected, and (**B**) forward scatter height and area assessed to allow selection of single cells.
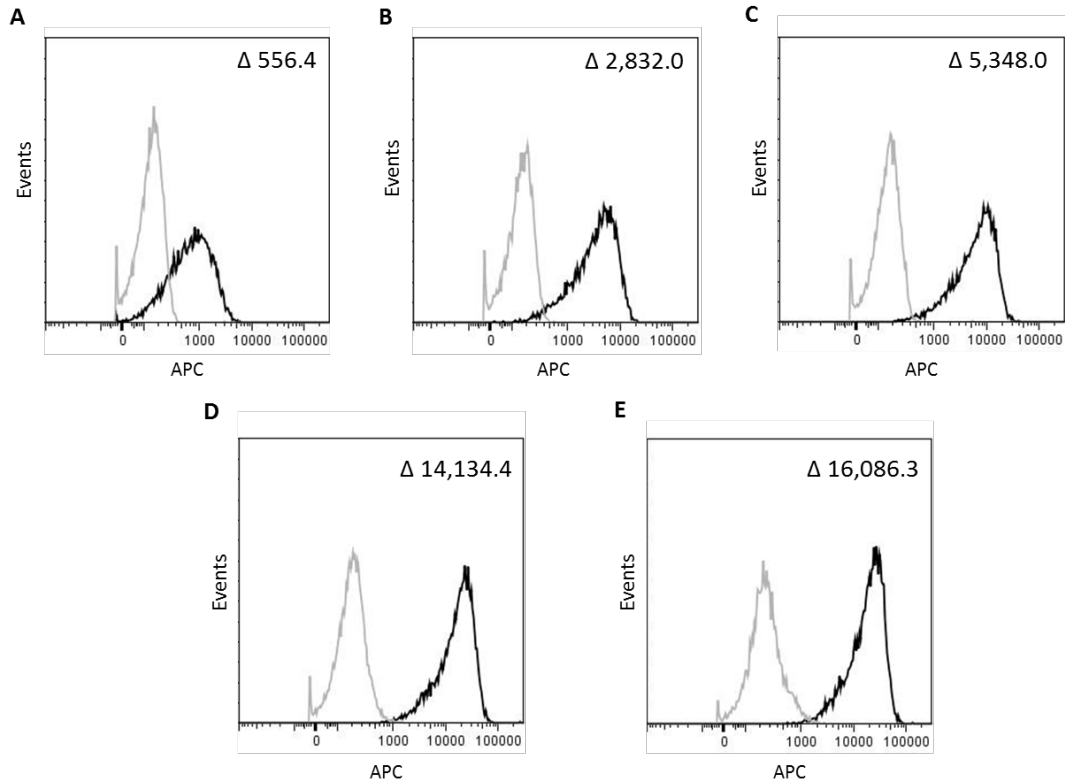
Figure 3-11: Primary HBECs display Orai1 cell surface expression.

Staining with Orai1 PAb (black graph) was compared with rabbit IgG antibody (grey graph) at (**A**) 0.1 µg/ml, (**B**) 0.5 µg/ml, (**C**) 1.0 µg/ml, (**D**) 5.0 µg/ml and (**E**) 10.0 µg/ml. Difference in geometric mean of fluorescence intensity (ΔGMFI) is displayed for each concentration. Data are expressed as increasing events (*y* axis) and units of the APC detection channel as a marker of fluorescence intensity (*x* axis).

A
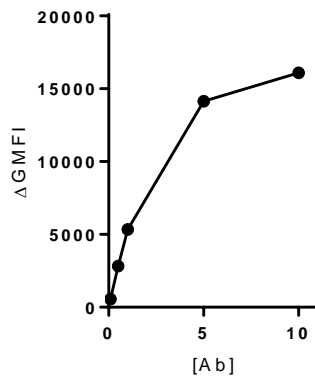
B
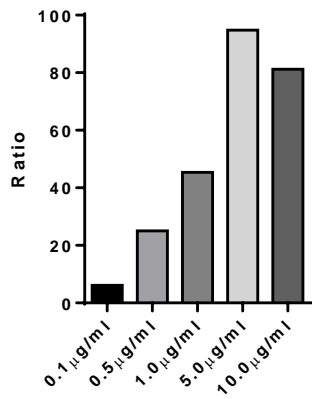
Figure 3-12: Difference in GMFI increases with antibody concentration.

(**A**) The difference in geometric mean of fluorescence intensity (ΔGMFI) increases with the concentration of antibody ([Ab]). (**B**) The ratio between Orai1 PAb and isotype control antibody GMFI at different antibody concentrations.

3.4   *Discussion*

These results demonstrate the expression of the Orai channel family in primary HBECs. qPCR, western blotting, and flow cytometry revealed the presence of Orai1, Orai2, and Orai3 mRNA, Orai1 and Orai2 protein, and Orai1 protein cell surface expression in primary HBECs. However, no characteristic Orai channel activity was recorded from HBECs in patch clamp experiments.

The expression and activity of Orai channels has been found in a wide variety of cell types including vascular smooth muscle cells (Zhang et al. 2014), T lymphocytes (Parekh et al. 2005), macrophages (Gao et al. 2010) and polarised secretory cells such as pancreatic and salivary gland acinar duct cells (Hong et al. 2011). Orai channels are also expressed in mast cells and play an important role in mast cell degranulation, which involves the exocytic release of preformed granules (Ashmole et al. 2012). This mechanism of exocytic degranulation is thought to be comparable to the mechanism by which goblet cells release specialised mucin granules (Davis et al. 2008). Therefore, investigating the expression of the Orai channel in bronchial epithelial cells offers insight into the potential role of the Orai channel in airway mucin secretion.

According to previous studies, the characteristic electrophysiological features of the Orai channel include the appearance of a current over 5 min as Ca^{2+} stores are depleted, a positive shift in reversal potential in physiological solutions, and strong inward rectification. The current mediated by the Orai channel is also sensitive to the specific blocker GSK-7975A (Ashmole et al. 2012, Ashmole et al. 2013, Derler et al. 2013). These characteristics were found in HLMCs, used as a positive control for this study. Orai channel activity in HLMCs was induced by intracellular IP3 and EGTA within 5 min, and the resulting current was blocked by 3 μM GSK-7975A. In addition, an increase in extracellular $[Ca^{2+}]$ produced a positive shift in reversal potential and increased inward current after 5 min with IP3 and EGTA, suggesting that depletion of intracellular Ca^{2+} stores had opened Orai channels.

In primary HBECs, however, IP3 and EGTA had no effect on membrane currents. GSK-7975A also had no effect, indicating an absence of Orai channel activity. No increase in the size of the inward current was seen in the presence of higher extracellular $[Ca^{2+}]$, indicating the current was absent rather than being too small

to be seen. Currents recorded from HBECs cultured under both submerged and differentiated ALI conditions failed to demonstrate any Orai characteristics. This implied that the differentiation status of the cells was not the cause of absent Orai activity.

The lack of Orai currents was investigated by examining whether Orai protein was expressed at the cell surface of primary HBECs. Western blot and qPCR demonstrated Orai1 and Orai2 protein and mRNA expression, respectively, in primary HBECs, but studies have demonstrated that the Orai channel requires exocytic insertion into the plasma membrane (Woodard et al. 2008). However, flow cytometry data with primary antibodies targeting the extracellular portion of Orai1 on non-permeablised HBECs suggests that Orai1 is already present at the cell surface. Consequently, internalisation of Orai protein is not the reason for the lack of Orai activity found in patch clamp experiments. Flow cytometry was only conducted on one HBEC donor due to limited availability of the cells, and should be repeated with more donors in future work. Western blot data failed to show Orai3 protein expression in primary HBECs.

Further investigations were conducted at the University of Oxford with Professor Anant Parekh. Under Professor Parekh's supervision, no characteristic Orai channel current was seen in primary HBECs. Rather than assuming that a lack of visible current was indicative of a lack of Orai activity, Professor Parekh suggested that the Orai current was too small and was being masked by other non-specific currents, such as those mediated by Cl^- channels. However, as demonstrated in **Figure 3-7**, replacing Cl^- with glutamate did not reveal characteristic Orai channel currents. Another suggestion was that the baseline current present was that of a TRP channel; this possibility coincides with previous reports of an interaction between Orai1 with TRPC proteins (Ong et al. 2007). The resulting currents reportedly exhibit different Ca^{2+} selectivity than the characteristic Orai current. Some TRP channels, such as TRPM5, are Ca^{2+}-permeable, and others are monovalent-selective cation channels that can be activated by store depletion (Perez et al. 2002, Hofmann et al. 2003). Consequently, Orai protein in primary HBECs could contribute to other store-operated channel currents, rather than forming Orai channels, and this should be investigated further.

For patch clamp experiments HLMCs were prepared by collecting an aliquot of cells from a purified culture. No treatment with trypsin-EDTA solution or centrifugation was required due to the suspension of the cells within culture medium. In contrast, primary HBECs adhere to culture flasks, and hence required treatment with trypsin-EDTA and subsequent centrifugation to obtain a cell pellet. This difference in cell preparation prior to patch clamp experiments could explain the presence of Orai activity in HLMCs but not in HBECs. Future work should include preparing HBECs by non-enzymatic methods, such as Cell Dissociation Solution (Sigma) which facilitates gentle dislodging of cells from culture flasks. In addition, HLMCs should be treated with trypsin-EDTA solution and centrifuged prior to patch clamp experiments to examine whether this treatment causes loss of Orai channel current.

The lack of Orai channel currents recorded from submerged and differentiated primary HBECs, in spite of mRNA and protein expression, suggests that further investigation into Orai expression in primary HBECs is required. Hence, the role of Orai in mucin secretion was not investigated in this study. In addition to optimising cell preparation prior to patch clamping, future experiments should investigate the presence of alternative currents in primary HBECs, such as those mediated by TRP channel activity.

Chapter 4:

K$_{Ca}$3.1 channel expression in human bronchial epithelial cells

4.1   Introduction

K_{Ca} channels play important physiological roles in a variety of cell types by regulating the membrane potential and modulating Ca^{2+} signalling. This allows them to indirectly regulate downstream pathways and cellular processes. In mast cells, $K_{Ca}3.1$ contributes to the regulation of granule exocytosis by potentiating Ca^{2+} influx (Duffy et al. 2004, Wulff et al. 2010). Although $K_{Ca}3.1$ expression has been reported in primary human renal epithelial cells and human airway epithelial cell lines (Calu-3, Nu-Li and Cu-Fi) (Devor et al. 1999, Albaqumi et al. 2008, Trinh et al. 2008), the role of $K_{Ca}3.1$ in mucin granule exocytosis from goblet cells has not yet been examined.

A useful pharmacological tool for the study of $K_{Ca}3.1$ channel activity are the $K_{Ca}3.1$ blockers TRAM-34 and ICA-17043, effective with high specificity at concentrations of 200 and 100 nM, respectively (Duffy et al. 2004, Wulff et al. 2001, Wulff et al. 2000, Roach et al. 2013, Roach et al. 2014). Elicitation of the $K_{Ca}3.1$ current can be achieved using the channel opener 1-EBIO. This compound is highly selective for, but not specific to, $K_{Ca}3.1$ channels (Devor et al. 1996, Singh et al. 2001, Jensen et al. 1998); 1-EBIO is also capable of opening $K_{Ca}2$ channels (Lam et al. 2013). The bee venom toxin apamin is capable of blocking $K_{Ca}2$ channels but has no effect on 1-EBIO-induced currents, which are inhibited by TRAM-34 (Wei et al. 2005, Stoneking et al. 2013). In addition, TRAM-34 does not inhibit $K_{Ca}2$ channel activity. Thus, 1-EBIO and TRAM-34 are suitable for identifying the presence of $K_{Ca}3.1$ currents.

1-EBIO induces $K_{Ca}3.1$ opening by enhancing the sensitivity of $K_{Ca}3.1$ channels to $[Ca^{2+}]_i$. Maximal K^+ currents can be induced using 100 μM 1-EBIO in the presence of low $[Ca^{2+}]_i$ (Duffy et al. 2004, Duffy et al. 2007, Pedersen et al. 1999). Activation of $K_{Ca}3.1$ channels in patch clamp experiments can also be achieved by utilising the dependency of $K_{Ca}3.1$ on $[Ca^{2+}]_i$. For instance, $K_{Ca}3.1$ currents can be recorded by patch clamping using the whole cell configuration in the presence of 1 μM Ca^{2+} within the pipette solution (Ghanshani et al. 2000, Nicolaou et al. 2007). In this study, only 1-EBIO was used to activate $K_{Ca}3.1$; high $[Ca^{2+}]_i$ can activate large Cl^- currents, and this could mask the current of interest. Moreover, the current elicited by 1-EBIO is well characterised.

4.2 Methods

4.2.1 In vitro cell culture

Primary HBECs were isolated from healthy control and asthmatic donors, and grown as submerged or differentiated ALI cultures (**Chapter 2, 2.2.1**). H292 cells were purchased from ATCC (**Chapter 2, 2.2.2**).

4.2.2 Quantification of K$_{Ca}$3.1 mRNA expression using qPCR

Total RNA was isolated from *in vitro* cell cultures and used for first strand cDNA synthesis (**Chapter 2, 2.3**). The quantity of K$_{Ca}$3.1 mRNA was measured using SYBR Green-based qPCR with commercially available K$_{Ca}$3.1 primers (Qiagen), alongside primers targeting the internal housekeeping gene, β-actin (Qiagen; **Chapter 2, 2.3.4**). Only experiments resulting in a clear, single PCR product band in an agarose gel of the appropriate fragment size were used; the expected product size with K$_{Ca}$3.1 and β-actin primers were 130 and 146 bp, respectively. PCR products were analysed by gel electrophoresis (**Chapter 2, 2.3.6**).

All expression data were corrected using the reference dye ROX and normalised to β-actin mRNA using the ΔC_T method (**Chapter 2, 2.3.5.1**). C_T values were of a log scale, base 2, and calculated per 10^3 β-actin mRNA.

4.2.3 Detecting K$_{Ca}$3.1 protein expression by western blotting

Expression of K$_{Ca}$3.1 protein was detected by western blotting (**Chapter 2, 2.4**). K$_{Ca}$3.1 protein was identified with primary polyclonal anti-K$_{Ca}$3.1 antibody (100 µg/ml; Biorbyt), labelled with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (0.05 µg/ml). Immunoreactive protein bands were visualised by applying Pierce ECL Western Blotting Substrate and detecting chemiluminescence. B-actin was used as a positive control for the western blot and was identified by primary monoclonal β-actin antibody HRP conjugate (7 ng/ml).

4.2.4 *Patch clamp electrophysiology*

The activity of the $K_{Ca}3.1$ channel was investigated using the whole cell patch clamp technique, using specific experimental solutions (**Chapter 2, 2.5**; **Table 4-1**). Prior to experiments, cells were prepared by treating with trypsin-EDTA solution and centrifuging to obtain a cell pellet, before resuspending in fresh media. Cells were incubated at 37°C for 20 min before beginning experiments.

Membrane currents of primary HBECs were evoked by applying voltage commands to a range of potentials (-120 to 100 mV) in 10 mV steps from a holding potential of 0 mV during a 100 ms pulse (**Figure 4-1A**). Upon reaching whole-cell status, cell contents became dialysed with the internal solution of the pipette (**Table 4-1**). Membrane currents were recorded from cells bathed in the standard external solution (this condition is herein referred to as "baseline"), and following the sequential addition of 100 µM 1-EBIO and 200 nM TRAM-34 to the experimental bath, with a final DMSO concentration of 0.1%. Application of DMSO alone did not affect 1-EBIO-induced currents. Characteristic electrophysiological features of $K_{Ca}3.1$ channels include an immediate appearance of the current when voltage steps are applied, and sustainment of this current throughout each 100 ms pulse. No decay in current is seen. Activation of $K_{Ca}3.1$ leads to a negative shift in the reversal potential and inward rectification from approximately 40 mV. An example of the raw currents recorded from primary HBECs in each of these experimental conditions is shown in **Figure 4-1B**.

A
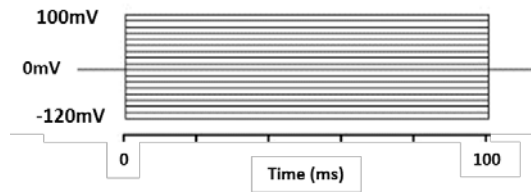
B
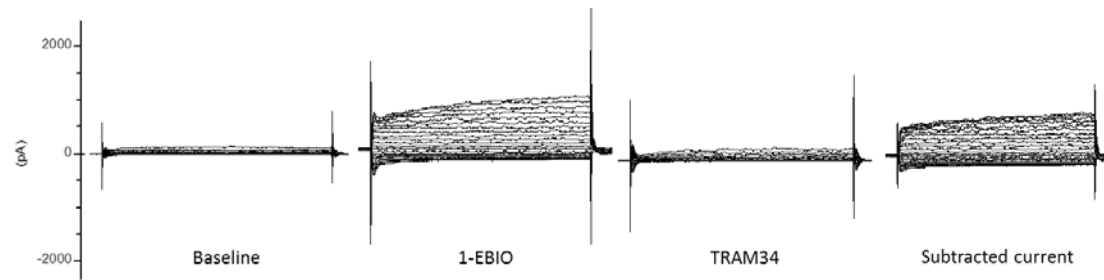
Figure 4-1: Electrophysiological properties of K$_{Ca}$3.1 channel currents.

(**A**) Voltage commands to a range of potentials in 10 mV steps applied to cells during whole-cell patch clamp experiments. (**B**) Raw current data recorded from HBECs at baseline, and following the addition of 100 μM 1-EBIO and 200 nM TRAM-34. The subtracted current data is achieved by subtracting the current recorded following the addition of TRAM-34 from the current induced by 1-EBIO, and is therefore representative of the functional K$_{Ca}$3.1 component of the recorded current.

External bath solution (mM)	Internal solution (mM)
NaCl, 140	KCl, 140
KCl, 5	$MgCl_2$, 2
$CaCl_2$, 2	HEPES, 10
$MgCl_2$, 1	Na^+-ATP, 2
HEPES, 10	GTP, 0.1

Table 4-1: Recipes for the standard external bath solution and the internal solution used for recording K_{Ca}3.1 channel activity.

The external bath solution was buffered to pH 7.3 with NaOH; the internal solution was buffered to pH 7.3 by KOH.

4.3 *Results*

4.3.1 *Expression of the K$_{Ca}$3.1 channel by HBECs*

4.3.1.1 Patient characteristics

In total, HBECs from 18 patients with asthma and 5 healthy controls were used for the following experiments. The patient clinical characteristics are displayed in **Table 4-2**.

4.3.1.2 Primary HBECs and H292 cells express K$_{Ca}$3.1 mRNA

K$_{Ca}$3.1 channel mRNA was detected at similar levels in asthmatic (*n* = 10) and healthy (*n* = 5) HBECs cultured in submerged conditions (**Figure 4-2A**). All RNA was collected from HBECs cultured at either passage 2 or 3. Analysis of PCR products using a 2% agarose gel confirmed the presence of a single product of 130 bp in all samples (**Figure 4-2C**), indicating that amplification of K$_{Ca}$3.1 channel mRNA during qPCR was specific. K$_{Ca}$3.1 primers were used alongside primers targeting the internal normaliser gene, β-actin, and produced a single product of 146 bp (**Figure 4-2D**). Similar levels of β-actin mRNA were detected in submerged HBECs, making it suitable for use as an internal normaliser gene (**Figure 4-2B**).

K$_{Ca}$3.1 channel mRNA was also expressed in differentiated HBEC ALI cultures originating from patients with asthma (*n* = 8). Agarose gel electrophoresis confirmed amplification of single products of the appropriate sizes in all reactions (**Figure 4-2C**). B-actin mRNA expression differed between submerged and ALI cultures, so prevented any comparison of K$_{Ca}$3.1 channel mRNA expression between submerged and differentiated HBECs.

The expression of K$_{Ca}$3.1 in H292 cells was assessed to determine whether this cell line would be a suitable model of HBECs for this study. K$_{Ca}$3.1 channel mRNA was detected in H292 cultures (*n* = 5), and β-actin was found to be a reliable housekeeping gene (**Figure 4-3**).

	Asthmatic *n* = 18	Healthy *n* = 5	P value
Disease severity	Severe	N/A	-
Age (y), mean ± SEM	50.9 ± 3.3	34.6 ± 7.4	0.0686
Sex (M/F)	9/9	3/2	0.6959
Asthma duration (y) , mean ± SEM	21.0 ± 4.7	N/A	-
Inhaled corticosteroid dose (μg), mean ± SEM (beclomethasone equivalents ‡)	1671 ± 125	0	**0.0002**
No. at BTS step 5	7	0	-
No. taking long-acting β-agonist	18	0	-
Exacerbations in last year, median (IQR)	2.0 (1.0 - 3.8)	N/A	-
Sputum eosinophil count (%), median (IQR)	5.3 (2.1 - 15.5)	0	**0.0107**
FEV1 (% predicted), mean ± SEM	68.9 ± 7.2	104.0 ± 3.3	**0.0103**
FEV1/FVC ratio (%), mean ± SEM	69.6 ± 4.2	84.6 ± 3.5	0.0904
Atopy (yes/no)	13/5	0/5	**0.0040**
Serum IgE (kU/L), median (IQR)	148.2 (64.8 - 313.1)	4.6 (3.1 - 16.9)	**0.0036**

Table 4-2: Clinical characteristics of donors recruited for collection of HBECs by bronchoscopy.

BTS, British Thoracic Society; *N/A*, not applicable; U/L, units per litre; IQR, interquartile range. ‡ Ratio for budesonide Turbuhaler calculated as 1.5; **bold** text highlights statistically significant P values. Data are presented as mean ± SEM or median (IQR).

A

B

C

Submerged HBECs

D

Differentiated HBECs
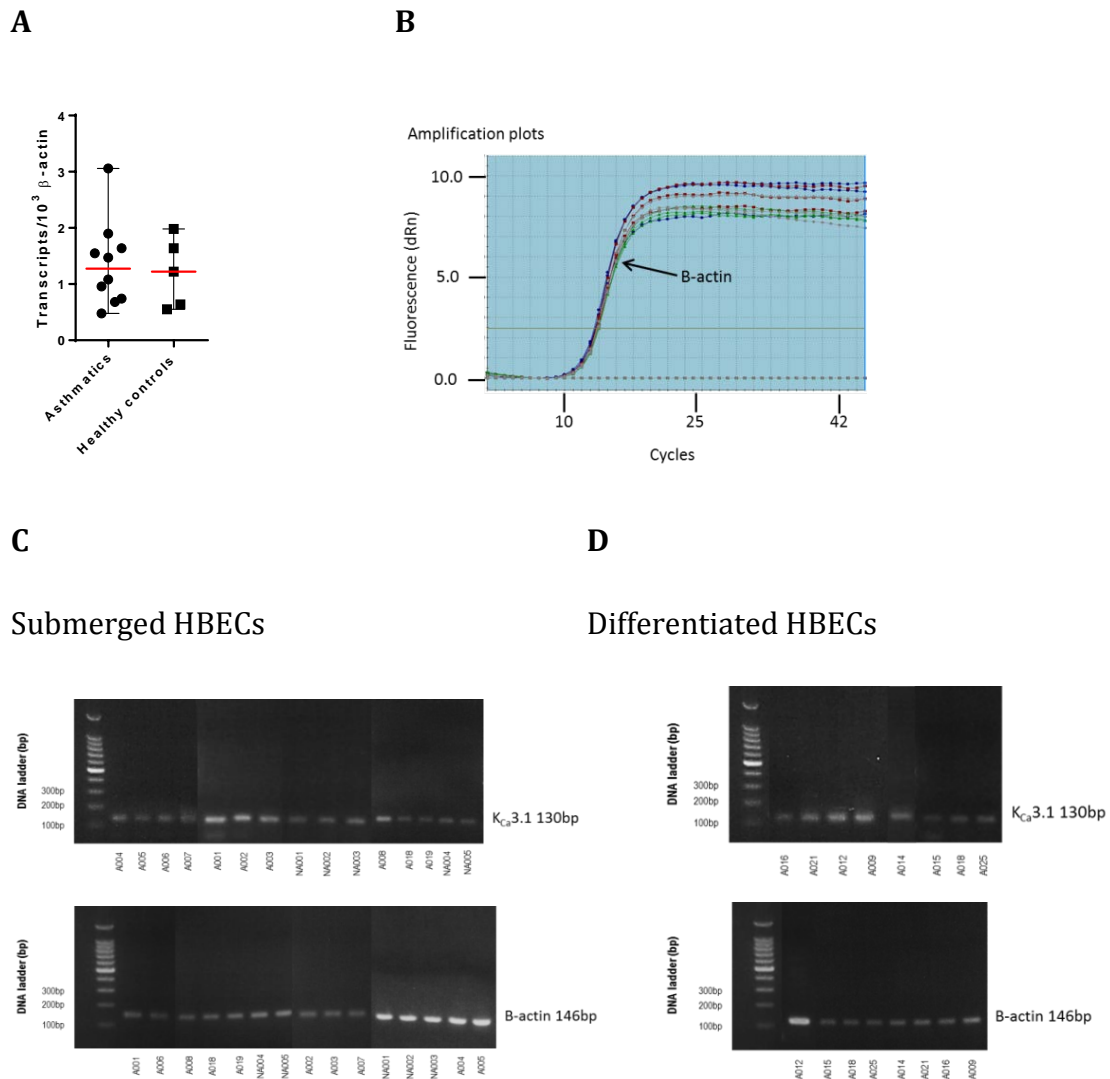
Figure 4-2: Primary HBECs express K$_{Ca}$3.1 channel mRNA.

(**A**) Comparison of K$_{Ca}$3.1 channel mRNA expression in submerged cells isolated from asthmatic ($n = 10$) and healthy ($n = 5$) donors. Expression of the gene of interest is presented per 10^3 β-actin mRNA. All data are expressed as median (range). Bar represents median of data points. (**B**) Amplification plots generated with β-actin primers from submerged HBECs. PCR products amplified with K$_{Ca}$3.1 and β-actin primers from (**C**) submerged ($n = 15$) and (**D**) differentiated ($n = 8$) HBECs.

A
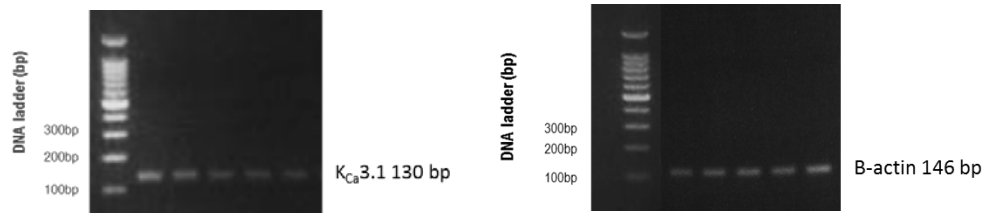
B
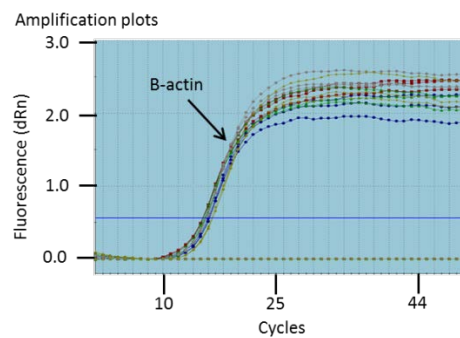
Figure 4-3: H292 cells express K$_{Ca}$3.1 mRNA.

(**A**) Analysis of PCR products amplified from H292 cultures (n = 5) with K$_{Ca}$3.1 and β-actin primers. (**B**) Amplification plots generated during PCR experiments conducted with β-actin primers.

4.3.1.3 Primary HBECs and H292 cells express K$_{Ca}$3.1 channel protein

K$_{Ca}$3.1 protein expression was detected by western blotting in HBEC lysates from 2 asthmatic and 1 healthy donors, and of H292 lysates. The predicted molecular weight of the K$_{Ca}$3.1 channel is 48 kDa, and staining with polyclonal anti-K$_{Ca}$3.1 antibody (Biorbyt) produced a single band of the target size in all primary HBEC donors and H292 cells (**Figure 4-4**). Staining for β-actin (42 kDa) was used as a positive control for the western blot and produced a single band at the approximate target protein size.

A
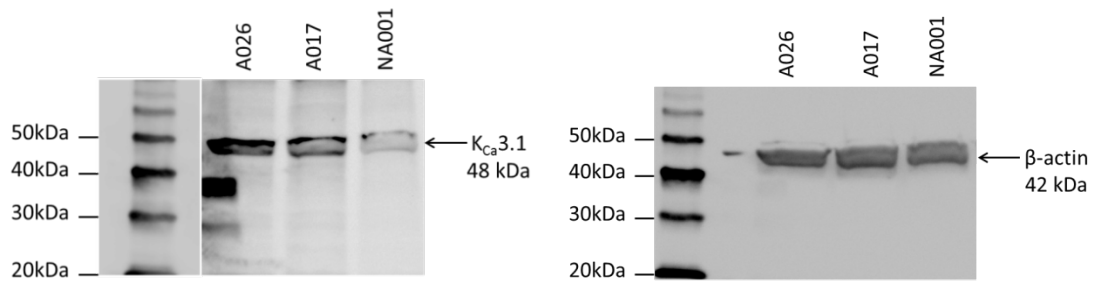
B
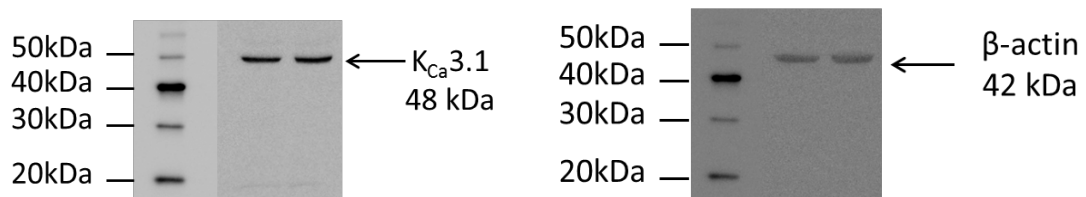
Figure 4-4: Primary HBECs and H292 cells express $K_{Ca}3.1$ protein.

Representative western blots using primary antibodies targeting $K_{Ca}3.1$ (48 kDa) and β-actin (42 kDa) protein in lysates of (**A**) submerged HBECs isolated from 2 asthmatic donors (A026 and A017) and 1 healthy control donor (NA001), and (**B**) H292 cells. Additional bands in (**A**), lane A026, are caused by bleed-over of the ladder at high exposures.

4.3.1.4 Primary HBECs exhibit K$_{Ca}$3.1 channel currents, which are larger in asthmatic cells

The electrophysiological activity of K$_{Ca}$3.1 in primary HBECs was investigated by patch clamp experiments to determine whether the channel was active in the cells, and whether any differences existed between healthy and asthmatic HBECs. All statistical significances were identified using the Friedman one-way ANOVA and Dunn's multiple comparisons test.

4.3.1.4.1 Asthmatic primary HBECs express K$_{Ca}$3.1 channel currents

Membrane currents were recorded from submerged HBECs (n = 20) from 8 asthmatic donors (**Figure 4-5**). The reversal potential of membrane currents at baseline was -20.0 ± 5.0 mV and became significantly hyperpolarised to -50.7 ± 2.6 mV by 1-EBIO (**Figure 4-5A**; *P < 0.0001). 1-EBIO caused a significant increase in the size of the outward current at 40 mV from 39.7 ± 11.6 pA to 357.5 ± 93.4 pA. This was reduced to 52.3 ± 19.9 pA by TRAM-34 (**Figure 4-5B**; #P < 0.0001). TRAM-34 also caused significant depolarisation of the membrane potential to -10.9 ± 3.3 mV (**Figure 4-5A**; *P < 0.0001). The subtracted K$_{Ca}$3.1 current exhibited a reversal potential approaching the equilibrium potential of K$^+$ (-62.6 ± 3.0 mV; **Figure 4-5D**).

K$_{Ca}$3.1 currents were also detected in primary HBECs of differentiated ALI cultures (n = 7) from 3 asthmatic donors (**Figure 4-6**). At baseline, membrane currents exhibited a reversal potential of -15.8 ± 2.2 mV. 1-EBIO significantly hyperpolarised the membrane potential to -54.9 ± 5.5 mV (**Figure 4-6A**; *P = 0.0151) and increased the size of the outward current at 40 mV from 18.5 ± 4.6 pA to 414.6 ± 148.3 pA (**Figure 4-6B**; #P = 0.0006). TRAM-34 inhibited the current induced by 1-EBIO to near-baseline levels, causing a reduction in the size of the outward current to 59.9 ± 26.6 pA (**Figure 4-6B**) and significant depolarisation of the membrane potential to -18.0 ± 4.8 mV (**Figure 4-6A**; **P = 0.0066). The subtracted K$_{Ca}$3.1 current exhibited a reversal potential approaching the equilibrium potential of K$^+$ (-71.5 ± 4.8 mV; **Figure 4-6D**).

Overall, the stronger outward currents induced by 1-EBIO, and the sensitivity of these currents to TRAM-34 suggest that the channel activity recorded in these experiments is that of K_{Ca}3.1.

A

B

C

D
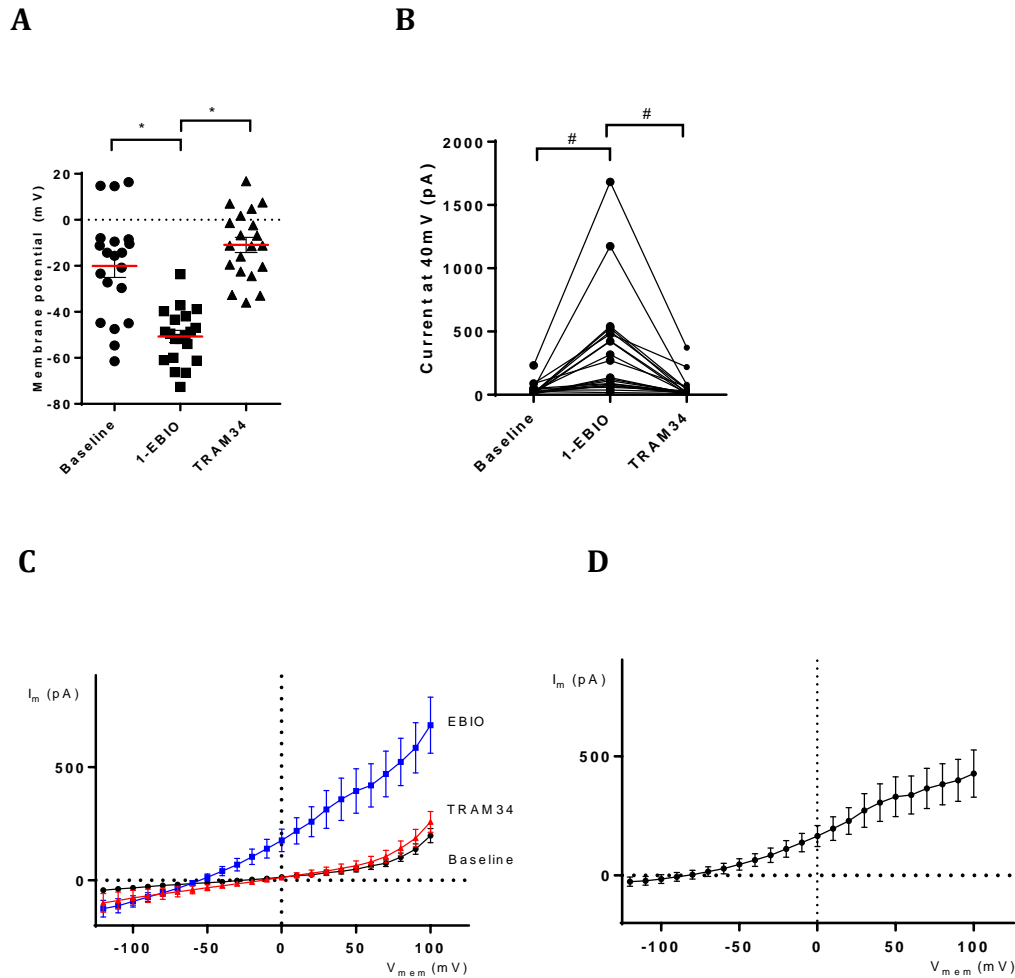
Figure 4-5: Asthmatic submerged HBECs express K$_{Ca}$3.1 currents.

Whole-cell membrane currents were recorded from submerged HBECs (n = 20 cells) from 8 asthmatic donors. Experiments demonstrate K$_{Ca}$3.1 activity in submerged asthmatic HBECs. (**A**) Reversal potential values recorded from each condition; *P < 0.0001. (**B**) Size of the current at 40 mV in each condition; #P < 0.0001. (**C**) I$_m$-V$_{mem}$ relationships of recordings at baseline (black graph), 1-EBIO (blue graph), and TRAM-34 (red graph). (**D**) Subtracted current (1-EBIO – TRAM-34). All values are mean ± SEM.

A

B

C

D
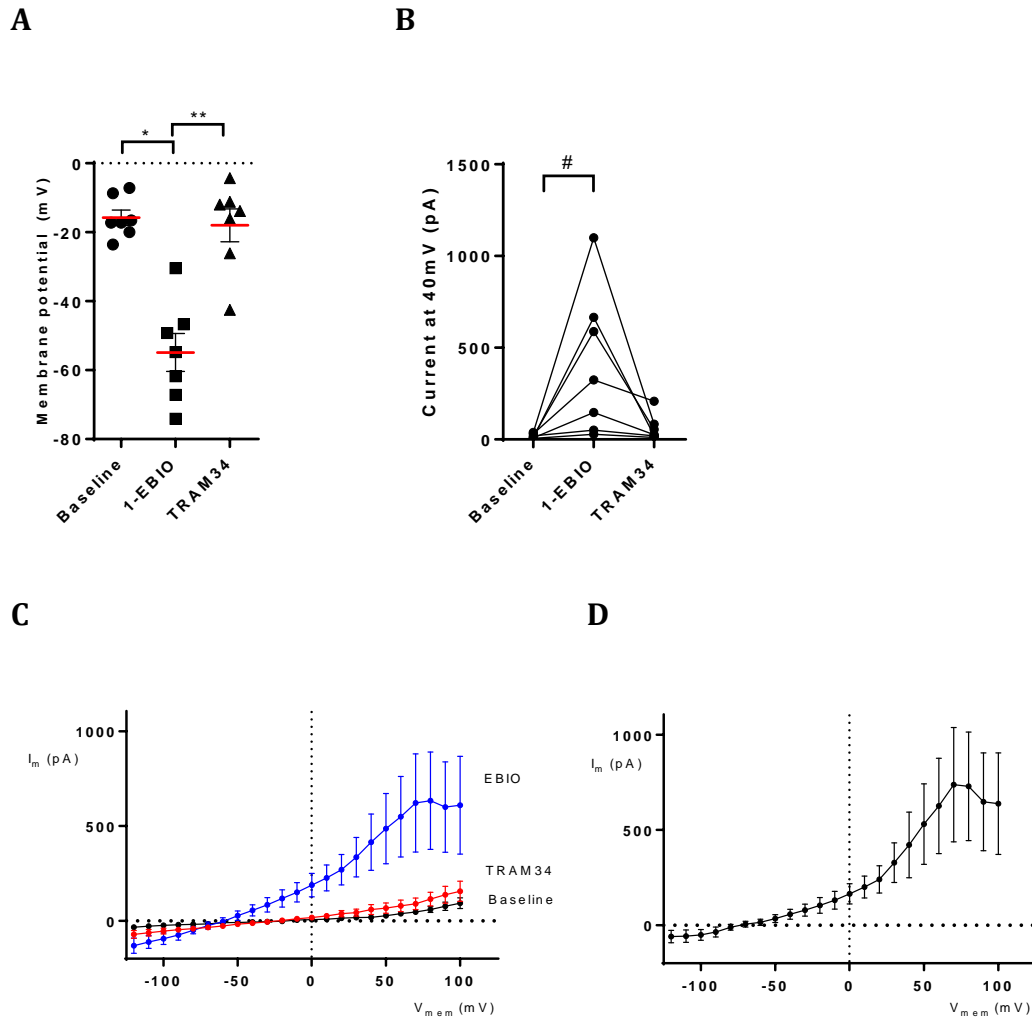
Figure 4-6: Asthmatic primary HBECs cultured at ALI express K$_{Ca}$3.1 currents.

Whole-cell membrane currents were recorded from differentiated HBECs (n = 7) from 3 asthmatic donors. Experiments demonstrated K$_{Ca}$3.1 activity in differentiated asthmatic HBECs. (**A**) Reversal potential values in each experimental condition; *P = 0.0019; **P = 0.0003. (**B**) Size of the current at 40 mV in each experimental condition; #P = 0.0346. (**C**) I$_m$-V$_{mem}$ relationships derived from whole-cell patch clamp recordings at baseline (black graph), 1-EBIO (blue graph), and TRAM-34 (red graph). (**D**) Subtracted current (1-EBIO – TRAM-34).

The electrophysiological characteristics of K$_{Ca}$3.1 currents were similar in submerged HBECs and differentiated ALI culture HBECs (**Figure 4-7**), as analysed by the Mann Whitney t-test. Similar numbers of HBECs from submerged cultures and differentiated ALI cultures responded to 1-EBIO and TRAM-34, suggesting that similar populations of K$_{Ca}$3.1-expressing cells are present within cultures of primary asthmatic HBECs (**Figure 4-7D**).

A

Baseline

B

1-EBIO

C

TRAM-34

D
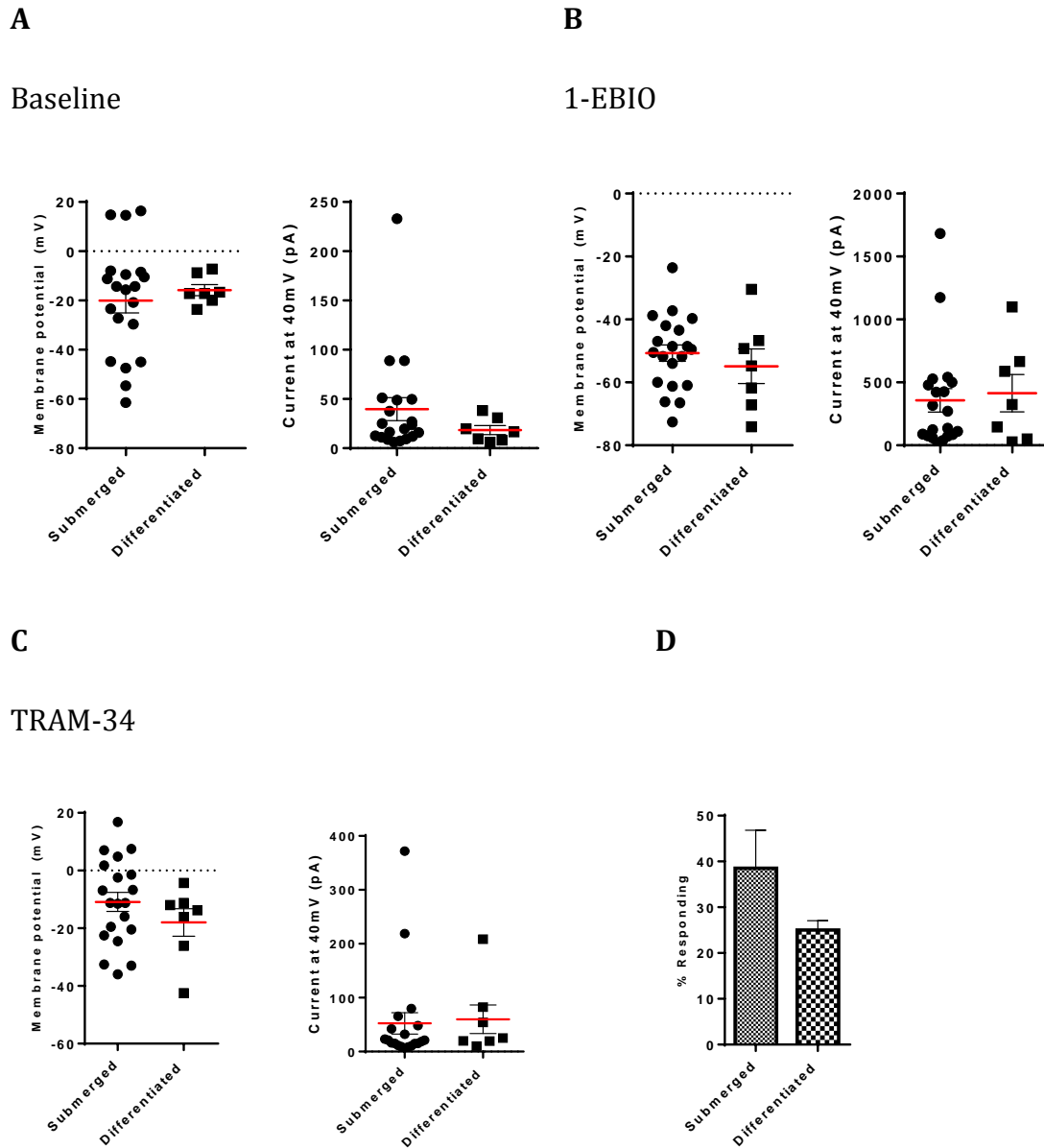
Figure 4-7: Submerged (*n* = 20) and differentiated (*n* = 7) HBECs exhibit K$_{Ca}$3.1 currents with similar characteristics.

Reversal potential values and the size of the outward current at 40 mV of submerged and differentiated HBECs at (**A**) baseline, (**B**) with 1-EBIO, or (**C**) after addition of TRAM-34. (**D**) Percentage of submerged and differentiated HBECs responding to 1-EBIO and TRAM-34; each data point represents an individual donor (submerged donors: *n* = 7; differentiated donors: *n* = 3). All data are expressed as mean ± SEM. Bar represents mean of data points.

4.3.1.4.2 Asthmatic HBECs exhibit larger K$_{Ca}$3.1 channel currents than healthy HBECs

K$_{Ca}$3.1 currents were recorded from submerged HBECs from both asthmatic donors (n = 20 cells from 8 donors) and healthy controls (n = 14 cells from 5 donors). All asthmatic donors were classified as severe asthmatics. All data were analysed by the Mann Whitney t-test.

At baseline, asthmatic and healthy HBECs displayed similar membrane currents (**Figure 4-8**). The reversal potentials (asthmatic: -20.0 ± 5.0 mV; healthy: -18.8 ± 7.4 mV) and size of currents at 40 mV were similar in asthmatic and healthy HBECs (asthmatic: 39.7 ± 11.6 pA; healthy: 27.4 ± 5.4 pA).

1-EBIO induced large outward currents and hyperpolarised membrane potentials in both asthmatic and healthy HBECs (**Figure 4-9**). Similar reversal potentials were seen in asthmatic (-50.7 ± 2.6 mV) and healthy HBECs (-52.5 ± 2.4 mV). However, the size of the outward current at 40 mV in asthmatic HBECs (357.5 ± 93.4 pA) was significantly larger (*P = 0.0329) than the size of the currents recorded from healthy HBECs (104.5 ± 20.7 pA; **Figure 4-9D**).

TRAM-34 inhibited 1-EBIO-induced currents of both asthmatic and healthy HBECs (**Figure 4-10**) and resulted in similar reversal potentials (asthmatic: -10.9 ± 3.3 mV; healthy: -11.2 ± 3.7 mV) and outward currents (asthmatic: 52.3 ± 19.9 pA; healthy: 30.1 ± 5.0 pA).

Analysis of the subtracted K$_{Ca}$3.1 channel components (1-EBIO – TRAM-34) revealed that both asthmatic and healthy HBECs displayed large outward currents with reversal potentials approaching the equilibrium potential of K$^+$ (**Figure 4-11A**; asthmatic: -62.6 ± 3.0 mV; healthy: -75.1 ± 4.1 mV). The size of the subtracted K$_{Ca}$3.1 current at 40 mV of asthmatic HBECs (305.2 ± 79.0 pA) was significantly larger than that of healthy HBECs (74.5 ± 19.6 pA; **Figure 4-11B**; #P = 0.0225). Similar percentages of asthmatic and healthy HBECs responded to 1-EBIO and TRAM-34 (**Figure 4-11C**).

A
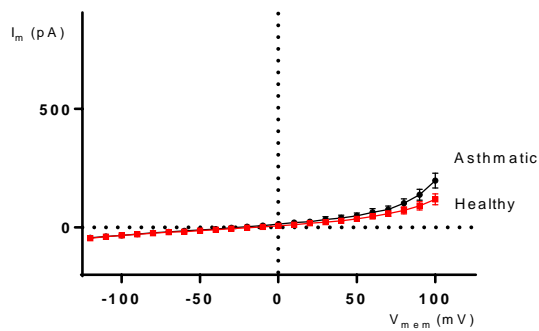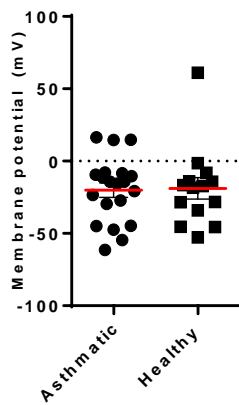
B   **C**
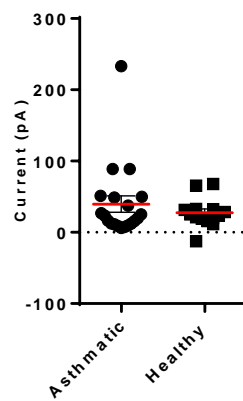
Figure 4-8: Asthmatic and healthy submerged HBECs display similar membrane currents at baseline.

(**A**) I$_m$-V$_{mem}$ relationship derived from whole-cell patch clamp recordings at baseline; asthmatic = black graph, healthy = red graph. Baseline values of (**B**) reversal potentials and (**C**) size of the outward current at 40 mV recorded from asthmatic and healthy HBECs.

A

Asthmatic

B

Healthy

C

D
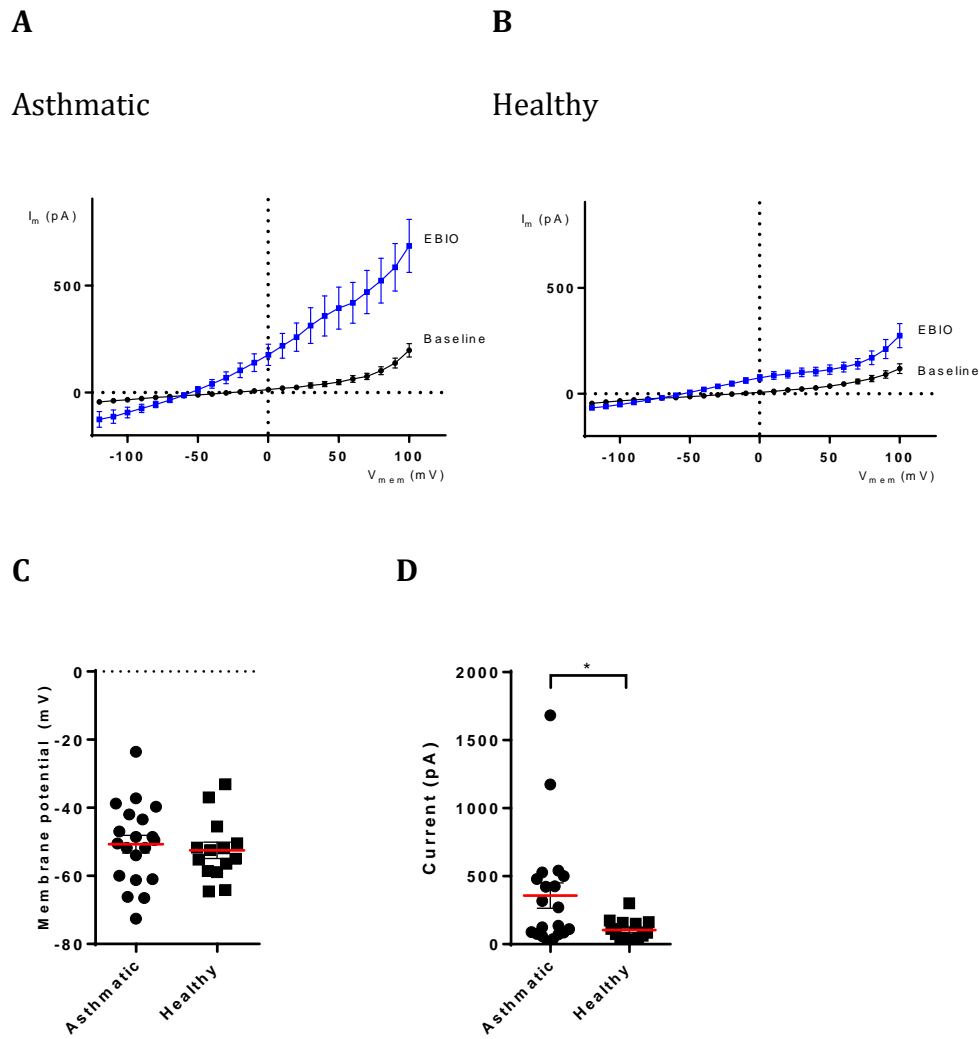
Figure 4-9: 1-EBIO induces larger outward currents in asthmatic HBECs compared with healthy HBECs.

I_m-V_{mem} relationship derived from recordings from (**A**) asthmatic and (**B**) healthy HBECs at baseline (black graph) and with 1-EBIO (blue graph). 1-EBIO-induced values of (**C**) reversal potentials and (**D**) the size of the outward current at 40 mV in asthmatic and healthy HBECs (*P = 0.0329).

A

Asthmatic

B

Healthy

C

D
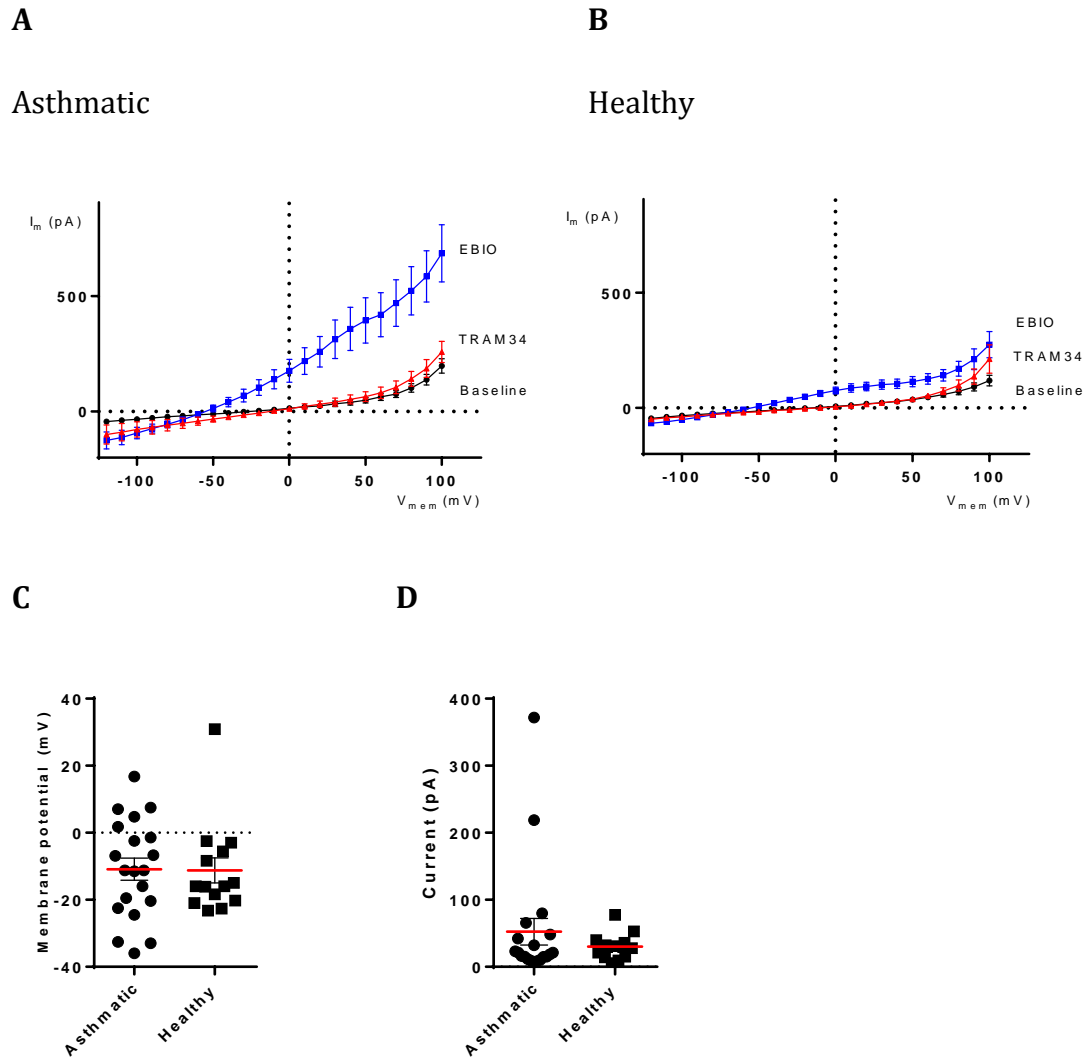
Figure 4-10: TRAM-34 inhibits 1-EBIO-induced currents in asthmatic and healthy HBECs.

I$_m$-V$_{mem}$ relationship derived from (**A**) asthmatic and (**B**) healthy HBECs at baseline (black graph) and following the sequential addition of 1-EBIO (blue graph) and TRAM-34 (red graph). TRAM-34-induced values of (**C**) reversal potentials and (**D**) the sizes of the outward currents at 40 mV recorded from asthmatic and healthy HBECs.

A
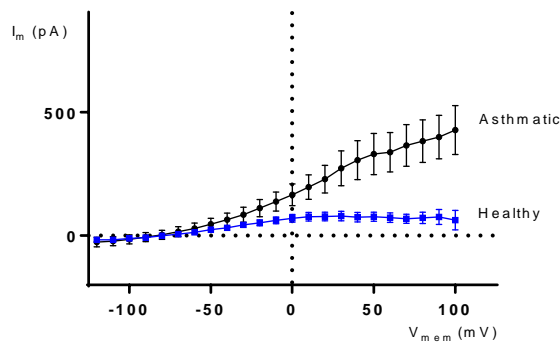
B
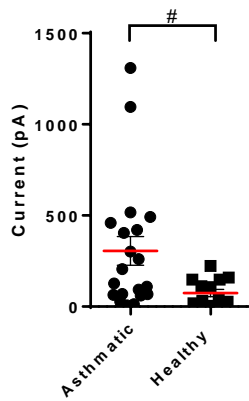
C
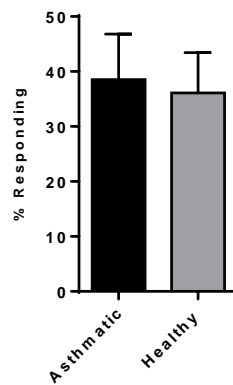
Figure 4-11: Asthmatic HBECs exhibit larger K$_{Ca}$3.1 currents than healthy HBECs.

(**A**) Comparison between subtracted K$_{Ca}$3.1 currents of asthmatic (black graph) and healthy (blue graph) HBECs. (**B**) Comparison between the size of the outward subtracted currents recorded at 40 mV in asthmatic HBECs and healthy HBECs (#P = 0.0225). (**C**) Percentage of asthmatic and healthy submerged HBECs responding to 1-EBIO and TRAM-34.

4.3.1.5 H292 cells exhibit K_{Ca}3.1 channel currents

Characteristic electrophysiological K_{Ca}3.1 activity was found in H292 cells.

At baseline, the membrane currents of H292 cells (*n* = 6) exhibited a reversal potential of -21.9 ± 3.7 mV. 1-EBIO induced significant hyperpolarisation of the membrane potential to -78.4 ± 2.4 mV (**Figure 4-12A**; *P = 0.0188), and significantly increased the size of the outward current at 40 mV from 29.8 ± 7.7 pA to 526.8 ± 95.7 pA (**Figure 4-12B**; #P = 0.0188). TRAM-34 inhibited this current to near-baseline levels, causing a significant reduction in the size of the current at 40 mV to 56.4 ± 20.1 pA (**Figure 4-12B**; #P = 0.0188) and significant depolarisation of the membrane potential to -19.4 ± 6.1 mV (**Figure 4-12A**; *P = 0.0188). Analysis of the subtracted K_{Ca}3.1 current of H292 cells showed that the large outward current had a reversal potential approaching the equilibrium potential of K^+ (-86.8 ± 3.7 mV; **Figure 4-12D**).

A

B

C

D
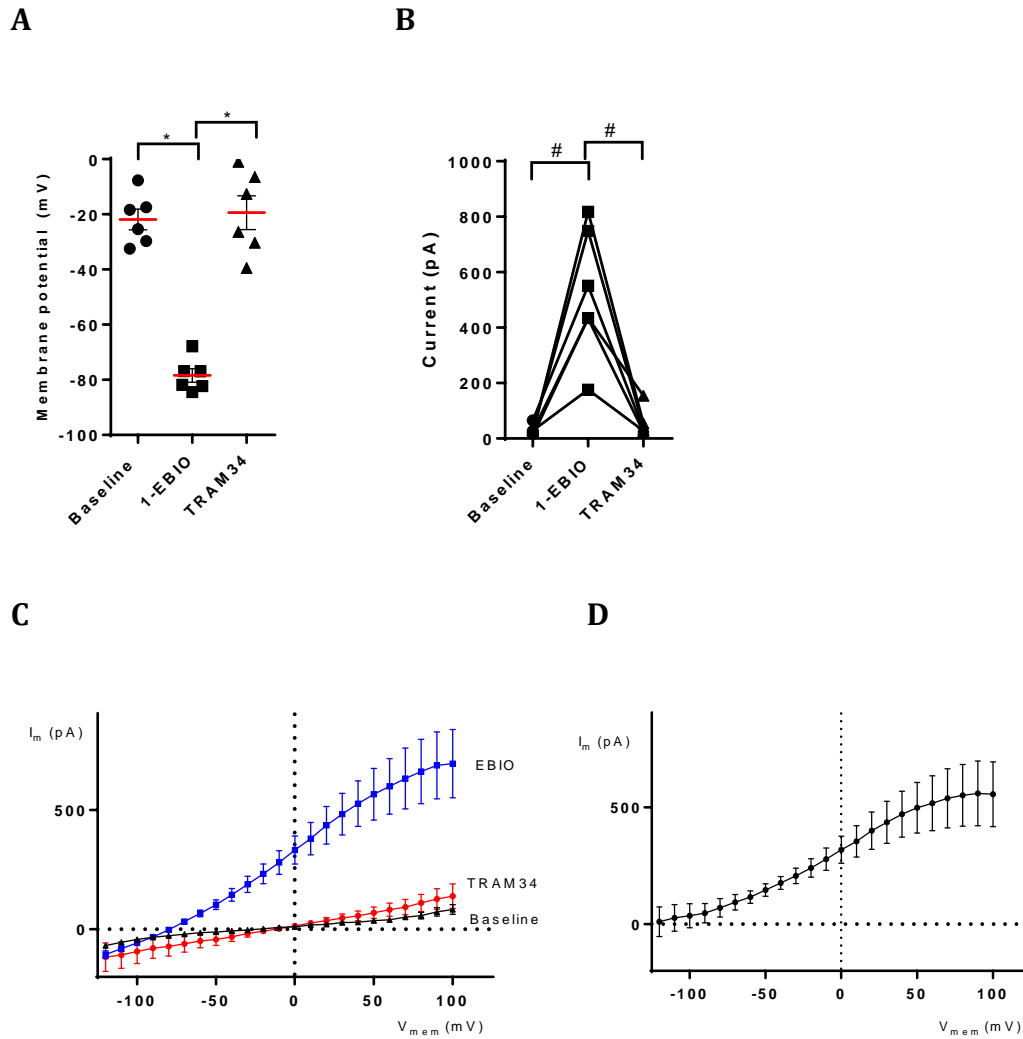
Figure 4-12: H292 cells express characteristic $K_{Ca}3.1$ channel currents.

Whole-cell membrane currents were recorded from H292 cells (*n* = 6) at baseline, and following the sequential addition of 100 μM 1-EBIO and 200 nM TRAM-34. (**A**) Reversal potential values (mV) of currents recorded from cells in each condition; *P = 0.0188. (**B**) Size of the current (pA) recorded at 40 mV from cells in each condition; #P = 0.0188. (**C**) I_m-V_{mem} relationships of recordings at baseline (black graph), 1-EBIO (blue graph), and TRAM-34 (red graph). (**D**) Subtracted current (1-EBIO – TRAM-34). All values are mean ± SEM.

4.4   *Discussion*

This is the first report of K_{Ca}3.1 expression in primary HBECs. Functional K_{Ca}3.1 currents were larger in HBECs isolated from patients with asthma compared to healthy volunteers.

The role of HBECs in asthma is paramount. As an interface between inhaled air and the immune system, the airway epithelium forms an essential protective barrier for the airways. The superficial position of goblet cells allows the airway epithelium to maintain the defensive barrier of the mucus layer, and respond to endogenous and exogenous triggers by rapidly secreting mucins. Capable of sequestering pathogens and facilitating their removal by mucociliary clearance, mucins form an indispensable part of the lungs' defence system. But, just as mucin secretion is vital for respiratory protection in healthy lungs; excessive mucin secretion facilitates the development of a harmful pathophysiological condition in asthmatic airways.

The exocytic release of preformed mucin granules from goblet cells is thought to be comparable to the mechanism of mast cell degranulation (Davis et al. 2008). In activated mast cells, granule exocytosis is regulated by intracellular Ca^{2+} signalling, mediated by store-operated Ca^{2+} entry. In HBECs, fluctuations in $[Ca^{2+}]_i$ accompany stimulated mucin secretion, and intracellular Ca^{2+} chelation diminishes mucin secretion (Kemp et al. 2004, Rossi et al. 2004, Bahra et al. 2004). In addition, mucin secretion from epithelial cell cultures is elicited by molecules capable of inducing store depletion, such as IP3, ionomycin and thapsigargin (Rossi et al. 2004, Abdullah et al. 1997). In mast cells, Ca^{2+} entry is accompanied by K_{Ca}3.1 opening, which potentiates Ca^{2+} influx by hyperpolarising the membrane, and subsequently leads to degranulation (Duffy et al. 2004, Wulff et al. 2010). The role of K_{Ca}3.1 in mediating the Ca^{2+} signalling-dependent pathway of granular exocytosis has been further implicated by findings that K_{Ca}3.1-deficient mice exhibit attenuated mast cell degranulation with reduced Ca^{2+} influx (Shumilina et al. 2008). The role of K_{Ca}3.1 in mucin granule exocytosis from goblet cells has not yet been examined. Consequently, investigating the expression of the K_{Ca}3.1 channel in HBECs offers insight into the potential role of K_{Ca}3.1 in airway mucin secretion and goblet cell function.

$K_{Ca}3.1$ expression is ubiquitous, with expression reported in a variety of cell types including fibroblasts (Roach et al. 2014), T lymphocytes (Nicolaou et al. 2007), and HLMCs (Cruse et al. 2006, Duffy et al. 2007). In the airway epithelium, $K_{Ca}3.1$ expression and activity has been reported in the viral transformed normal lung epithelial cell line NuLi-1, and in the cystic fibrosis cell line CuFi (Trinh et al. 2008, Klein et al. 2009), but its role in mucin secretion has not been explored.

In this study, results demonstrate $K_{Ca}3.1$ mRNA and protein expression in primary HBECs isolated from both patients with asthma and healthy controls, and in HBECs cultured under both submerged and differentiated ALI culture conditions. The $K_{Ca}3.1$ current in submerged HBECs was similar to currents recorded from HBECs isolated from ALI cultures, implying that the differentiation status of the cells did not affect $K_{Ca}3.1$ activity. No comparison between $K_{Ca}3.1$ mRNA expression in submerged and differentiated cells could be made due to differences in the level of housekeeping gene expression. The small size of the ALI cultures prevented western blot analysis of $K_{Ca}3.1$ protein in differentiated HBECs.

qPCR data revealed similar levels of $K_{Ca}3.1$ mRNA expression in asthmatic and healthy HBECs, and patch clamp experiments found that comparable percentages of asthmatic and healthy HBECs exhibited $K_{Ca}3.1$ activity. However, the size of the $K_{Ca}3.1$ current in asthmatic HBECs was significantly larger than the $K_{Ca}3.1$ current recorded from healthy HBECs. This indicates an interesting difference between healthy and asthmatic HBECs, and should be investigated further. In addition, as shown in **Figure 4-9**, 1-EBIO-induced currents recorded from asthmatic HBECs exhibited greater variation in comparison to healthy HBECs. This may indicate that $K_{Ca}3.1$ activity may differ in HBECs isolated from different subtypes of asthma.

Culturing HBECs under submerged conditions causes de-differentiation of the cells into a basal-like phenotype, and emersion to ALI promotes the formation of a pseudo-stratified, differentiated culture (Prytherch et al. 2011). When submerged and differentiated cultures were lysed for RNA and protein collection, individual HBEC phenotypes were not separated. Consequently, $K_{Ca}3.1$ mRNA and protein expression detected in this study represents total $K_{Ca}3.1$ expression per culture, rather than identifying $K_{Ca}3.1$ expression in specific HBEC phenotypes. Similarly, patch clamp experiments did not distinguish between the different phenotypes of

HBECs, unless cells displayed visible cilia. As a result, these results do not demonstrate that goblet cells exhibit K$_{Ca}$3.1 currents, or whether the difference in current size was present in goblet cells isolated from healthy controls or asthmatic patients. This study aims to investigate the potential role of K$_{Ca}$3.1 in mucin secretion. Hence, further experiments should be conducted to discover whether isolated goblet cells express K$_{Ca}$3.1, and whether fundamental differences are exhibited by goblet cells from healthy and asthmatic donors. The expression of K$_{Ca}$3.1 by goblet cells has been addressed in Chapter 6 by immunohistochemical detection of K$_{Ca}$3.1 in the bronchial epithelium of GMA-embedded biopsies. Using this method, the expression of K$_{Ca}$3.1 was localised to specific cell types.

Chapter 5:

Developing assays for the quantification of mucins from *in vitro* cultures

5.1   *Introduction*

Mucins are the major solid components of mucus and their biochemical properties confer hydration, pathogen sequestration, and other protective mechanisms to the mucus layer. Mucins are large glycoproteins that are either secreted by airway epithelial cells or remain tethered to the airway surface. Their large size, inherent stickiness, complex nature and the viscous gels they form make the detection and quantification of mucins challenging. Different methods and techniques have been developed and are reported in the literature. In this Chapter, a selection of these methods were trialled and optimised for the quantification of mucins produced and secreted from *in vitro* airway epithelial cell cultures. In addition, MUC5AC mRNA expression was examined in primary HBEC and H292 cultures.

5.2   *Methods*

5.2.1   *In vitro cell culture*

Primary HBECs were isolated bronchial brushings obtained from healthy control and severe asthmatic donors, and grown as submerged or differentiated ALI cultures (**Chapter 2, 2.2.1**). H292 cells were grown as submerged or ALI cultures (**Chapter 2, 2.2.2**). Purified HLMCs were isolated and activated with anti-FcεRI antibodies for 1 or 16 h, and supernatants were collected (**Chapter 2, 2.2.3**). HLFs were grown from resected lung tissue from lung cancer patients (**Chapter 2, 2.2.4**).

5.2.2   *Quantification of MUC5AC mRNA expression using qPCR*

Total RNA was isolated from *in vitro* cell cultures and used for first strand cDNA synthesis (**Chapter 2, 2.3**). The quantity of mRNA encoding the MUC5AC mucin polypeptide domain was measured using qPCR with TaqMan probes, alongside probes targeting the housekeeping gene ribosomal 18S (**Chapter 2, 2.3.3**). The expected fragment size with MUC5AC and 18S TaqMan probes were 83 and 187 base pairs (bp), respectively. PCR products were analysed by gel electrophoresis (**Chapter 2, 2.3.6**).

5.3   *Results*

5.3.1   *Primary HBEC ALI cultures and H292 cells express MUC5AC mRNA*

To select an *in vitro* model of the human airway epithelium capable of producing and secreting mucins, MUC5AC mRNA expression was examined in H292 cells and in primary HBECs from 9 patients with asthma and 4 healthy controls. The patient clinical characteristics are displayed in **Table 5-1**.

5.3.1.1   HBEC ALI cultures and H292 cells express MUC5AC mRNA

MUC5AC mRNA was not detected in the majority of submerged HBEC cultures isolated from patients with asthma (n = 3) and healthy controls (n = 4; **Figure 5-1A**). Analysis of PCR products of MUC5AC TaqMan probe reactions on a 2% agarose gel revealed a single product of 83 bp in only 1 asthmatic donor (A008). MUC5AC TaqMan probes were used alongside probes targeting the internal normaliser gene, ribosomal 18S, and a single product of approximately 187 bp was found in all samples (**Figure 5-1B**). In contrast, MUC5AC mRNA was detected in differentiated HBEC ALI cultures from asthmatic donors (n = 7) alongside 18S mRNA (**Figure 5-1B**). In addition, H292 cells cultured in submerged conditions (n = 5) also expressed MUC5AC mRNA (**Figure 5-1C**). These findings suggest that asthmatic primary HBECs cultured at ALI and H292 cells are suitable models for future experiments investigating MUC5AC expression. No healthy HBEC ALI cultures were available for study.

	Asthmatic *n* = 9	Healthy *n* = 4	P value
Disease severity	Severe	N/A	-
Age (y), mean ± SEM	52.6 ± 5.7	35.3 ± 9.5	0.2303
Sex (M/F)	5/4	3/1	0.5060
Asthma duration (y) , mean ± SEM	26.0 ± 7.8	N/A	-
Inhaled corticosteroid dose (µg), mean ± SEM (beclomethasone equivalents ‡)	1714 ± 190	0	**0.0030**
No. at BTS step 5	4	0	-
No. taking long-acting β-agonist	9	0	-
Exacerbations in last year, median (IQR)	2.0 (1.0 - 4.0)	N/A	-
Sputum eosinophil count (%), median (IQR)	2.8 (1.8 - 9.0)	0	**0.0364**
FEV1 (% predicted), mean ± SEM	66.6 ± 11.5	104.8 ± 4.1	0.0727
FEV1/FVC ratio (%), mean ± SEM	73.1 ± 6.9	83.0 ± 4.0	0.5273
Atopy (yes/no)	13/5	0/5	**0.0261**
Serum IgE (kU/L), median (IQR)	195.0 (35.6 - 618.3)	4.6 (3.1 - 16.9)	**0.0238**

Table 5-1: Clinical characteristics of donors recruited for collection of HBECs by bronchoscopy.

BTS, British Thoracic Society; *N/A*, not applicable; U/L, units per litre; IQR, interquartile range. ‡ Ratio for budesonide Turbuhaler calculated as 1.5; **bold** text highlights statistically significant P values. Data are presented as mean ± SEM or median (IQR).

A

Submerged HBECs
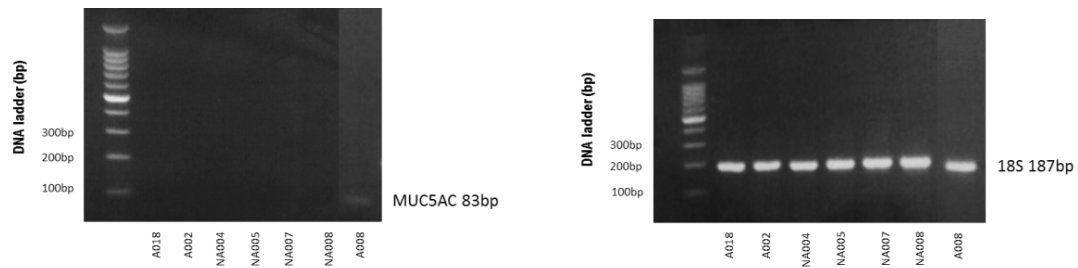
B

ALI culture HBECs
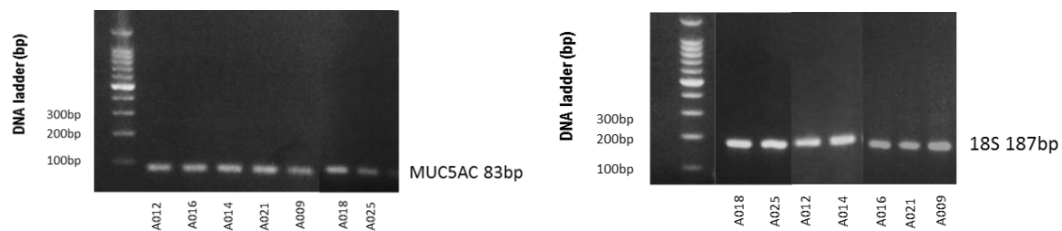
C

H292 cells
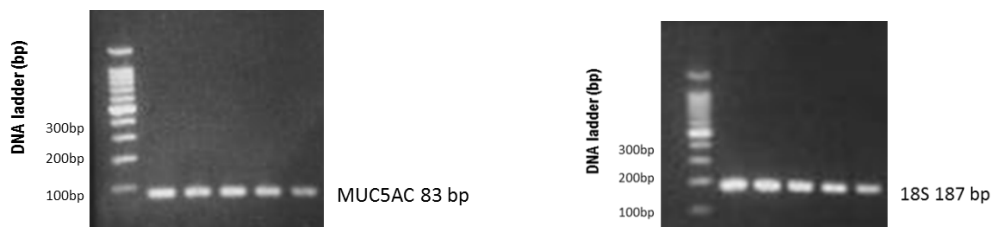
Figure 5-1: MUC5AC mRNA is expressed in H292 cultures and HBEC ALI cultures, but not in submerged HBEC cultures.

Analysis of PCR products amplified with MUC5AC and 18S TaqMan probes with (**A**) submerged HBEC RNA and (**B**) HBEC ALI culture RNA. Donors denoted with 'A' represented asthmatic HBECs, whereas donors denoted with 'NA' represented healthy, non-asthmatic HBECs. (**C**) MUC5AC mRNA was also detected in H292 cells cultured in submerged conditions ($n = 5$).

5.3.2 *Finding and optimising a method for quantifying in vitro mucin production*

To investigate whether $K_{Ca}3.1$ is involved in upregulating mucin production by HBECs, a method was needed to quantify the amount of stored mucins within the cultures and the amount of mucins secreted from the cultures. The following work aims to find and optimise a method suitable for quantifying the mucin content of samples collected from *in vitro* airway epithelial cell cultures.

5.3.2.1 *Immunohistochemical detection of mucins in H292 ALI culture cross-sections*

Immunohistochemical staining of paraffin-embedded H292 ALI cultures with MUC5AC-specific antibodies allows visualisation of intracellular MUC5AC. MUC5AC is a marker of goblet cells and demonstrates intracellular mucin accumulation (Stewart et al. 2012). A limitation of this method is the inability to assess secreted mucins; only intracellular mucins are stained. This method was optimised to provide a semi-quantitative method of analysing the intracellular mucin content of cells under experimental conditions.

ALI culture specimens were embedded into paraffin wax for immunohistochemistry. This method of embedding provides good antigen presentation and morphology preservation, and also allows the delicate cultures to be orientated in the wax so that a cross-sectional view of the culture can be cut and seen in stained sections. Wax sections were cut at a thickness of 4 μm, which prevented co-localisation of target antigens in sequential sections.

5.3.2.1.1 Preparation and staining of ALI culture specimens

ALI cultures were isolated from the Transwell using a sterile scalpel. A central strip of the culture was cut out and fixed in 10% neutral-buffered formalin (NBF; 40% formaldehyde, distilled water, NaH_2PO_4 and anhydrous NaH_2PO_4) for 4 h at room temperature. NBF stabilised cellular proteins and components and preserved cell culture morphology. Formalin-based fixation is useful for obtaining good ultrastructural preservation while keeping antigen masking to a minimum. When

fixed, soluble proteins become insoluble and are secured in a condition of stasis, thereby preserving the state and phenotype of the cells. ALI cultures were stored in 70% industrial methylated spirits (IMS) at room temperature; storage in alcohol limited nuclear and cytoplasmic shrinkage and minimised morphological distortion.

Fixed cultures required dehydration prior to embedding due to the immiscibility of paraffin with water (**Table 5-2**). ALI cultures were dehydrated in a series of alcohol baths to gradually displace the water content of the specimens. However, due to the immiscibility of paraffin wax with alcohol, an intermediate solvent, xylene, was used to progressively displace the alcohol within the dehydrated specimen. Once thoroughly infiltrated by xylene, specimens were incubated in wax to allow substitution of xylene by wax. A final wax block containing the specimen was produced by filling a plastic mould with liquid wax and carefully positioning the ALI culture slice in an upright position until the wax solidified.

Sections of paraffin-embedded ALI cultures were cut using a microtome (Leica) and floated onto a water bath heated to 42°C. Sections were collected on charged Colorfrost Plus glass slides (Fisher Scientific) and left to air-dry on a heated slide rack (60°C), before being baked overnight (maximum 18 h) at 37°C to promote secure adherence of the sections to the slides. Baking for longer than 18 h resulted in loss of antigenicity. Slides were deparaffinised and rehydrated to allow efficient interactions between antibodies and antigens. For deparaffinisation, slides were immersed in a series of xylene and alcohol baths and then in deionised water (**Table 5-3**). Antigen retrieval was conducted by heating slides in 400 ml 0.01 M citric acid buffer (pH adjusted to 6 with NaOH) for approximately 5 min in a microwave until the solution started to boil. Slides were carefully removed from the heated buffer and submerged in 2 sequential baths of deionised water. Slides were stained immediately to prevent them from drying out, which can increase the risk of non-specific antibody-antigen interactions and background staining.

Slides were stained with 20 μg/ml primary monoclonal MUC5AC antibody (MUC5AC MAb; Millipore) (**Chapter 2, 2.7.2**). After counterstaining with Mayer's haematoxylin, slides were dehydrated by immersing in baths of IMS and xylene, as

listed in **Table 5-3** in the reverse order. Dehydrated slides were covered by DPX mountant (Sigma) and a glass coverslip.

5.3.2.1.2 Semi-quantitative analysis of $K_{Ca}3.1$ and MUC5AC immunostaining in the bronchial epithelium

The amount of MUC5AC immunostaining in each ALI culture cross-section was analysed by a semi-quantitative thresholding method (**Chapter 2, 2.7.4**).

The total area of each culture cross-section was identified and measured using Cell^F software. Final threshold values were selected by calculating the lower and upper limit of HSI values from 10 specimens. All ALI cultures were subsequently measured using this final threshold. Thresholding values were representative of positive MUC5AC immunostaining and expressed as an average of the percentage of the total area of epithelium per culture (MUC5AC area fraction %). Prism 5 software (GraphPad Software) was used for statistical analysis. Immunohistochemistry data were expressed as median (range).

5.3.2.1.3 Paraffin-embedded H292 ALI culture cross-sections display MUC5AC immunoreactivity

H292 ALI cultures were treated with 10 or 100 ng/ml rh-AREG alongside the vehicle control (PBS-0.1% BSA). Staining with 20 µg/ml MUC5AC MAb revealed MUC5AC immunoreactivity in the ALI cultures (**Figure 5-2A**). All sections were counter-stained with Mayer's haematoxylin to visualise cell nuclei. 20 µg/ml mouse isotype control antibody (Dako) resulted in no immunoreactivity. Multi-layered structures of the cell cultures were seen when H292 cells were maintained at ALI for 24, 48 and 72 h (**Figure 5-2B**). To maintain consistency throughout experiments with H292 ALI cultures the following experiments were conducted after H292 cells had been maintained at ALI for 48 h.

Time (min)	Solution
15	70% IMS
15	90% IMS
15	100% IMS
15	100% IMS
30	100% IMS
45	100% IMS
20	Xylene
20	Xylene
45	Xylene

Table 5-2: Processing sequence for ALI culture specimens.

The processing of specimens involves the replacement of water with alcohol (dehydration), followed by the displacement of alcohol by the clearing agent, xylene. After the final xylene solution change specimens are infiltrated by wax. Each solution type represents a fresh solution change.

Time (min)	Solution type
2	Xylene
2	Xylene
2	100% IMS
2	100% IMS
2	70% IMS
-	Deionised water

Table 5-3: Deparaffinisation and rehydration sequence of paraffin-embedded ALI culture sections on glass slides.

Each solution type represents a fresh solution change.

A

20 µg/ml MUC5AC MAb
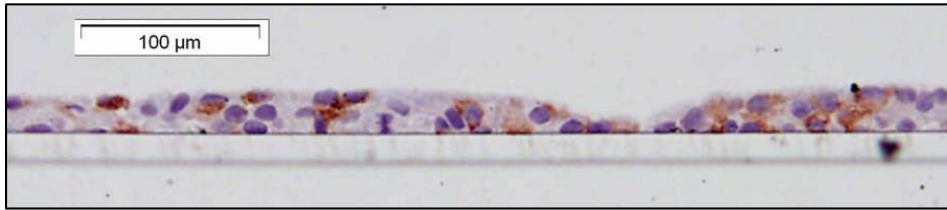
20 µg/ml isotype control
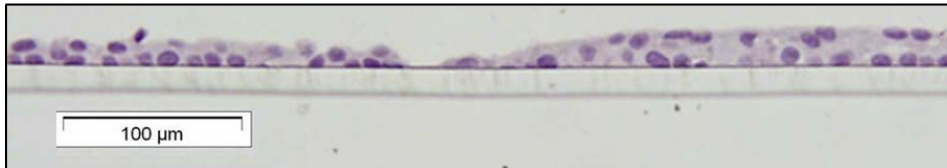
B

24 h
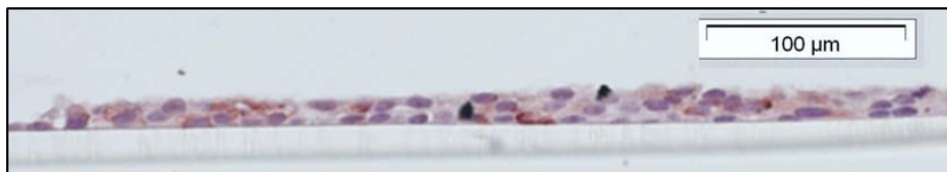
48 h
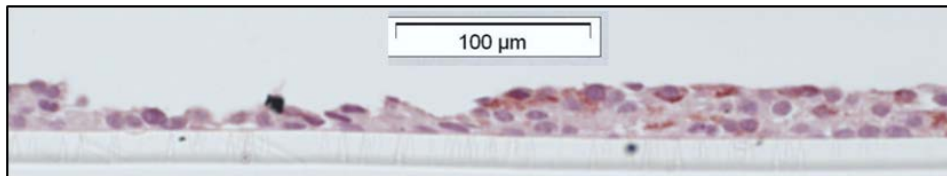
72 h
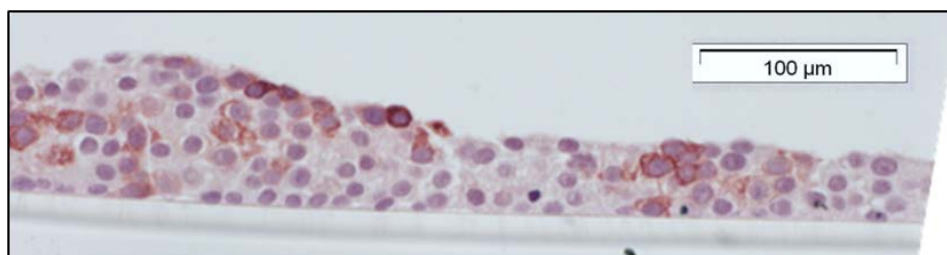
Figure 5-2: MUC5AC immunoreactivity is evident in H292 ALI cultures.

Paraffin-embedded H292 ALI culture labelled with (**A**) 20 µg/ml MUC5AC MAb or isotype control antibody. (**B**) Multi-layered structures of H292 cultures were visible after 24 h, 48 h, and 72 h. All sections were counter-stained with Mayer's haematoxylin counterstain. Bar represents 100 µm.

5.3.2.1.4 rh-AREG upregulates intracellular MUC5AC immunostaining in H292 cell ALI cultures

H292 ALI cultures (n = 3) were incubated for 24 h with 10 or 100 ng/ml rh-AREG alongside a vehicle control (PBS-0.1% BSA). Light microscopy images (**Figure 5-3A**) and thresholding analysis (**Figure 5-3B**) of MUC5AC immunostaining demonstrated that rh-AREG upregulated MUC5AC expression in a concentration-dependent manner. The greatest difference was seen in cultures treated with 100 ng/ml rh-AREG. Consequently, H292 ALI cultures were stimulated with 100 ng/ml rh-AREG for 24 h in future experiments. These findings demonstrate that quantification of MUC5AC immunostaining is a useful semi-quantitative method for assessing the intracellular mucin content of stimulated versus unstimulated H292 ALI cultures.

A

Unstimulated

10 ng/ml rh-AREG
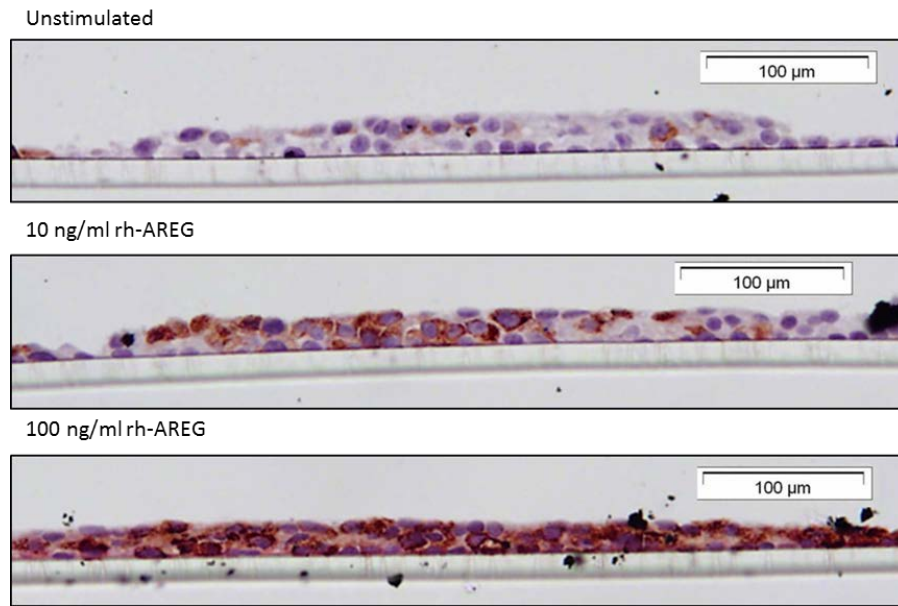
100 ng/ml rh-AREG

B
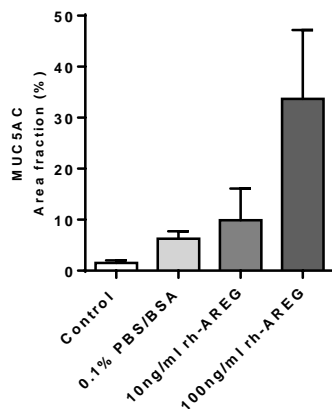
Figure 5-3: rh-AREG upregulates MUC5AC immunostaining in H292 ALI cultures after 24 h ($n = 3$).

H292 ALI cultures were treated vehicle control (PBS-0.1% BSA), 10 ng/ml or 100 ng/ml rh-AREG for 24 h. (**A**) Light microscopy images revealed upregulation of MUC5AC immunostaining with 10 and 100 ng/ml rh-AREG in a concentration-dependent manner. (**B**) Percentage of MUC5AC immunostaining (MUC5AC area fraction) within H292 ALI cultures. Data is expressed as median (range).

5.3.2.1.5 Method limitations

Firstly, no secreted mucins could be seen when staining cross-sections of paraffin-embedded H292 ALI cultures. Consequently, immunohistochemical detection of mucins is only suitable for assessing intracellular mucin expression. Supplementary assays will be required to analyse mucin secretion from the cultures.

Secondly, the fragile nature of the ALI cultures made each specimen particularly susceptible to damage (**Figure 5-4A**), and consequently made experiments difficult to reproduce. Further optimisation of this method could involve using additional steps to protect the cultures during the embedding process. For instance, the aqueous gel HistoGel (Thermo Scientific) could be used to minimise damage to the cultures.

Thirdly, of 11 staining runs, 5 were unsuccessful and failed to produce coloured end product when stained with MUC5AC MAb (**Figure 5-4B**). While this may suggest lack of MUC5AC in these specimens, repeat runs conducted on another day often achieved good staining. This suggested that rather than culture variability, reproducibility of this technique was unreliable and inconsistent. In addition, the small size of the specimens limited the number of sections that could be cut for repeated staining.

The method of antigen retrieval was optimised in an attempt improve the reproducibility of this method. Initially, slides were heated for 10 min in citric acid buffer in a microwave. However, prolonged heating often led to detachment of the sections from the slides. Reducing the heating time to 5 min minimised this loss of sections but often meant that sections were not heated for long enough to achieve optimal antigen retrieval, and resulted in no visible coloured end product. Decreasing the volume of citric acid buffer allowed boiling point to be reached in a shorter amount of time but limited the number of slides that could be heated simultaneously. Here, reproducibility was also limited when batches of slides had to be heated separately. The final optimised method of antigen retrieval involved heating slides in 400 ml citric acid buffer for 5 min, but still failed to produce staining on regular occasions.

A
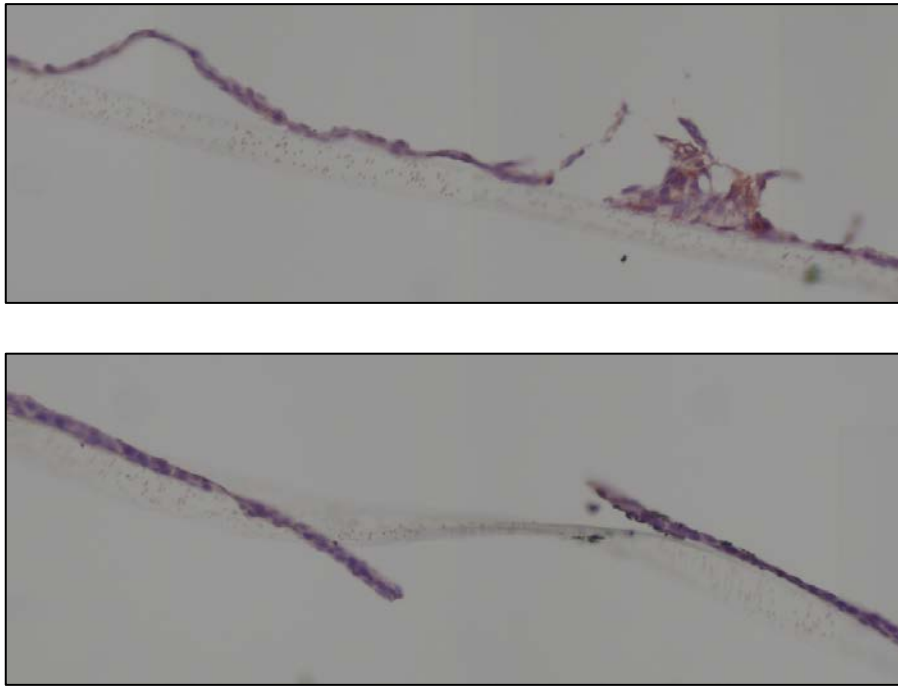
B
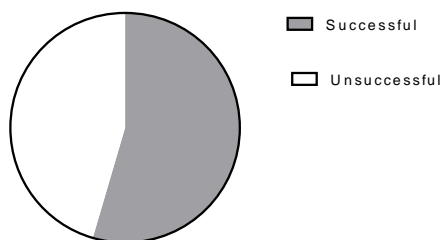
Figure 5-4: Immunohistochemical analysis of intracellular MUC5AC in H292 ALI cultures was difficult to reproduce.

(**A**) H292 ALI culture sections were easily damaged. (**B**) 46% of staining runs (n = 11) had to be repeated due to lack of coloured end product. Repeats of the staining runs often resulted in successful staining, suggesting that the reproducibility of this technique was low.

5.3.2.2 The PAS assay is not suitable for quantifying the mucin content of in vitro samples

The periodic acid-Schiff's reagent (PAS) staining in a microtiter plate assay was tested as a potential method for the detection of mucins within samples collected from *in vitro* airway epithelial cultures. If successful, this assay would be useful for detecting the quantity of mucins secreted by airway epithelial cultures, thus accompanying immunohistochemistry data. The PAS stain is commonly used in histology for visualising mucins (Dabbagh et al. 1999, Mantle et al. 1978, Grainge et al. 2011) and has been developed in previous studies for the quantitative analysis of carbohydrates such as mucin glycoproteins in solution (Mantle et al. 1978, Kilcoyne et al. 2011). Periodic acid oxidises the hydroxyl groups on the sugar residues of each mucin molecule to yield aldehyde groups, which subsequently react with Schiff's reagent to produce a characteristic pink/purple colour. According to Kilcoyne et al., the colour can be detected by plate readers at 550 nm wavelengths (Kilcoyne et al. 2011).

Potential advantages of this method include the use of N-acetylgalatosamine (GalNAc; Sigma) as a commercially available positive control; GalNAc residues have been identified as common components of the glycosylated mucin molecule (Sheehan et al. 2004). In addition, only a small volume of each sample is required for the assay (Kilcoyne et al. 2011). One disadvantage of this assay is that chemical staining with PAS may also detect non-mucin glycoproteins within unknown samples, and therefore may not distinguish between mucins and non-specific carbohydrate macromolecules.

5.3.2.2.1 Detection of mucins with PAS assay

Mucin glycoproteins were detected as described previously (Mantle et al. 1978, Kilcoyne et al. 2011). 120 µl of 0.06% periodic acid in 7% acetic acid was added to 25 µl of sample in a sealed 96-well plate and incubated for 1.5 h at 37°C. Colour development was achieved by adding 100 µl Schiff's reagent (Sigma) for 5 min at room temperature. Absorbance was read at 550 nm using a Perkin Elmer EnSpire plate reader.

5.3.2.2.2 Optimising sample preparation

GalNAc (Sigma), provided as a lyophilised powder and resuspended in PBS in serial dilutions, produced a concentration-dependent increase in absorbance (n = 2; **Figure 5-5A**). This indicated that GalNAc was a suitable positive control for the assay.

Initially, H292 lysates were harvested using trypsin-EDTA solution and centrifuging (1000 rpm, 5 min). Cells were resuspended in 1 ml media, and then lysed by repeated freeze-thaw. In the first experiment, comparing unstimulated cells with media alone, no upregulation in absorbance was seen (**Figure 5-5B**). When the cells were resuspended in a smaller volume of 100 μl media prior to lysis, higher absorbance was detected (**Figure 5-5C**). This suggested that resuspending cells in a smaller volume of media prior to lysis improved the detection of mucins.

Secreted mucins were harvested by collecting all media from each well, herein referred to as supernatants. For experiments, 1 ml media per well was used. However, supernatants collected from unstimulated H292 cells produced lower absorbance than media alone, used as a negative control for the assay (**Figure 5-5D**). To test whether media was creating a false positive result, FBS- and glutamine-free media was used instead, and appeared to eliminate this false-positive effect in supernatants (**Figure 5-5E**).

To examine whether the PAS assay could detect upregulation of mucin production in response to stimulation, H292 cells were stimulated with rh-AREG for 24 h, similar to immunohistochemistry experiments. Lysates of H292 cells stimulated with rh-AREG produced higher absorbance compared to unstimulated cells, although the differences were not significant (n = 3; **Figure 5-6A**). These findings suggested the assay detected an increase in mucins. However, non-mucin-expressing fibroblasts and submerged HBECs produced high absorbance in comparison with H292 cells (n = 3; **Figure 5-6B**), suggesting non-specific absorbance was produced. Furthermore, purified mucin from bovine submaxillary glands (Sigma), which exhibit a plethora of sugar chains (Kobayashi et al. 2004, Savage et al. 1990, Tsuji et al. 1986), was not detected by the assay (**Figure 5-6C**). These findings suggest that the PAS microtiter plate assay does not specifically

detect mucins in *in vitro* samples. Further optimisation and validation of this technique is required.

A
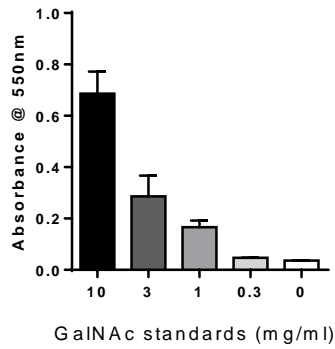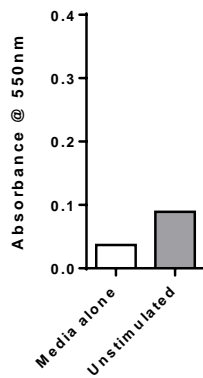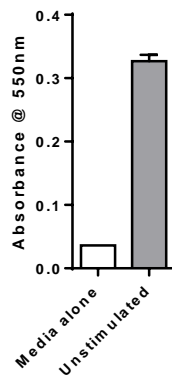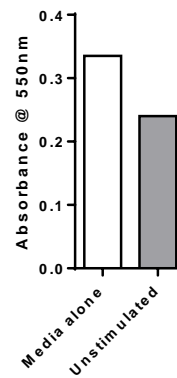
B **C** **D** **E**
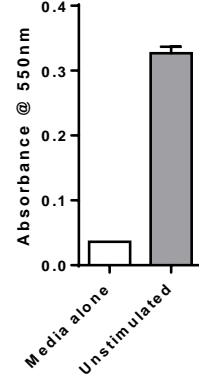
Figure 5-5: Optimising the PAS assay for mucin detection.

H292 cells were grown to confluence in 6-well plates and stimulated for 24 h with 100 ng/ml rh-AREG. (**A**) A concentration-dependent increase in absorbance at 550 nm was seen with half-log dilutions of GalNAc. Improved detection was seen in unstimulated cell lysates generated in (**C**) 100 μl media (n = 2) in comparison with (**B**) 1 ml media (n = 1). (**D**) Media supplemented with FBS and glutamine produced high absorbance, causing a false positive result (n = 1), whereas (**E**) FBS- and glutamine-free media produced low absorbance. Each sample was run in triplicate.

A

B

C
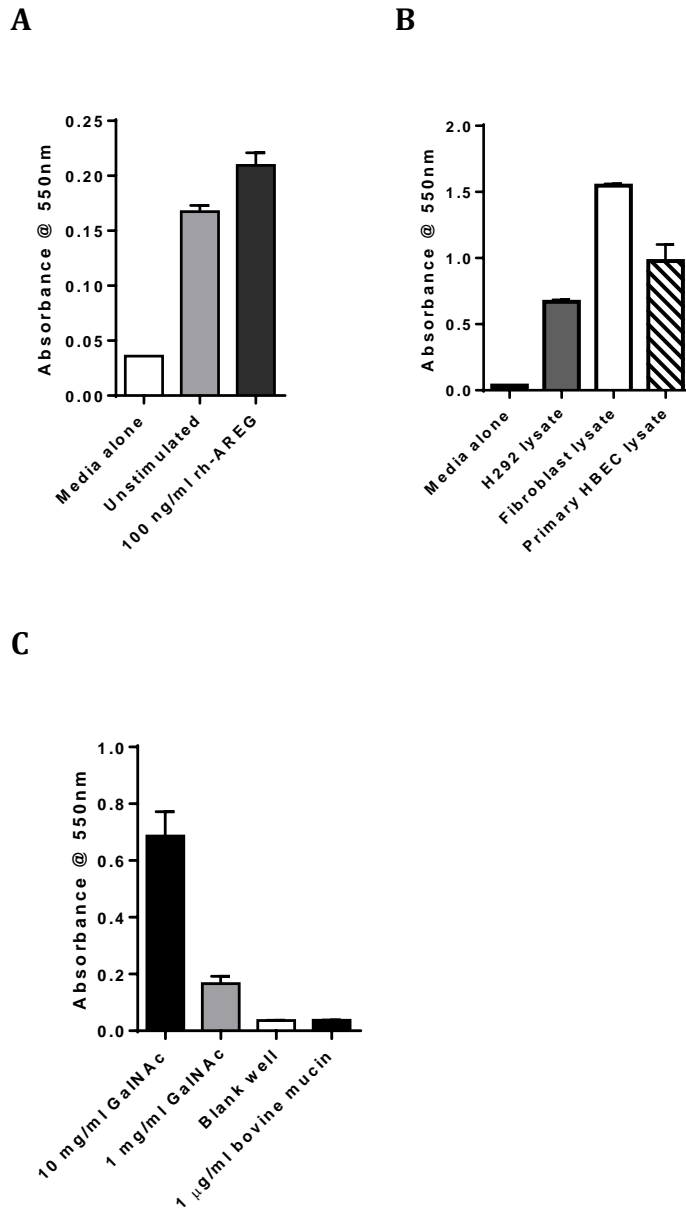
Figure 5-6: The PAS microtiter plate assay is not specific for mucin detection.

(**A**) Higher absorbance was seen in lysates of H292 cells treated with 100 ng/ml rh-AREG for 24 h in comparison with unstimulated cells (*n* = 3). (**B**) Fibroblast and submerged HBEC lysates generated from similar cell densities produced higher absorbance in comparison with unstimulated H292 lysates (*n* = 3). (**C**) 1 μg/ml purified mucin from bovine submaxillary glands (Sigma) produced no absorbance in the assay in comparison with GalNAc standards (*n* = 2).

5.3.2.3 Western blotting is not suitable for detecting mucin secretion from HBEC ALI cultures

Western blotting has been described as a method for detecting and quantifying mucins secreted from HBEC ALI cultures (Thornton et al. 1996b, Thornton et al. 1995, Harrop et al. 2013). To test this method, washes of the apical surface of HBEC ALI cultures were collected to harvest secreted mucins. The mucin content of these washes was analysed by agarose gel electrophoresis and western blotting. All reagents and antibodies were kindly provided by Professor David Thornton of the University of Manchester. All experiments were conducted in Professor Thornton's lab under the supervision of Dr Karine Rousseau.

MUC5AC mucins were targeted by MAN5AC-I, a rabbit polyclonal anti-serum raised against the peptide sequence within the non-tandem repeat region of MUC5AC (Thornton et al. 1996b, Thornton et al. 1995, Harrop et al. 2013). MUC5B mucins were detected by MAN5B-I, a rabbit polyclonal anti-serum raised against a region of MUC5B mucin subunit. Using antibodies to target the mucin polypeptide subunit is particularly valuable; the mucin subunit comprises an essential part of the core protein of the mucin macromolecule and is representative of the primary mucin gene product (Thornton et al. 1995).

5.3.2.3.1 Collection and preparation of samples

Primary HBECs were cultured to ALI to induce ciliogenesis and mucus production, as visualised using a light microscope. Secreted mucins were collected by incubating PBS on the apical surface of the cultures for 1 h at 37°C, as described previously (Kreda et al. 2007, Harrop et al. 2013); these samples are herein referred to as apical washes. PBS was gently pipetted over the culture to collect all secreted mucins and washes were stored at -80°C with protease inhibitor cocktail (Sigma; 1/20 dilution).

MUC5AC and MUC5B standards were used as a positive control for the western blots and were kindly provided by Professor Thornton. Standards were prepared alongside apical washes as follows. 40 µl of standards and 160 µl of samples were incubated with 25 mM (1.54 mg/ml) DTT in 1X urea reduction buffer with loading

dye (1 in 10 dilution of 10X stock containing 15.4 mg/ml DTT; **Table 5-4**) for 10 min at 100°C. DTT reduced mucin macromolecules by disrupting the disulphide bonds between adjacent mucin monomers, subsequently breaking up the large mucin polymer and allowing the separation of mucin molecules by gel electrophoresis according to individual monomer size. A urea-based reduction buffer was used due to the chaotropic nature of urea; urea facilitated the denaturation of mucin macromolecules by interfering with non-covalent intramolecular interactions. Thus, urea improved the solubility of the hydrophobic regions of the mucin molecule.

5.3.2.3.2 Sample electrophoresis

0.7% agarose gels were made using 1X Tris acetate-EDTA (TAE) buffer containing 0.1% SDS. The gels allowed separation of high molecular weight proteins under denaturing conditions. The gel tank was filled with Running Buffer (**Table 5-4**), and then samples were loaded into the wells in duplicate for simultaneous staining with both MAN5AC-I and MAN5B-I primary antibodies. The gel was run for 3 h at 60 V or 17 h at 30 V.

5.3.2.3.3 Sample transfer and membrane blotting

Separated samples were vacuum-blotted onto 0.45 μm nitrocellulose membrane (Protran BA85 Whatman) using a VacuGene Blotting Pump (GE Healthcare) (Thornton et al. 1995). The membrane was placed into a vacuum tray attached to the pump and submerged in 4X SSC Buffer (**Table 5-4**). The gel was washed in 4X SSC Buffer and placed on top the membrane, ensuring a complete vacuum was created. The gel and membrane were submerged in 4X SSC Buffer, used as a hybridisation buffer to control stringency of washing following hybridisation, reduce non-specific staining, and to prevent drying of the gel during vacuum transfer. The vacuum pump was run at 40-60 mbar for 1.5 h to allow transfer of the samples onto the membrane.

5.3.2.3.4 Protein detection

The membrane was cut in half to allow duplicates of all standards and samples to be probed separately with either MAN5AC-I or MAN5B-I antibodies. Membranes were incubated with Blocking Buffer (**Table 5-4**) for 30 min at room temperature to inhibit non-specific protein-antibody interactions, and then incubated for 2 h at room temperature with primary antibodies, diluted in Wash Buffer by 1/2000 (**Table 5-4**). Unbound antibodies were removed by washing 3 times for 5 min. Membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Abcam) for 30 min at room temperature. Unbound antibody was removed by washing (3 x 5 min). Bound antibodies were detected by incubating membranes with alkaline phosphatase buffer (**Table 5-4**) until colour development was visualised (no longer than 20 min, according to Dr Rousseau). Finally, membranes were rinsed with tap water and left to dry overnight at room temperature.

Buffer	Recipe
1X urea reduction buffer	12.1 g Tris HCl
	1.86 g EDTA
	Make up to 1 L with 6 M urea
	Adjust pH to 8 using HCl
10X urea reduction buffer (with loading dye and DTT)	25 ml 20X urea reduction buffer
	10 ml 50X TAE
	5 ml 10% SDS
	1 spatula Bromophenol blue
	15.4mg/ml dithiothreitol (DTT)
	Make up to 50 ml with glycerol
Running Buffer	1X Tris acetate-EDTA buffer
	See below
1X Tris acetate-EDTA buffer	50X stock purchased from Fisher Scientific
	Dilute to 1X with pure water
20X saline-sodium citrate (SSC) Buffer (diluted to 4X with pure water)	175.25 g NaCl
	88.2 g Na citrate
	Make up to 1L with pure water
	Adjust pH to 7 with NaOH
Blocking Buffer	5 g milk powder
	Make up to 100 ml with 1X Wash Buffer (see below)
Wash Buffer (20X TBST) (diluted to 1X with pure water)	250 ml 2 M Tris HCl (pH 8)
	438.8 g NaCl
	25 ml Tween-20 (Sigma)
Alkaline phosphatase buffer	12.1 g Tris HCl
	5.8 g NaCl
	1.016 g $MgCl_2$ $6H_2O$
	Adjust pH to 9.5 with HCl

Table 5-4: List of solutions for the detection of mucins by agarose gel electrophoresis and western blotting.

5.3.2.3.5 Western blotting did not detect mucins in apical washes of HBEC ALI cultures

Unlike previous studies (Harrop et al. 2013), no colour development was achieved following incubation with the alkaline phosphatase buffer. In conclusion, western blotting did not detect mucins in the apical washes of HBEC ALI cultures.

Several problems could explain the lack of mucins detected by this method: inefficient collection of mucins from the ALI cultures; poor mucin secretion by the ALI cultures; lack of specificity of primary antibodies for mucins produced by ALI cultures; or insufficient mucins within samples required to satisfy the minimum detection limit of the primary antibodies.

Optimisation of sample collection was attempted by incubating the apical surface of ALI cultures with 0.2 or 1 ml PBS for either 30 min or 2 h. Smaller volumes of PBS were used to obtain more concentrated samples of mucins were collected, but failed to produce staining. In addition, samples were stored with or without the protease inhibitor cocktail to optimise mucin collection. However, western blots failed to detect mucins within these samples. Incubations of >1 h often caused loss of cells from the culture surface and disrupted ALI culture morphology. In the majority of cases, this eventually led to the development of leaks, loss of confluence, and cell death. As a result, multiple samples could not be collected from ALI cultures over an extended period of time, as has been reported previously (Harrop et al. 2013). These problems might be caused by poor quality of the cultures. Consequently, further work should investigate methods of improving the quality of HBEC ALI cultures.

5.3.2.4 ELISAs are not suitable for quantifying the mucin content of in vitro samples

Enzyme-linked immunosorbent assays (ELISAs) have previously been used to determine the quantity of MUC5AC in lysates, supernatants and apical washes of primary HBEC and H292 cultures (Harrop et al. 2013). Consequently, ELISAs would be useful for analysing the quantity of mucins secreted from *in vitro* airway epithelial cell cultures; immunohistochemistry only provides data for intracellular mucin expression. ELISAs utilise the specificity of antibodies targeting sequences of the mucin polypeptide subunit, thus identifying the presence of the core protein of the mucin macromolecule (Thornton et al. 1995).

To detect the presence of mucins in samples collected from H292 cultures, a primary monoclonal anti-human MUC5AC antibody (Millipore) was used (herein referred to as MUC5AC MAb). H292 cell cultures were used for this work due to the unlimited supply of the cell line and the well-documented expression and secretion of mucins from H292 cells (Okumura et al. 2005, Takami et al. 2012, Iwashita et al. 2010).

5.3.2.4.1 Initial preparation of samples

H292 cells were cultured in 6-well plates until confluent. Mucin production was upregulated by treating the cells with rh-AREG at 10 or 100 ng/ml for 24 h (Okumura et al. 2005) alongside the vehicle control (PBS-0.1% BSA). Secreted mucins were collected by harvesting all media from each well and storing at -80°C before analysis (referred to as supernatants). Intracellular mucins were collected by treating the cells with trypsin-EDTA solution, centrifuging (1000 rpm, 5 min) to obtain a cell pellet, and lysing by resuspending the cell pellet in fresh media and conducting 3 cycles of freezing in liquid nitrogen and thawing at 37°C. Cell debris was removed by centrifuging the lysates (5000 rpm, 5 min) and discarding the cell pellet. These samples will herein be referred to as lysates.

5.3.2.4.2 Immunoassay of MUC5AC protein

MUC5AC was detected by ELISA as described previously (Takeyama et al. 1999, Takeyama et al. 2001, Parker et al. 2012, Thavagnanam et al. 2011). All reagents are listed in **Table 5-5**. 100 μl of sample was incubated with 100 μl Coating Buffer at 37°C overnight in a sealed Maxisorp Nunc 96-well plate (Fisher Scientific). Unbound samples were discarded by washing 3 times with Wash Buffer at room temperature for 15 sec. Non-specific protein-antibody interactions were inhibited by incubating the plate with 100 μl Blocking Buffer for 1 h at room temperature. Blocking Buffer was discarded and the plate was washed 3 times. The plate was incubated with 50 μl of MUC5AC MAb (diluted in Blocking Buffer) per well at room temperature for 1 h. The plate was washed 3 times and then incubated with 100 μl of secondary biotinylated rabbit-anti mouse antibody (Dako) for 1 h at room temperature. Unbound secondary antibody was removed by washing the plate 3 times. 100 μl streptavidin and horseradish peroxidase (Av-HRP) solution (R&D Systems) was added to each well and incubated for 20 min at room temperature. The plate was washed 3 times to remove unbound Av-HRP. Finally, colour was developed with 3,3',5,5'-tetramethylbenzidine (TMB; BD Biosciences), which acts as a substrate for HRP and produces a blue product. The reaction was stopped with 2 N H_2SO_4, resulting in a yellow colour. Absorbance was read at 450 nm using an EnSpire plate reader (Perkin Elmer).

Buffer	Recipe
Coating Buffer	*For 100 ml:* 1 capsule of carbonate-bicarbonate buffer (Sigma) Dissolved in 100 ml pure water
Wash Buffer	*For 500 ml:* 500 ml 1X Dulbecco's phosphate-buffered saline (PBS; Invitrogen) 250 µl Tween20 (Sigma)
Blocking Buffer	*For 100 ml:* 100 ml 1X D-PBS 1 g bovine serum albumin (BSA)

Table 5-5: List of reagents for MUC5AC ELISA.

5.3.2.4.3 Further optimisation of the ELISA is needed

Several steps of the sample preparation and immunoassay were modified to optimise the ELISA for the specific detection of MUC5AC within the samples.

Low absorbance was detected in supernatants collected from 6-well plates with 2 ml media per well. Consequently, the volume of media was reduced to 1 ml to increase the concentration of mucins in each sample. Similarly, lysates, which were originally generated by resuspending pellets in 1 ml media, were subsequently produced with 350 μl media.

Preliminary experiments were conducted with H292 supernatants (n = 3) and lysates (n = 2) collected from unstimulated cells to determine the specificity of the primary antibody for the target antigen. Samples were stained with MUC5AC MAb alongside a mouse isotype control antibody (Dako) at either 5 or 10 μg/ml. Staining of supernatants with both isotype antibody and primary MUC5AC MAb produced similar absorbance values (**Figure 5-7A**). A greater difference between isotype and primary antibody staining was seen with lysates (**Figure 5-7B**). Initially, this was thought to suggest that greater specificity of MUC5AC MAb binding was achieved when staining lysates. To examine whether the assay could detect varying quantities of MUC5AC in lysate samples, lysates (n = 4) were added to the ELISA in serial dilutions of 1/2 and 1/4. However, staining of the diluted samples resulted in no distinct difference in absorbance with MUC5AC MAb, and similar staining was produced with the isotype antibody (**Figure 5-7C**).

A comparison between stimulated and unstimulated H292 samples was conducted to examine whether the absorbance values in the isotype control wells were simply background absorbance, and whether a greater difference between the isotype and primary antibody could be seen when mucin expression was increased. No difference was seen in supernatants collected from H292 cells stimulated with 100 ng/ml rh-AREG in comparison with unstimulated cells (**Figure 5-8A**). This could suggest that no mucins were secreted from the cultures. However, no difference was seen in lysates collected from H292 cells stimulated with 100 ng/ml rh-AREG in comparison with unstimulated cells (**Figure 5-8B**). This conflicted with immunohistochemistry data, which demonstrated a concentration-dependent increase in intracellular mucin expression with rh-AR

stimulation (**Figure 5-3A**). In addition, a high level of absorbance remained when staining with the isotype antibody (**Figure 5-8**).

Unlike previous studies (Takeyama et al. 1999, Takeyama et al. 2001, Parker et al. 2012, Thavagnanam et al. 2011), ELISAs with MUC5AC MAb did not show specific detection of MUC5AC in unstimulated and stimulated H292 supernatants or lysates. Similar staining with primary and isotype antibodies suggests that primary antibody binding was non-specific. The similarity in primary antibody and isotype control absorbance values also indicates that high levels of background staining are inherent in the ELISA. Further optimisation of the method is required to produce an ELISA with high specificity and sensitivity.

A

B

C
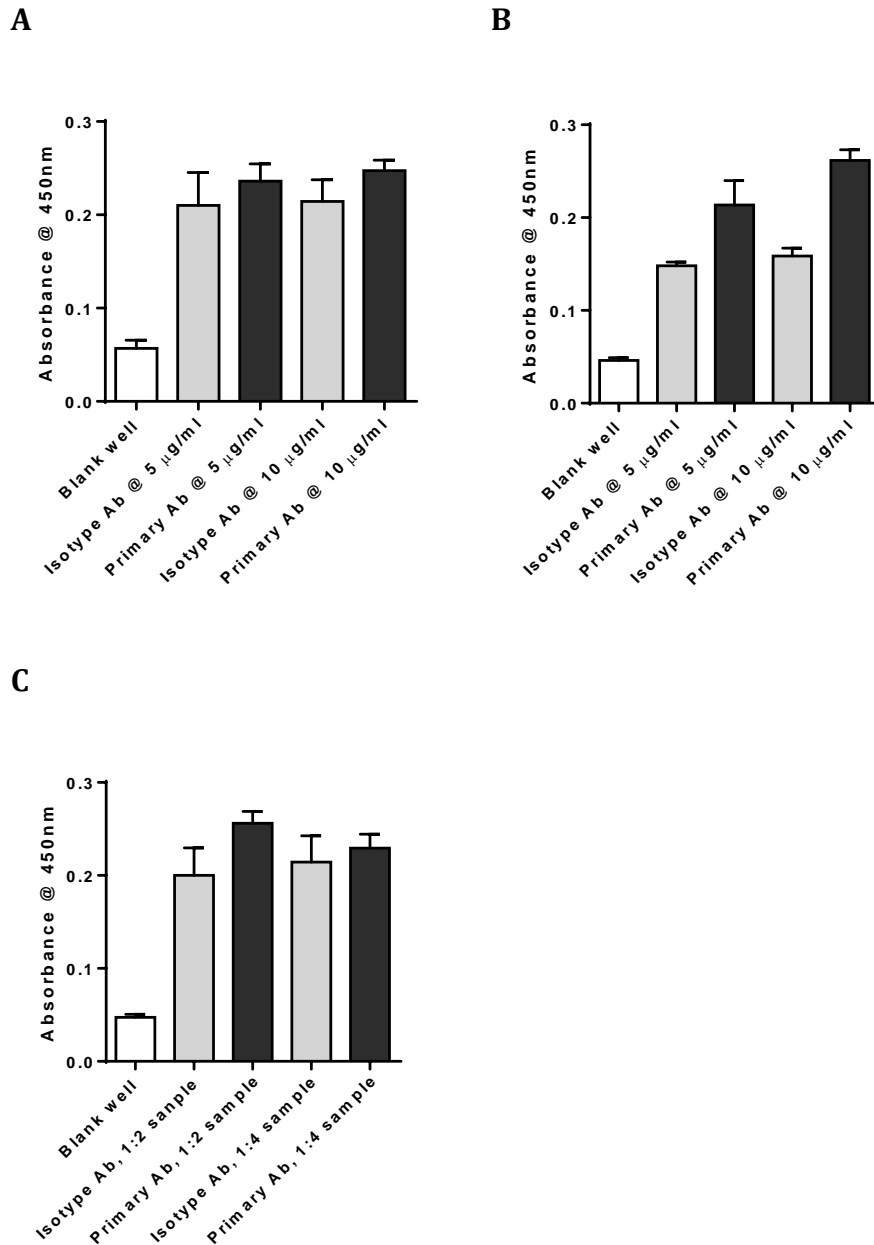
Figure 5-7: Preliminary ELISA experiments to optimise antibody concentrations.

(**A**) Supernatant samples (*n* = 3) and (**B**) lysate samples (*n* = 3) stained with mouse isotype control (Isotype Ab) and primary MUC5AC (Primary Ab) antibodies at 5 µg/ml and 10 µg/ml. (**C**) Similar absorbance values were obtained with isotype and primary MUC5AC antibodies (10 µg/ml) in ELISA experiments with H292 lysate samples diluted 1:2 or 1:4 (*n* = 4). Blank well = coating buffer only. All data are expressed as mean ± SEM.
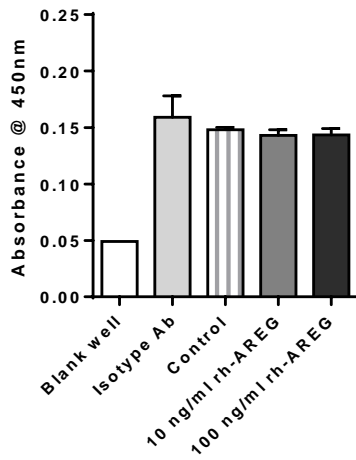
A
B
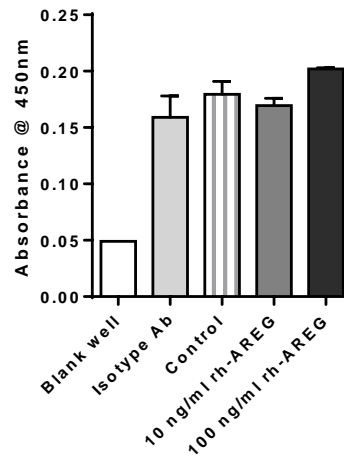
Figure 5-8: MUC5AC ELISA does not distinguish between unstimulated and stimulated H292 cells (*n* = 3).

Similar absorbance values were obtained with mouse isotype control and primary MUC5AC antibodies (10 μg/ml) in ELISA experiments with (**A**) supernatants and (**B**) lysates of H292 cells stimulated with 10 or 100 ng/ml rh-AREG for 24 h.

5.3.2.5 ELLA with biotinylated lectin from *Helix pomatia*

The enzyme-linked lectin assay (ELLA) is a semi-quantitative method for the detection of mucins in *in vitro* samples from primary HBECs and human epithelial cell lines (Poachanukoon et al. 2014, Harrop et al. 2012, Devine et al. 1991, Hewson et al. 2004), and employs a similar method similar to that of ELISAs. Previous studies have used ELLA to quantify secreted mucins in media harvested from submerged H292 cultures (Poachanukoon et al. 2014, Berger et al. 1999a). Accordingly, preliminary work began by analysing the mucin content of supernatants and lysates of H292 cells cultured in 6-well plates by ELLA.

Mucins were identified using a biotinylated lectin from the snail *Helix pomatia* (Sigma). Lectins are oligomeric proteins and exhibit saccharide-binding sites with affinities for specific glycoconjugates. The *Helix pomatia* lectin is a 70 kDa hexamer and recognises terminal alpha *N*-GalNAc residues, such as those found on mucins (Poachanukoon et al. 2014, Harrop et al. 2012, Devine et al. 1991, Hewson et al. 2004). *Helix pomatia* lectin binds to purified mucin from bovine submaxillary glands (Sigma) (Hewson et al. 2004, Brooks 2000, Latella et al. 1996, Dwek et al. 2001), which was used as a positive control in the following experiments. Unlike antibody-based ELISAs, lectin-binding assays do not distinguish between specific mucin gene products. Consequently, ELLA provides a semi-quantitative method of assessing the total mucin content of unknown samples rather than determining specific quantities of mucin subtypes.

5.3.2.5.1 Detection of mucins with ELLA

Mucins were detected as described previously (Hewson et al. 2004). All reagents and recipes are listed in **Table 5-6**. 100 µl of each sample was incubated with 100 µl Coating Buffer at 37°C for 2 h in a sealed Maxisorp Nunc 96-well plate (Fisher Scientific). Unbound samples were discarded and the plate was washed 3 times with Wash Buffer at room temperature. Non-specific lectin binding was inhibited by incubating with 100 µl Blocking Buffer for 1 h at 37°C. The plate was washed 3 times and then incubated with 100 µl of lectin (Sigma; diluted in Blocking Buffer) at 37°C for 1 h. Unbound lectin was removed by washing the plate 3 times. 100 µl of Av-HRP solution (R&D Systems; 1/200 dilution in Blocking Buffer) was added to

each well and incubated for 20 min at room temperature. Unbound Av-HRP was removed by washing the plate 3 times. Finally, colour reaction was developed with TMB substrate (BD Biosciences) and stopped with 2 N H_2SO_4. Absorbance was read at 450 nm using an EnSpire plate reader (Perkin Elmer).

5.3.2.5.2 Mucin from bovine submaxillary glands is a useful positive control for ELLA

Standards of mucin from bovine submaxillary glands (herein referred to as bovine mucin) were prepared in PBS. A concentration-dependent linear response was detectable with 50 µg/ml lectin (**Figure 5-9**). Hence, 50 µg/ml lectin was used for subsequent analysis of *in vitro* samples.

Buffer	Recipe
Coating Buffer	1 capsule of carbonate-bicarbonate buffer (Sigma) Dissolved in 100 ml pure water
Wash Buffer	500 ml 1X Dulbecco's phosphate-buffered saline (PBS; Invitrogen) 250 μl Tween20 (Sigma)
Blocking Buffer	100 ml Wash Buffer (see above) 1 g gelatine

Table 5-6: List of recipes for ELLA with lectin from *Helix pomatia*.
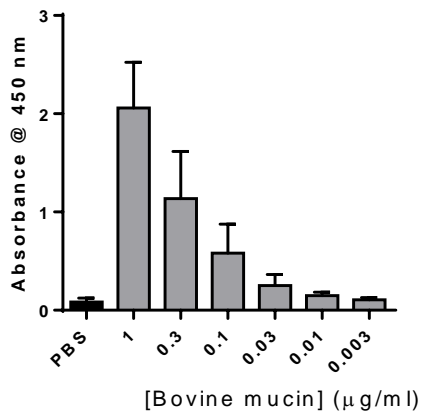
Figure 5-9: Bovine mucin is a suitable positive control for ELLA.

A linear and concentration-dependent response of bovine mucin standards was observed with 50 μg/ml lectin. Data are representative of 3 separate preparations of bovine mucin standards (*n* = 3) at different concentrations run in triplicate with ELLA. Results are expressed as median (range). [Bovine mucin] = concentration of bovine mucin.

5.3.2.5.3 ELLA detects rh-AREG-induced mucin content in H292 ALI culture lysates and washes

Initially, H292 cells (n = 4) were cultured in 6-well plates until confluent and treated for 24 h with either vehicle control (PBS-0.1% BSA) or 100 ng/ml rh-AREG. Secreted mucins were harvested by collecting all media from each well and storing immediately at -80°C (herein referred to as supernatants). No significant difference was seen between unstimulated supernatants and supernatants of cells treated with 100 ng/ml rh-AREG (**Figure 5-10A**), suggesting the assay did not detect mucins within the supernatants of submerged cultures. This could be due to the low level of mucins secreted by the cells, or the dilution of secreted mucins by the total volume of media used for the experiment.

To improve the detection of secreted mucins, H292 cells were cultured at ALI (n = 3) and apical washes were collected by incubating the apical surface of the ALI cultures with warm PBS for 5 min at room temperature and gently pipetting the PBS over the surface 3 times to maximise mucin collection. Washes were stored at -80°C. A concentration-dependent increase in absorbance was found in washes collected from unstimulated ALI cultures compared to cultures treated with 100 ng/ml rh-AREG for 24 h (*P = 0.0141; Friedman one-way ANOVA with Dunn's multiple comparisons test; **Figure 5-10B**). For future experiments, secreted mucins were detected by collecting apical washes from H292 ALI cultures.

Initially, lysates were prepared by incubating H292 ALI cultures (n = 3) with trypsin-EDTA solution and centrifuging cells (1000 rpm, 5 min) to obtain a cell pellet. Cells were resuspended in 350 μl serum- and glutamine-free media (sufficient for analysing each sample in triplicate). Cells were lysed in 3 freeze-thaw cycles with liquid nitrogen and heating at 37°C. Cell debris was removed by centrifuging lysates (5000 rpm, 5 min) and collecting the supernatant. No difference was seen between unstimulated and stimulated cells (**Figure 5-10C**), suggesting that upregulation of intracellular mucins had not been detected by the assay. This was in contrast to immunohistochemistry findings (**Figure 5-3**). This method of lysate preparation may have facilitated mucin degradation and thus prevented their detection.

To optimise lysate preparation, ALI cultures (n = 5) were detached from Transwells with a scalpel and lysed by placing in 350 µl sterile water and freezing at -80°C. A significant difference was found between unstimulated and 100 ng/ml rh-AREG lysates (**P = 0.0287; Friedman one-way ANOVA with Dunn's multiple comparisons test; **Figure 5-10D**). This method of lysate preparation was used for future experiments.

To test the specificity of ELLA, fibroblasts (HLF; n = 4) were lysed by freezing a cell pellet in 350 µl water at -80°C. Before lysing, cells were counted using a haemocytometer to ensure that the cell density was similar to that of the H292 ALI cultures. Unstimulated H292 lysates (n = 4) exhibited significantly higher absorbance than fibroblast lysates (***P = 0.0286; Mann-Whitney t-test; **Figure 5-10E**). These results indicate that detection of mucins by ELLA is specific.
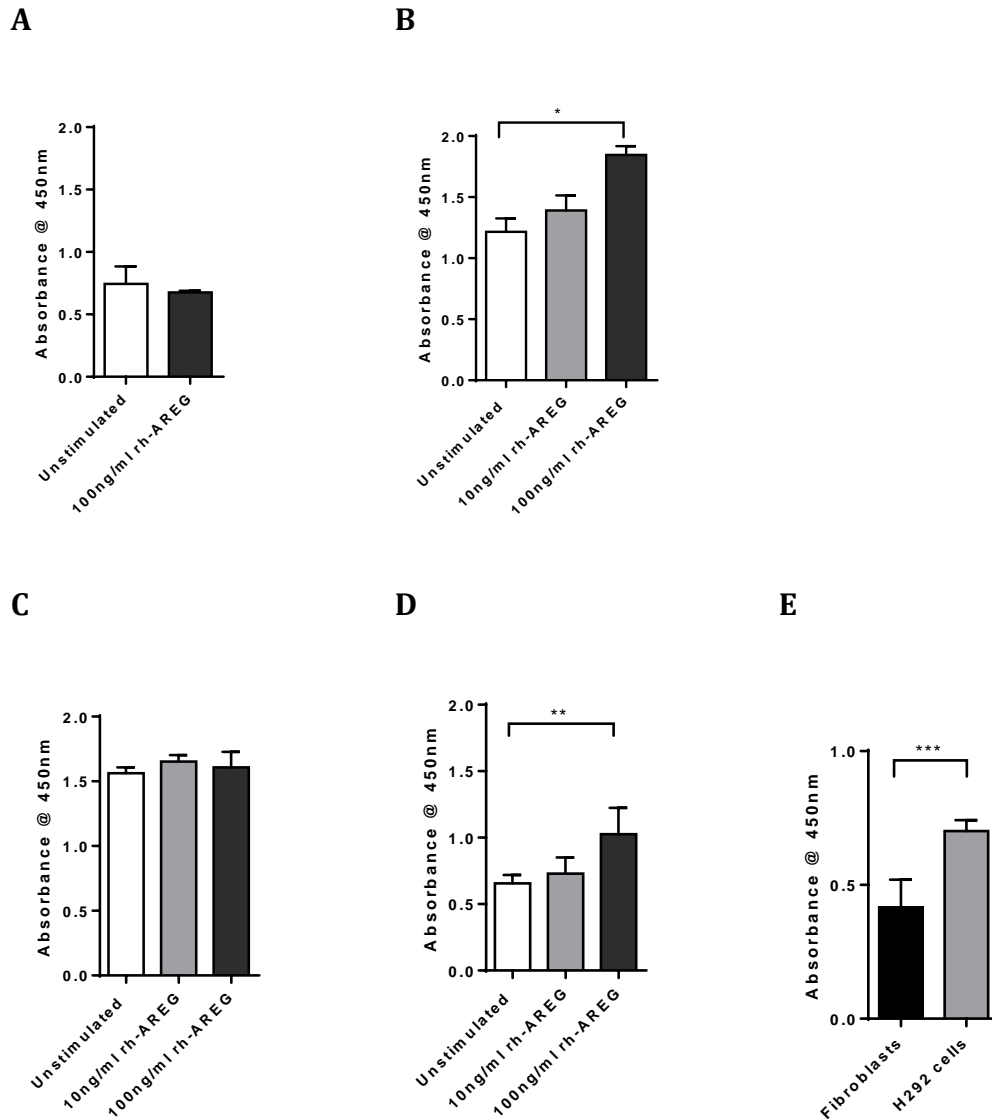
Figure 5-10: ELLA detects rh-AREG-induced mucin content in apical washes and lysates of H292 ALI cultures.

ELLA did not detect a change in absorbance from (**A**) supernatants collected from H292 cells in 6-well plates (n = 4), but found a significant difference in (**B**) apical wash samples from H292 ALI cultures (n = 4; *P = 0.0141). ELLA did not detect a difference in absorbance from (**C**) lysates prepared by trypsin-EDTA detachment of cells and freeze-thaw cycles in FBS- and glutamine-free media. In contrast, a significant difference in absorbance was seen in (**D**) lysates prepared by freezing ALI cultures in water (**P = 0.0287). (**E**) Comparison between fibroblast and H292 lysates (n = 4; ***P = 0.0286). Detection of mucins is presented as absorbance at 450 nm. Data are expressed as median (range).

5.3.2.5.4 ELLA does not detect upregulation of mucin production by other secretagogues

Previous studies have found that activation of P2Y$_2$ purinoceptors by ATP for 10 to 20 min elicits mucin secretion from HBEC ALI cultures (Jones et al. 2012, Davis 2002), measured by immunoslot blot. However, incubation of H292 ALI cultures with 10 and 100 μM ATP failed to upregulate the mucin content detected by ELLA in the apical washes or lysate samples (n = 1; **Figure 5-11A**). This could be due to a lack of P2Y$_2$ receptor expression; P2Y$_2$ receptor expression on H292 cells is unknown, and is absent on other cell lines such as Calu-3 (Kreda et al. 2007).

HLMCs express a variety of mediators and cytokines capable of inducing mucus secretion. H292 ALI cultures were incubated for 24 h with supernatants collected from HLMCs activated for either 1 h or 16 h with anti-FcεRI antibodies (**Chapter 2, 2.2.3.1**). No increase in ELLA absorbance was induced by HLMC supernatants in H292 ALI culture apical washes or lysates (**Figure 5-11B**). Consequently, rh-AREG was used to upregulate mucin production and secretion from H292 ALI cultures in future experiments.

A
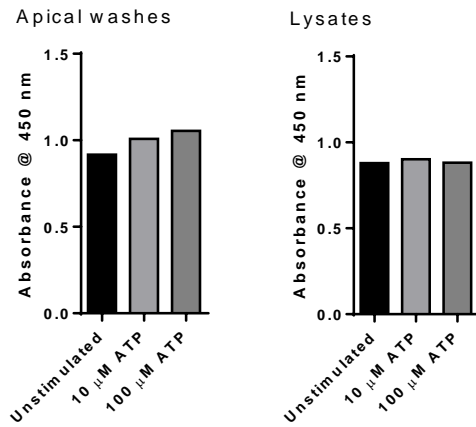
B
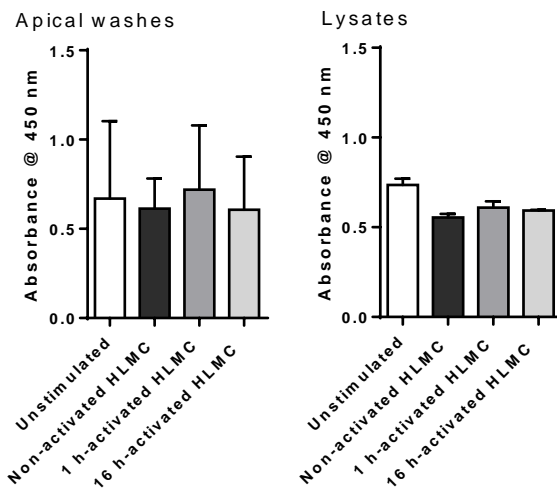
Figure 5-11: ELLA does not detect changes with activated HLMC supernatants or ATP.

ELLA did not detect a change in absorbance of apical washes or lysates of H292 ALI cultures incubated for 24 h with (**A**) 10 or 100 μM ATP (*n* = 1) or (**B**) supernatants collected from HLMCs activated for 1 h or 16 h with anti-FcεRI antibody (*n* = 4). Data are expressed as mean (range).

5.4   *Discussion*

This Chapter demonstrates that immunohistochemical staining of paraffin-embedded ALI culture cross-sections allowed visualisation of intracellular mucins, providing a semi-quantitative method of assessing intracellular mucin expression in response to stimulation by rh-AREG. In addition, this Chapter found that ELLA provides a semi-quantitative method of analysing the mucin content of apical washes and lysates of ALI cultures, thus indicating the level of intracellular and secreted mucins within *in vitro* airway epithelial cell cultures.

Primary HBECs were cultured as either submerged or differentiated ALI cultures, and their ability to express MUC5AC mRNA was assessed by qPCR. Similarly, the expression of MUC5AC mRNA in H292 cells was examined. Primary HBEC ALI cultures and H292 cultures were found to express MUC5AC mRNA, suggesting they are suitable models for studying mechanisms of airway epithelial mucin production and secretion. Submerged primary HBEC cultures did not express detectable MUC5AC mRNA.

Previous studies have utilised a variety of methods for quantifying mucin production and secretion *in vitro*, including the PAS microtiter plate assay (Mantle et al. 1978, Kilcoyne et al. 2011), western blotting (Thornton et al. 1996b, Thornton et al. 1995, Harrop et al. 2013), ELISAs (Takeyama et al. 1999, Takeyama et al. 2001, Parker et al. 2012, Thavagnanam et al. 2011), and ELLAs (Poachanukoon et al. 2014, Harrop et al. 2012, Devine et al. 1991, Hewson et al. 2004). In addition, immunohistochemistry with mucin-specific antibodies allows visualisation of intracellular MUC5AC, a marker of goblet cells (Stewart et al. 2012).

Immunohistochemical analysis of paraffin-embedded H292 ALI culture cross-sections with MUC5AC MAb allowed visualisation of intracellular MUC5AC. Thresholding analysis permitted quantification of the staining, and was compared across different experimental conditions. Overall, this method provided a semi-quantitative method of analysing the intracellular mucin content of cells under specific conditions. Limitations of this method include the inability to assess secreted mucins and the high failure rate of staining runs. The fragile nature of the ALI cultures made them extremely susceptible to damage during fixing, processing

and embedding. Paraffin-embedded sections could also be broken when staining, resulting in an incomplete section on the slide unsuitable for analysis. HistoGel (Thermo Scientific) was used to provide a protective coating for the cultures prior to embedding, but was susceptible to becoming brittle or deformed, and frequently made specimens unusable. Despite the limitations of this method, a concentration-dependent increase in MUC5AC immunostaining was observed in H292 ALI cultures treated with 10 or 100 ng/ml rh-AREG for 24 h. Ideally, this method should be used alongside another assay suitable for quantifying secreted mucins.

The PAS microtiter plate assay was assessed as a method of detecting mucins within supernatants and lysates of H292 cultures. Although previous studies have reported that the assay is suitable for the quantitative analysis of carbohydrates such as mucin glycoproteins in solution (Mantle et al. 1978, Kilcoyne et al. 2011), no significant difference was seen between unstimulated and stimulated cells. More importantly, mucin from bovine submaxillary glands did not exhibit any reactivity in the assay, and strong reactivity was seen with non-mucin-expressing cells, such as fibroblasts. Consequently, this assay was not selected for use in this study.

Western blotting was also examined as a method of mucin quantification. Apical washes were collected from primary HBEC ALI cultures, as described in the literature (Harrop et al. 2013), and analysed at the University of Manchester under the supervision of Professor Thornton. Anti-MUC5AC and anti-MUC5B primary antibodies and MUC5AC and MUC5B standards were kindly provided by Professor Thornton. Unfortunately, no immunoreactive bands were visualised in gels with either antibody. There are several possible explanations for this. First of all, the apical washes might not contain sufficient mucins, and so might not fall within the detection limits of the antibodies. This could be attributable to poor quality of ALI cultures, or simply because mucin production by the cultures is too low for detection by this method. Attempts to optimise mucin collection and minimise sample volume to increase mucin concentration did not improve detection. Minimising sample volume prevented running each sample in duplicate, so gels could not be repeated and samples could not be stained with both antibodies. Secondly, collection of surface mucins by washing apical surfaces might have been

inefficient; mucins may have remained adhered to the culture surface. Thirdly, the antibodies might not have had specificity for the mucins within the apical washes – although this is unlikely, since previous studies have successfully identified mucins collected from primary HBEC cultures by western blotting with these antibodies (Harrop et al. 2013). Finally, while working at the University of Manchester, purified MUC5AC and MUC5B mucins used as positive controls provided by Professor Thornton failed to produce immunoreactive bands. This could suggest that a problem with the western blot at the time, rather than a lack of mucins within the samples.

If western blotting was used in future work, the quality and robustness of the ALI cultures should be improved; incubating with PBS for more than 5 min caused visible damage to the culture morphology and confluence, indicating poor quality. In addition, future experiments should examine whether stimulating ALI cultures with secretagogues to increase the quantity of mucins could improve mucin detection. To increase the content of mucins loaded onto the gel multiple washes could be collected and pooled into a single sample; although it should be noted that this would significantly increase the number of individual ALI cultures required for each experiment. In conclusion, the western blot method requires further optimisation before being used for the detection of mucins in apical secretions from primary HBEC ALI cultures.

The use of ELISAs for quantifying mucin production and secretion by airway epithelial cultures has been reported in the literature. Consequently, ELISAs were optimised for the detection of mucins in this study. Staining with mouse isotype control antibody produced high absorbance, similar to that achieved with primary MUC5AC MAb. This suggested that binding of antibodies to samples was non-specific. Staining with a range of antibody concentrations and dilutions of samples failed to sufficiently increase the difference between primary antibody and isotype control antibody absorbance. This could be due to low mucin expression in the samples. However, when samples collected from stimulated cells were compared with samples from unstimulated cells, no change in absorbance was seen, and absorbance with the isotype control antibody remained high. This is likely due to

the inherent "stickiness" of mucins. Consequently, the ELISA was not suitable for this study.

A significant limitation encountered during optimising the ELISA was the lack of positive control for the assay. A positive control is essential for validating a working assay by indicating a known positive result. No recombinant human MUC5AC is commercially available, but previous studies have used pooled apical washings of primary HBEC ALI cultures and purified human mucin standards as positive controls (Kemp et al. 2004, Hewson et al. 2004, Goswami et al. 1994). Future work should include the development of a reliable and reproducible positive control for the ELISA.

A negative control would also be useful for validating mucin assays such as ELISAs. For instance, a MUC5AC knockdown cell line could provide a valuable negative control; adenoviruses encoding short hairpin-RNA (shRNA) directed against MUC5AC could abolish MUC5AC expression in well-characterised mucin-expressing cell lines such as HT-29 cells or H292 cells (Harrop et al. 2013). Knockdown efficiency could be confirmed by qPCR and mass spectrometry, thus providing a suitable negative control for MUC5AC ELISAs.

Similarly to ELISAs, ELLA was limited by a lack of human mucin positive control. However, a concentration-dependent change in absorbance was seen with mucin from bovine submaxillary glands and demonstrated specific detection of mucins. Consequently, bovine mucin standards were used as a positive control for the ELLA. For future work, the assay would benefit from a negative control, as mentioned above. The suitability of ELLA for this study was shown by the significant difference seen between unstimulated cells and cells treated with rh-AREG. Furthermore, the specificity of the assay was supported by low reactivity with fibroblasts, a non-mucin-expressing cell type. Optimising the collection of washes and lysates demonstrated that the assay was capable of detecting both intracellular and secreted mucins.

An important observation during this Chapter was the inability to detect mucin upregulation in cultures stimulated with either ATP or with HLMC supernatants using ELLA. While this could be attributed to the fact that neither ATP nor HLMC

supernatants induced mucin upregulation, this does not agree with the literature (Okumura et al. 2005, Jones et al. 2012, Davis 2002). This could suggest that further validation of this assay and use of H292 cells as a model of airway mucin secretion are required, and should be considered in future work. The effect of ATP and HLMC supernatants on mucin production should also be assessed by immunohistochemistry; due to the success of MUC5AC immunostaining in paraffin-embedded ALI cultures, immunohistochemistry data could have aided interpretation of these ELLA results. A lack of response to HLMC supernatants could also be attributed to low levels of HLMC mediators and cytokines following HLMC activation. Future work could include assessment of HLMC degranulation by histamine assay to ensure HLMC activation had occurred.

The findings of this Chapter highlight the suitability of immunohistochemistry and ELLA for the semi-quantitative analysis of mucin production and secretion from *in vitro* airway epithelial cell cultures. In addition, this Chapter indicates areas for future work when optimising other mucin assays, including the development of positive and negative controls. Currently, there is no gold standard for mucin quantification, nor any commercially available ELISA kits or purified human mucin standards. The methods assessed in this Chapter do not represent the only methods available for mucin quantification. For instance, studies have reported the effectiveness of in-cell Western (ICW) for detecting proteins in epithelial layers maintained on permeable filters (Mukherjee et al. 2013). ICW is a fluorescence-based immuno-blot semi-quantitative assay, and permits detection of proteins in fixed epithelial cells *in situ*. In addition, FM1-43 might be useful. FM1-43 is a fluorescent styryl dye used for visualising exocytosis and movement of secretory granules, most commonly within motor nerve terminals (Amaral et al. 2011). However, for the purposes of this study, FM1-43 might not be suitable. Although the dye could provide a useful method of measuring mucin granule exocytosis, FM dyes reportedly induce store-operated Ca^{2+} entry (Li et al. 2009). Quinacrine-based confocal microscopy is another potential method that could be investigated for this work; quinacrine labels intracellular granules. However, quinacrine localisation is associated with ATP content of granules, and consequently may not provide a specific method of identifying mucin granules (Akopova et al. 2012, Pangrsic et al. 2007).

Future work could also investigate the use of proteomic approaches to identify mucin glycoproteins within and secreted from *in vitro* cultures. In Mucins: Methods and Protocols, Kesimer and Sheehan present a method of mass spectrometric analysis of mucin proteins (McGuckin et al. 2012). In this approach, mucus samples can be fractionated by density gradient centrifugation and treated with specific proteases to form a complex mixture of proteins and peptides. Different fractions can then be assessed by a combination of high performance liquid chromatography and mass spectrometry. By this method, specific mucin core peptides within concentrated samples can be separated by liquid chromatography and subsequently identified by mass spectrometry. This approach would not only facilitate mucin quantification, but also the identification of different mucin species.

Chapter 6:

The role of K_{Ca}3.1 in mucin production and secretion

6.1   *Introduction*

This Chapter investigates the role of $K_{Ca}3.1$ in mucin production and secretion from *in vitro* airway epithelial cell cultures. As demonstrated in Chapter 5, primary HBEC ALI cultures and H292 cells express MUC5AC mRNA, and hence were used for the following experiments. Also shown in Chapter 5 was the ability of rh-AREG to upregulate MUC5AC mRNA expression in H292 cells and the mucin content of H292 lysates and apical washes. Thus, rh-AREG was used as a model of stimulated mucin secretion from H292 cells in this study.

Amphiregulin (AREG) is a growth factor protein with significant homology to epithelial growth factor (EGF), but with a lower affinity for EGFR than EGF (Adam et al. 1995). AREG is produced and secreted by mast cells following their activation by cross-linking of FcεRI (Wang et al. 2005, Okumura et al. 2005, Polosa et al. 1999), and is also produced by HBECs by cleavage of a membrane-bound ligand (Polosa et al. 1999, Hartl et al. 2008). Elevated expression of AREG in mast cells correlates significantly with the extent of goblet cell hyperplasia in biopsies isolated from patients with asthma (Okumura et al. 2005), and AREG concentrations are upregulated in the sputa of asthmatic patients; this increase in AREG is transient, occurring during an acute attack (Enomoto et al. 2009). Previous studies have demonstrated that treatment of H292 cells with 10 ng/ml rh-AREG or AREG secreted by activated human mast cells results in elevated MUC5AC mRNA expression after 24 h (Okumura et al. 2005). In addition, exposure to 1 to 100 ng/ml AREG increases MUC5AC protein in *in vitro* HBEC cultures, identified by immunocytochemistry and immunofluorescence (Enomoto et al. 2009). Consequently, AREG is an important molecule in asthma, capable of contributing to over-expression of respiratory mucins, and is likely to play a role in airway mucus hypersecretion.

Mucin secretion from primary HBEC ALI cultures was also induced by rh-IL-13 in this Chapter. IL-13 is a Th2 cytokine and a potent inducer of mucin secretion and goblet cell hyperplasia (Dabbagh et al. 1999, Zhu et al. 2011). Animal models overexpressing lung-specific IL-13 display an inflammatory response with mucous cell metaplasia and mucus hypersecretion (Zhu et al. 1999, Zhu et al. 2011), and IL-13-treated mice exhibit increased MUC5AC mRNA expression and MUC5AC mucins

in bronchoalveolar lavage fluid (Zuhdi Alimam et al. 2000). Over-expression of IL-13 has been reported in bronchial tissue and induced sputum from patients with severe asthma (Saha et al. 2008). In addition, *in vitro* studies have demonstrated that IL-13 upregulates mucin production and secretion in primary HBEC cultures (Kanoh et al. 2011, Harrop et al. 2013, Thavagnanam et al. 2011).

In this Chapter, the role of $K_{Ca}3.1$ in mucin production and secretion by H292 cells was examined due to the importance of $K_{Ca}3.1$ in modulating Ca^{2+} signalling and mast cell degranulation, and the significance of Ca^{2+} signalling in mucin production and secretion. $K_{Ca}3.1$ blockade was achieved using TRAM-34 and ICA-17043, as demonstrated in Chapter 4. Both drugs were dissolved in DMSO, and this was used in experiments as a negative control at a final concentration of 0.1%.

6.2 *Methods*

6.2.1 *In vitro cell culture*

H292 cells were grown as submerged or ALI cultures (**Chapter 2, 2.2.2**). Primary healthy HBECs (Lonza) were differentiated to ALI culture to induce ciliogenesis and mucus production, and were a gift from Dr Rob Hirst at University Hospitals, Leicester.

6.2.2 *Quantification of MUC5AC mRNA expression using qPCR*

Total RNA was isolated from *in vitro* cell cultures and used for first strand cDNA synthesis (**Chapter 2, 2.3**). The quantity of mRNA encoding the MUC5AC mucin polypeptide domain was measured using qPCR with TaqMan probes, alongside probes targeting the housekeeping gene ribosomal 18S (**Chapter 2, 2.3.3**). PCR products were analysed by gel electrophoresis (**Chapter 2, 2.3.6**).

All expression data were corrected using the reference dye ROX and normalised to 18S mRNA using the ΔC_T method (**Chapter 2, 2.3.5.1**). For relative quantification to assess the fold change caused by different treatment conditions, the $\Delta\Delta C_T$ method was used (**Chapter 2, 2.3.5.2**). Expression of the gene of interest (MUC5AC) is expressed as relative expression (i.e. fold-change above control).

6.2.3 *Detecting $K_{Ca}3.1$ and MUC5AC expression in bronchial biopsies*

Immunohistochemistry was used for the immunoenzymatic identification of $K_{Ca}3.1$ and MUC5AC in GMA-embedded bronchial biopsy specimens collected by fiberoptic bronchoscopy (**Chapter 2, 2.7**). $K_{Ca}3.1$ was identified using primary polyclonal anti-KCNN4 antibody (5 µg/ml; Sigma); MUC5AC was identified using primary monoclonal anti-MUC5AC antibody (20 µg/ml; Millipore). Primary antibodies were used alongside rabbit IgG (BD Biosciences) and mouse IgG1 (Dako) isotype control antibodies at corresponding concentrations to identify cross-reactivity of the primary antibodies.

6.2.3.1 *Co-localising $K_{Ca}3.1$ and MUC5AC within the bronchial epithelium*

Cells staining positive for both MUC5AC and $K_{Ca}3.1$ within the bronchial epithelium of biopsies isolated from asthmatic patients and healthy controls were co-localised in sequential sections (**Chapter 2, 2.7.3**). A minimum of 6 areas of epithelium per donor were counted. Values obtained for each individual donor represent the total average of cells positive for both target antigens within all areas of epithelium per donor biopsy. Results of each donor group are expressed as a percentage of MUC5AC-positive epithelial cells displaying $K_{Ca}3.1$ immunostaining. Each percentage is presented as median ± IQR of the values obtained for each individual donor of the group.

6.2.3.2 *Semi-quantitative analysis of $K_{Ca}3.1$ and MUC5AC immunostaining in the bronchial epithelium*

The amount of positive staining in the epithelium of each biopsy was assessed using a thresholding technique based on detection limits for hue, saturation and intensity (HSI) of immunostaining (**Chapter 2, 2.7.4**). The median of the lower and upper limit of the HSI values were calculated from 10 biopsies from severe asthmatic patients and healthy controls. All epithelial sections within each bronchial biopsy were blinded and measured using this final threshold.

Thresholding values were representative of positive MUC5AC or $K_{Ca}3.1$ immunostaining and expressed as an average of the percentage of the total area of

bronchial epithelium per biopsy (area fraction %). Prism 5 software (GraphPad Software) was used for statistical analysis. All data are expressed as median ± interquartile range.

6.2.4   *Quantifying the mucin content of lysates and apical washes by ELLA*

The quantity of mucins within apical washes and lysates of H292 ALI cultures was determined by ELLA (**Chapter 5, 5.3.2.5**). Apical washes were collected by incubating the surface of H292 ALI cultures with warm PBS for 5 min, and gently washing the PBS over the culture to maximise collection of mucins. Lysates were harvested by removing the ALI culture from the Transwell and storing in pure water at -80°C.

6.2.5   *Detecting intracellular mucins in H292 and HBEC ALI cultures*

Intracellular mucins were identified by immunohistochemistry and staining with MUC5AC MAb (**Chapter 5, 5.3.2.1**). MUC5AC immunostaining was used as an indicator of goblet cells in HBEC cultures, and as a marker of intracellular mucin expression. Positive immunostaining in H292 cultures was identified by the semi-quantitative thresholding method (**Chapter 5, 5.3.2.1**). Results are expressed as MUC5AC area fraction %.

Positive immunostaining in primary HBEC ALI cultures was identified by counting the number of MUC5AC-positive cells in each section of each culture (**Chapter 2, 2.9**). Results are expressed as the number of MUC5AC-positive cells per μm^2, calculated by dividing the total number of stained cells per culture by the total area of the culture section.

6.2.6   *Experimental design: examining the role of K$_{Ca}$3.1 in mucin production and secretion*

To examine whether K$_{Ca}$3.1 has a role in rh-AREG-induced mucin production in H292 cells, MUC5AC mRNA expression in H292 cells in the presence or absence of K$_{Ca}$3.1 blockers was assessed by qPCR, and the mucin content of apical washes and

lysates of H292 ALI cultures was analysed by ELLA and immunohistochemistry. For qPCR, H292 cells were grown to confluence in 6-well plates and treated with 10 ng/ml rh-AREG (R&D Systems) for 24 h. For ELLA or immunohistochemistry, H292 cells were grown into ALI cultures and maintained for 48 h before treating with rh-AREG for 24 h. RNA, apical washes and lysates were harvested after 24 h.

To investigate the role of K$_{Ca}$3.1 in rh-IL-13-induced mucin production in primary HBECs, HBEC ALI cultures were treated with 10 ng/ml rh-IL-13 for 12 days. On Day 0, 3, 5, 7, 9 and 12, fresh media with rh-IL-13 were added and apical washes were collected. On Day 12, ALI cultures were collected for embedding into paraffin.

For K$_{Ca}$3.1 blockade, cultures were treated with 200 nM TRAM-34 or 100 nM ICA-17043, with a final DMSO concentration of 0.1%, for 30 min prior to stimulation with rh-AREG or rh-IL-13. K$_{Ca}$3.1 blockers were used alongside 0.1% DMSO as a negative control.

6.3   *Results*

6.3.1   *K$_{Ca}$3.1 and MUC5AC are expressed in human bronchial epithelial tissue*

6.3.1.1 *Patient characteristics*

In total, biopsies from 10 healthy controls, and 3 mild, 6 moderate and 10 severe asthmatics were used for the following work. The clinical characteristics of all asthmatic patients and healthy controls can be found in **Table 6-1**.

Table 6-1– see separate PDF

Table 6-1– see separate PDF

6.3.1.2 *K$_{Ca}$3.1 and MUC5AC immunoreactivity are evident in human bronchial epithelium*

MUC5AC (**Figure 6-1**) and K$_{Ca}$3.1 (**Figure 6-2**) immunoreactivity were evident in the bronchial epithelium of asthmatic and healthy bronchial biopsy tissue. The isotype control antibodies produced no immunoreactivity. MUC5AC immunoreactivity was restricted to the bronchial epithelial layer; no staining of the surrounding tissue was seen. In contrast, K$_{Ca}$3.1 immunoreactivity was not restricted to the bronchial epithelial layer and was detected in the surrounding cells such as smooth muscle cells. K$_{Ca}$3.1 immunoreactivity was detected throughout the bronchial epithelial layer rather than being restricted to goblet cells alone. This suggests that K$_{Ca}$3.1 expression is not unique to goblet cells, and therefore may have several functions in the physiology of the bronchial epithelium. K$_{Ca}$3.1 immunostaining appeared to be denser in the basolateral region of the epithelial cells, supporting previous studies (Devor et al. 1999, Rufo et al. 1996).
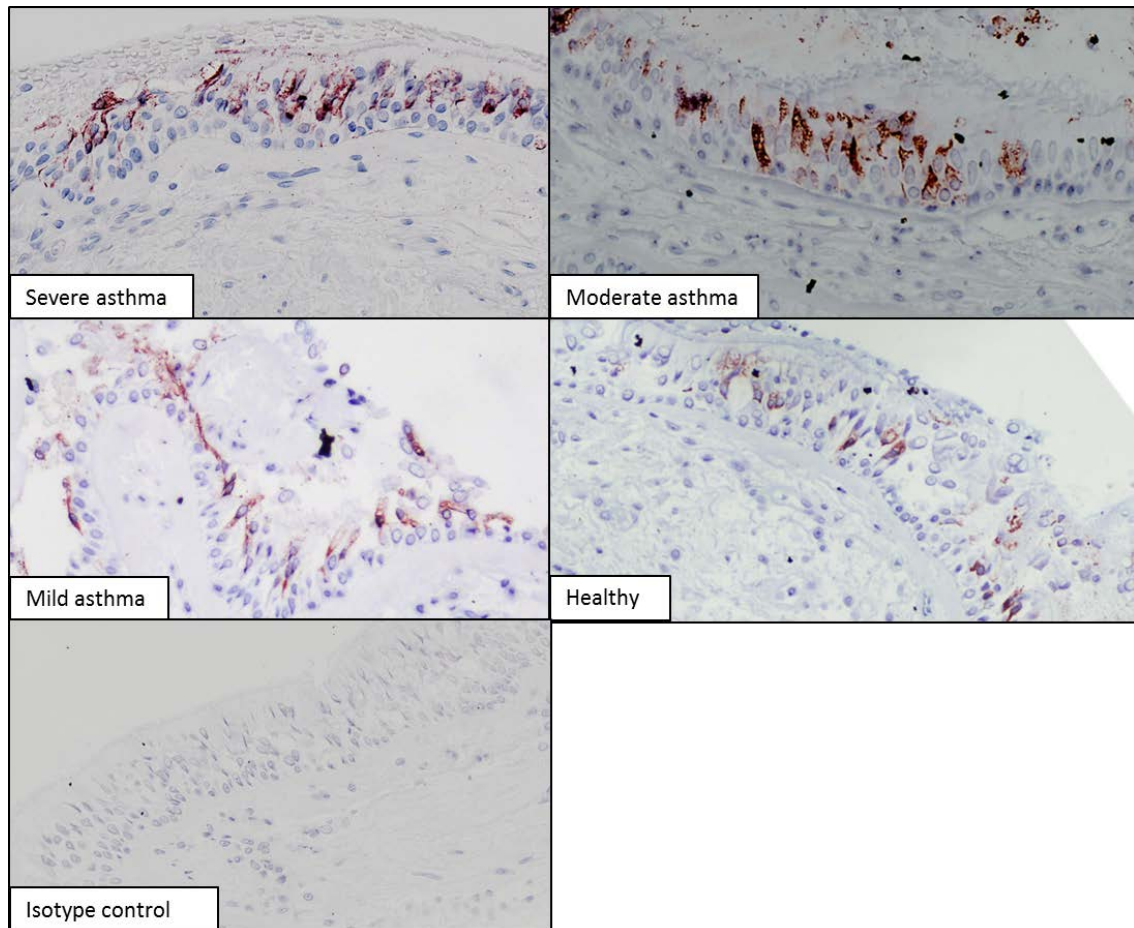
Figure 6-1: MUC5AC immunostaining is evident in HBECs in asthmatic and healthy bronchial biopsies.

MUC5AC immunostaining with primary monoclonal anti-MUC5AC antibody (Millipore) at 20 µg/ml was detected in the epithelium of bronchial biopsies isolated from patients with severe, moderate and mild asthma and in healthy control subjects. No immunoreactivity was seen with 20 µg/ml mouse IgG1 isotype control antibody. All sections were counter-stained with Mayer's haematoxylin.
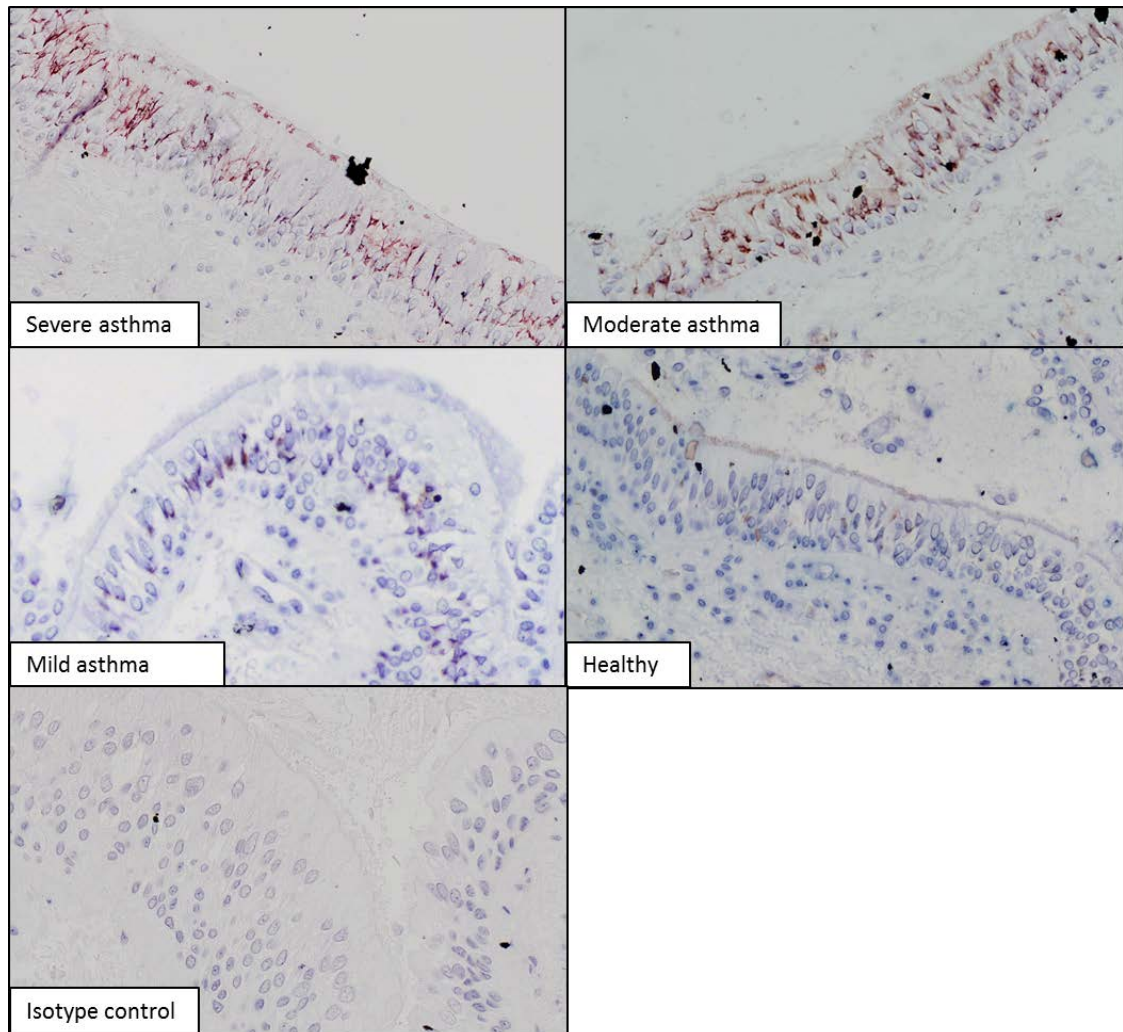
Figure 6-2: K$_{Ca}$3.1 immunostaining is present in HBECs in asthmatic and healthy bronchial biopsies.

K$_{Ca}$3.1 immunostaining with primary polyclonal anti-KCNN4 antibody at 5 µg/ml (Sigma) was found in the epithelium of bronchial biopsies isolated from patients with severe, moderate and mild asthma and in healthy control subjects. No immunoreactivity was seen with 5 µg/ml rabbit IgG isotype control antibody.

6.3.1.3 K_{Ca}3.1 channel and MUC5AC protein co-localise within the bronchial epithelium

MUC5AC and K_{Ca}3.1 antigens co-localised within bronchial epithelial tissue in similar percentages in patients with severe (47.5 (37.4 – 54.7)), moderate (41.8 (37.3 – 48.0)) and mild (39.5 (31.2 – 56.2)) asthma, and in healthy controls (44.1 (32.7 – 49.3)) (**Figure 6-3**). No significant difference was found between the 4 groups (Kruskal-Wallis ANOVA). Data presented as median (IQR).

6.3.1.4 Levels of K_{Ca}3.1 and MUC5AC immunostaining are higher in severe asthmatic bronchial epithelium

Significantly higher levels of K_{Ca}3.1 immunostaining were detected in the bronchial epithelium of patients with asthma compared with healthy controls (**Figure 6-4A**; *P = 0.0108; Mann Whitney *t*-test). Further analysis found a significant difference between severe asthma and mild asthma and severe asthma and healthy control groups and (**Figure 6-4B**; **P = 0.0081; ***P = 0.0017; Kruskal-Wallis ANOVA with Dunn's multiple comparisons test).

MUC5AC immunostaining was also significantly elevated in the bronchial epithelium of asthmatic patients compared with healthy controls (**Figure 6-4A**; #P = 0.0155), and analysis of the individual groups revealed a significant difference in between the severe asthmatic and healthy control groups (**Figure 6-4B**; ##P = 0.0343).

A significant correlation was found between K_{Ca}3.1 and MUC5AC immunostaining in the bronchial epithelium of all donors (**Figure 6-5A**; P = 0.0008; Spearman non-parametric correlation). When healthy control data were excluded, a significant correlation was still seen between K_{Ca}3.1 and MUC5AC (**Figure 6-5B**; P = 0.0211). These findings suggest a relationship may exist between K_{Ca}3.1 and MUC5AC in health and asthma, and in the different severities of asthma.

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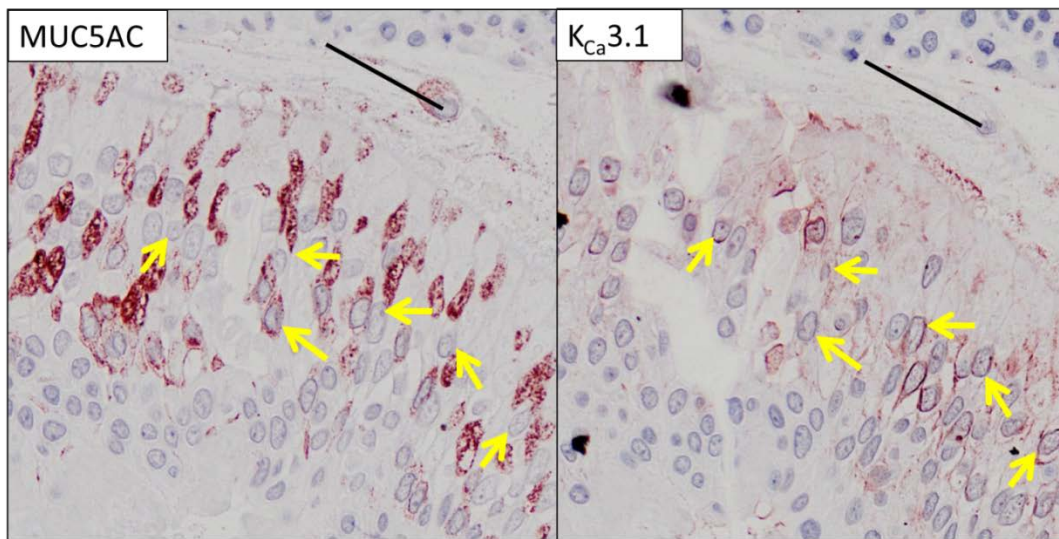
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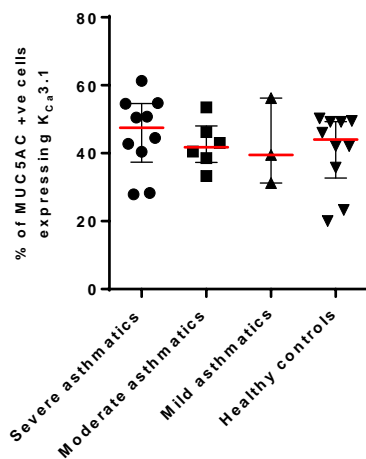
Figure 6-3: MUC5AC and $K_{Ca}3.1$ co-localise in bronchial epithelial tissue.

(**A**) $K_{Ca}3.1$ and MUC5AC immunostaining were co-localised in bronchial epithelial cells; yellow arrows highlight cells exhibiting $K_{Ca}3.1$ and MUC5AC expression in sequential sections of a severe asthmatic bronchial biopsy. (**B**) Percentage of cells labelled with MUC5AC (MUC5AC +ve cells) displaying $K_{Ca}3.1$ channel immunoreactivity in bronchial epithelial tissue of severe (n = 10), moderate (n = 6) and mild (n = 3) asthmatics, and healthy subjects (n = 10). Data is expressed as median (interquartile range).

A

B

C

D
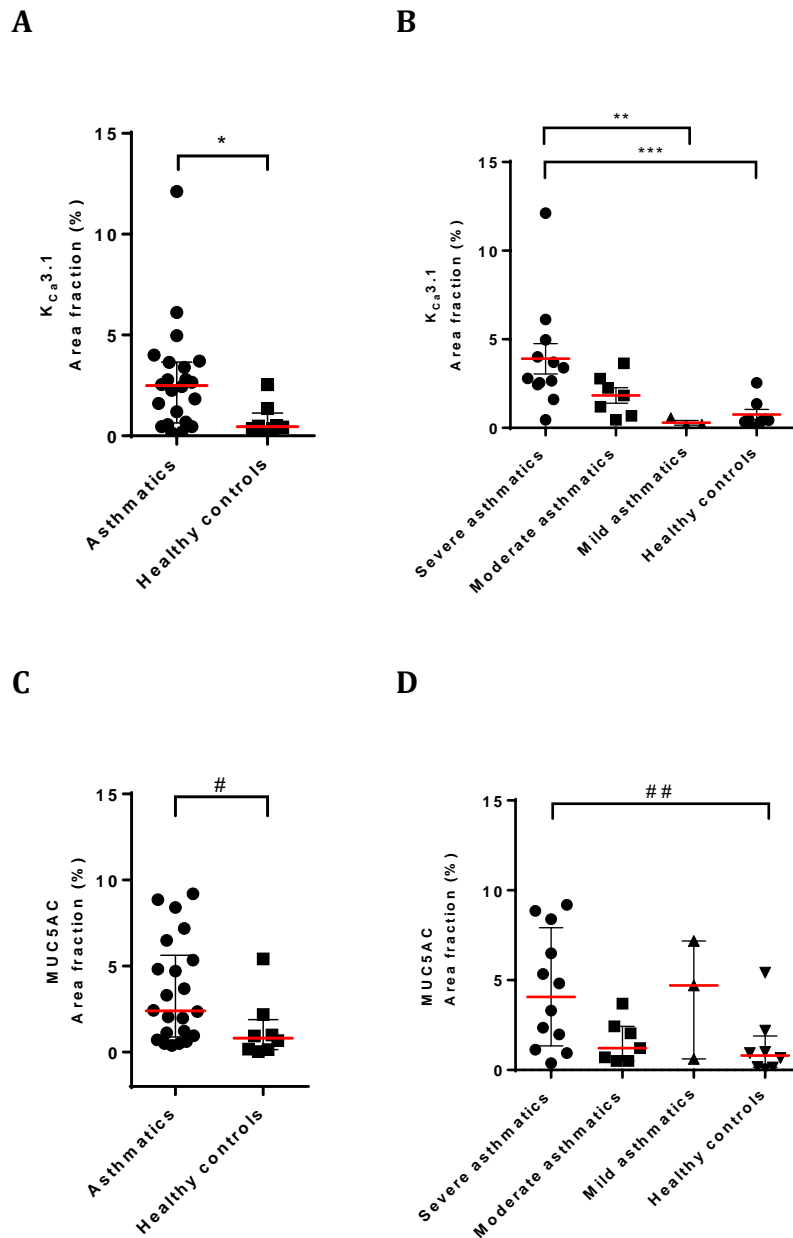
Figure 6-4: K$_{Ca}$3.1 and MUC5AC immunoreactivity are higher in severe asthmatic bronchial epithelium.

Percentage of (**A**) K$_{Ca}$3.1 and (**C**) MUC5AC immunostaining (% Area Fraction) within the bronchial epithelium of asthmatic patients (n = 19) compared with healthy controls (n = 10); *P = 0.0108; #P = 0.0155. Percentage of (**B**) K$_{Ca}$3.1 and (**D**) MUC5AC immunostaining within the bronchial epithelium of severe, moderate, and mild asthmatics and healthy controls; **P = 0.0021; ***P = 0.0017; ##P = 0.0343.

A
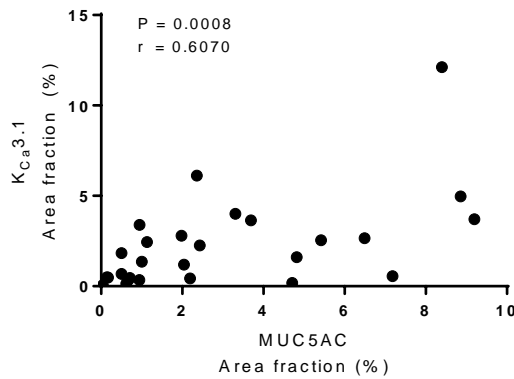
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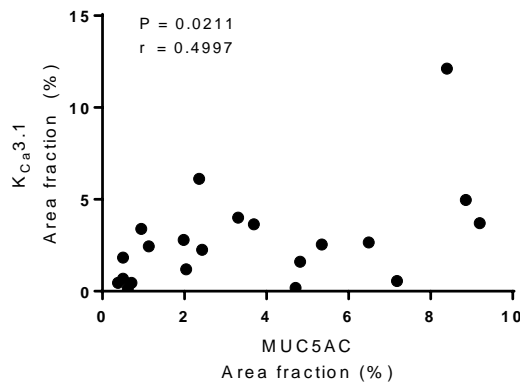
Figure 6-5: $K_{Ca}3.1$ and MUC5AC correlate in healthy and asthmatic bronchial epithelial tissue.

Correlation between the percentage of $K_{Ca}3.1$ channel and MUC5AC immunostaining within the bronchial epithelium of (**A**) all asthmatic and healthy donors (P = 0.0008) and (**B**) of patients with severe, moderate or mild asthma (P = 0.0211).

6.3.2 K_{Ca}3.1 channel activity does not modulate rh-AREG-induced mucin production in H292 cells

6.3.2.1 K_{Ca}3.1 activity does not regulate rh-AREG-induced MUC5AC mRNA expression in H292 cells

No significant upregulation of MUC5AC mRNA was seen in H292 cells with 10 or 100 ng/ml rh-AREG after 6 h. However, after 24 h, 10 ng/ml (*P = 0.0099) and 100 ng/ml (**P = 0.0278) rh-AREG significantly upregulated MUC5AC mRNA expression in H292 cells (**Figure 6-6A**). Accordingly, the 24 h time-point and 10 ng/ml rh-AREG concentration were used for the next experiments.

H292 cells were incubated for 30 min with 200 nM TRAM-34 before being stimulated with 10 ng/ml rh-AREG for 24 h. This ensured complete blockade of K_{Ca}3.1 prior to stimulation. TRAM-34 had no effect on rh-AREG-induced MUC5AC mRNA upregulation (**Figure 6-6B**).

6.3.2.2 rh-IL-13 does not upregulate MUC5AC mRNA expression in H292 cells

MUC5AC mRNA expression in H292 cells was not upregulated by rh-IL-13 after 6 h or 24 h (**Figure 6-7**). Consequently, stimulation of H292 cells with rh-IL-13 was not used for experiments.

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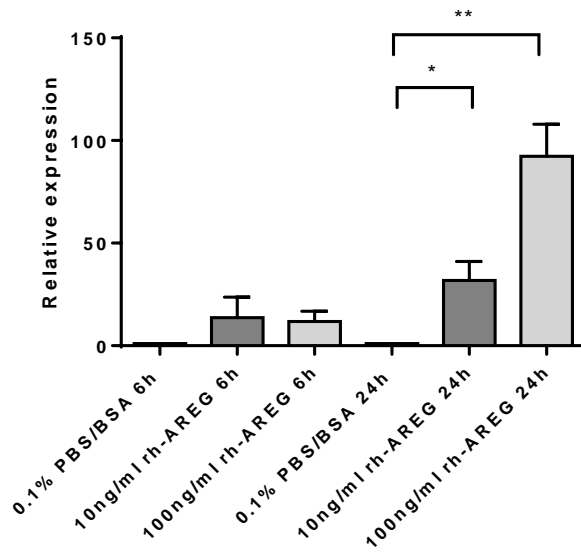
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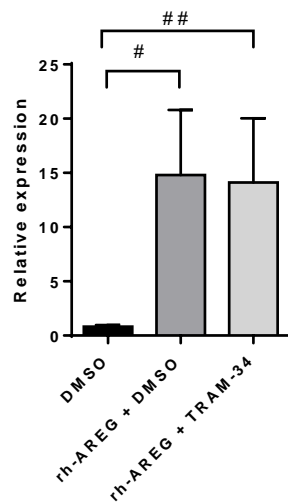
Figure 6-6: $K_{Ca}3.1$ blockade has no effect on rh-AREG-induced MUC5AC mRNA upregulation.

(**A**) rh-AREG upregulates MUC5AC mRNA expression in H292 cells after 24 h in a dose-dependent manner; *P = 0.0099; **P = 0.0278. (**B**) 200 nM TRAM-34 did not affect rh-AREG-induced MUC5AC expression (n = 6); #P = 0.0078; ##P = 0.0419. Expression of the gene of interest is presented as relative expression (fold change above control, where control = 1). All data are expressed as mean ± SEM.

A
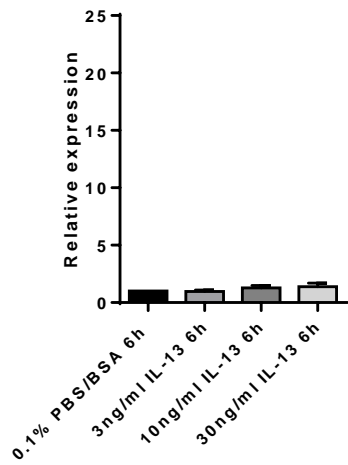
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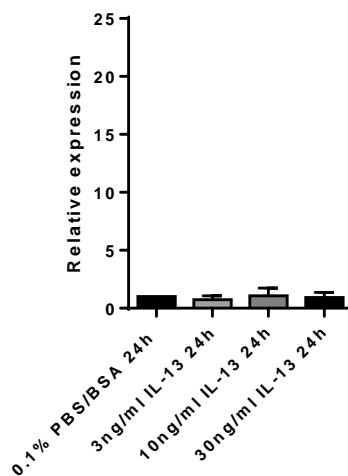
Figure 6-7: rhIL-13 does not upregulate MUC5AC mRNA expression in H292 cells.

H292 cells were incubated with 3, 10 and 30 ng/ml rh-IL-13 for 6 h or 24 h. Total RNA was extracted from the cells (n = 3 individual experiments). All data are expressed as mean ± SEM.

6.3.2.3 K_{Ca}3.1 blockade does not inhibit rh-AR-induced upregulation of mucin intracellular content or secretion

Analysis of H292 ALI culture lysates by ELLA (**Figure 6-8A**; $n = 6$) showed that 100 ng/ml rh-AREG significantly upregulated intracellular mucin content after 24 h (*P = 0.0313), but K_{Ca}3.1 blockers (200 nM TRAM34 and 100 nM ICA-17043) had no effect on this upregulation.

Similarly, although 100 ng/ml rh-AREG for 24 h significantly increased absorbance in apical washes collected from H292 ALI cultures (**Figure 6-8B**; $n = 6$; **P = 0.0274), K_{Ca}3.1 blockers did not affect this rh-AREG-induced mucin secretion, as measured by ELLA. This was supported by findings that 200 nM TRAM-34 did not inhibit the rh-AREG-induced increase in MUC5AC immunostaining in paraffin-embedded H292 ALI cultures after 24 h (**Figure 6-8C**; $n = 3$).

A
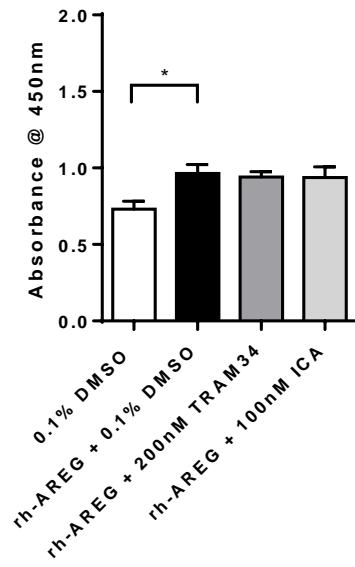
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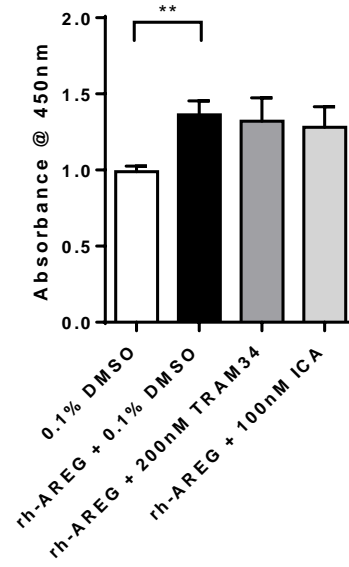
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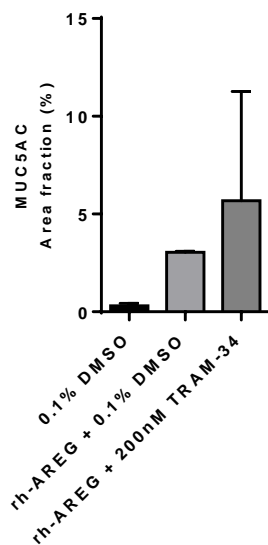
Figure 6-8: $K_{Ca}3.1$ does not modulate rh-AREG-induced upregulation of intracellular mucin content or mucin secretion after 24 h.

100 ng/ml rh-AREG for 24 h significantly upregulated (**A**) intracellular mucin content and (**B**) mucin secretion ($n = 6$) from H292 cells, as detected by ELLA, but this was not affected by 200 nM TRAM34 or 100 nM ICA-17043. *P = 0.0313; **P = 0.0274. (**C**) rh-AREG-induced increase in MUC5AC immunostaining (area fraction %) of H292 ALI cultures was not inhibited by $K_{Ca}3.1$ blockade.

6.3.3 *K$_{Ca}$3.1 does not modulate H292 cell proliferation*

To examine whether K$_{Ca}$3.1 activity was involved in goblet cell proliferation, and thus hyperplasia in asthma, the effect of K$_{Ca}$3.1 blockers on H292 proliferation was examined. Proliferation of H292 cells was induced by serum after 24 and 72 h, but TRAM-34 did not inhibit serum-induced proliferation (**Figure 6-9**).

6.3.4 *K$_{Ca}$3.1 activity does not modulate rh-IL-13-induced mucin production*

6.3.4.1 *K$_{Ca}$3.1 blockade does not inhibit rh-IL-13-induced MUC5AC mRNA expression in HBECs*

Treatment of healthy HBEC ALI cultures ($n = 2$) for 12 days with 10 ng/ml rh-IL-13 induced upregulation of MUC5AC mRNA expression (**Figure 6-10A**). Simultaneous administration of 200 nM TRAM-34 for 12 days had no effect on rh-IL-13-induced MUC5AC mRNA expression (**Figure 6-10B**; $n = 3$).

6.3.4.2 *ELLA does not detect IL-13-induced mucin secretion from HBEC ALI cultures*

To investigate the effect of rh-IL-13 on mucin production and secretion, MUC5AC-positive cells were counted in paraffin-embedded HBEC ALI cultures after 12 days with 10 ng/ml rh-IL-13 ($n = 2$). Only a small number of cells exhibited MUC5AC immunostaining, but an increase was seen with rh-IL-13 (**Figure 6-11A**).

Apical washes collected from healthy HBEC ALI cultures on Day 0, 3, 5, 7, 9 and 12 were analysed by ELLA for mucin content ($n = 2$; **Figure 6-11B**). Despite a small increase in the number of MUC5AC-positive cells, no change in absorbance, representative of mucin content, was found in washes harvested from cultures stimulated with 10 ng/ml rh-IL-13 across the 12 days. This suggested that either rh-IL-13 did not induce mucin secretion from HBEC ALI cultures, or ELLA was not sufficiently sensitive to detect IL-13-induced mucin secretion. Consequently, the role of K$_{Ca}$3.1 in rh-IL-13-induced mucin secretion was not investigated in this study, and the effects of rh-IL-13 in the presence of K$_{Ca}$3.1 blockers were not examined.

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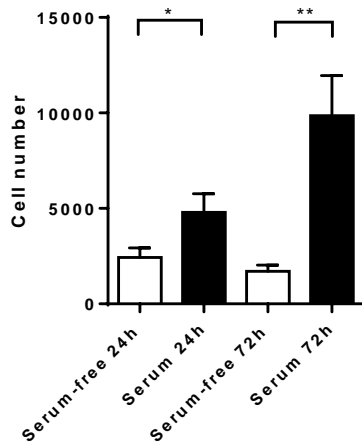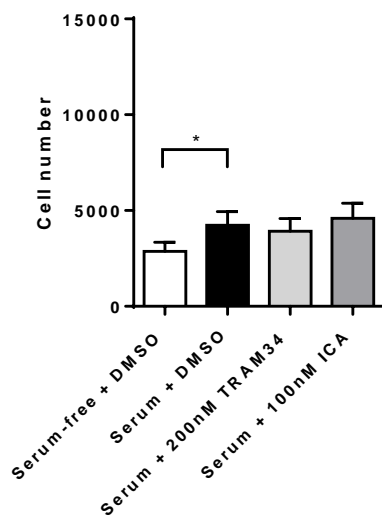
B   **C**
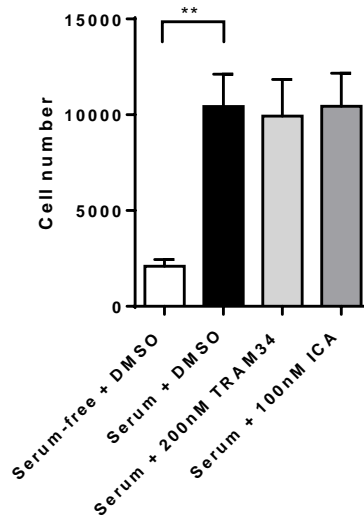
Figure 6-9: $K_{Ca}3.1$ does not modulate serum-induced H292 cell proliferation.

(**A**) Serum induced H292 cell proliferation after 24 and 72 h (n = 4); *P = 0.0079; **P = 0.0225. 200 nM TRAM-34 had no effect on serum-induced proliferation after (**B**) 24 h (n = 8; *P = 0.0025) or (**C**) 72 h (n = 4; **P = 0.0101).

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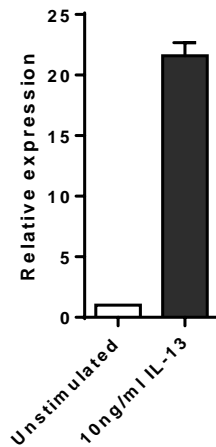
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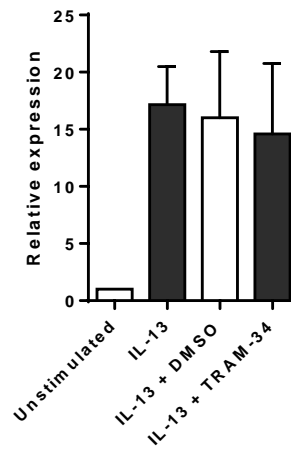
Figure 6-10: K_{Ca}3.1 blockade does not inhibit rh-IL-13-induced MUC5AC mRNA expression in healthy HBEC ALI cultures.

(**A**) Treatment of healthy HBEC ALI cultures (*n* = 2) with 10 ng/ml rh-IL-13 for 12 days caused upregulation of MUC5AC mRNA. (**B**) TRAM-34 did not affect rh-IL-13-induced MUC5AC mRNA expression after 12 days (*n* = 3).

A
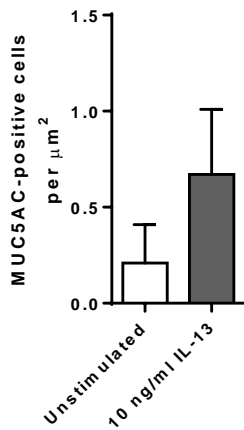
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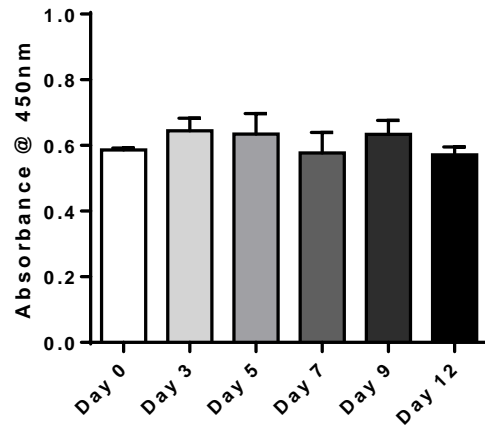
Figure 6-11: ELLA did not detect an increase in mucin secretion from healthy HBECs treated for 12 days with rh-IL-13.

(**A**) 10 ng/ml rh-IL-13 increased the number of MUC5AC-positive cells per μm^2 of ALI culture. (**B**) No difference in the absorbance at 450 nm in ELLA with apical wash samples was seen in cultures treated with 10 ng/ml rh-IL-13 across the 12 days.

6.4   *Discussion*

This is the first time the role of K_{Ca}3.1 has been investigated in mucin production and secretion from *in vitro* epithelial cell cultures. The data demonstrate that K_{Ca}3.1 blockade does not affect rh-AREG-induced MUC5AC mRNA expression, intracellular mucin content, or mucin secretion from H292 cells.

Mucus hypersecretion plays an important role in the pathophysiology of asthma, and chronic mucus hypersecretion is an indicator of FEV_1 decline in patients with asthma (Lange et al. 1998, Thomson et al. 2013, de Marco et al. 2006). In addition, plugging of the airways is present in acute severe asthma and in fatal cases of asthma (Aikawa et al. 1992, Bullen 1952, Houston et al. 1953, Durward et al. 2000, Kuyper et al. 2003). Goblet cell hyperplasia is a characteristic histological attribute of the asthmatic bronchial epithelium and is directly associated with disproportionate mucus production in asthma (Aikawa et al. 1992, Fahy 2002, Ordonez et al. 2001). Mechanisms of goblet cell granule release are considered to be comparable to those utilised by HLMCs; in activated HLMCs, Ca^{2+} signalling and K_{Ca}3.1 activity promote granule exocytosis. Until this study, the role of K_{Ca}3.1 in mucin secretion had not been explored. As demonstrated in Chapter 2, primary HBECs isolated from bronchoscopy brushings express K_{Ca}3.1, and functional K_{Ca}3.1 currents were larger in asthmatic HBECs compared to healthy HBECs. This suggests a potentially interesting difference in K_{Ca}3.1 activity between healthy and diseased epithelium. Consequently, investigating the role of the K_{Ca}3.1 channel in mucin production and secretion offers insight into the potential use of K_{Ca}3.1 as a therapeutic target for the treatment of mucus hypersecretion in asthma.

Immunohistochemistry revealed K_{Ca}3.1 and MUC5AC expression in the epithelium of GMA-embedded bronchial biopsies. MUC5AC immunostaining was confined to goblet cells, whereas K_{Ca}3.1 immunostaining was visible throughout the epithelium and nearby tissue. This supported previous findings of K_{Ca}3.1 expression in human ASM cells (Chachi et al. 2013). Identifying K_{Ca}3.1 and MUC5AC co-expression in sequential sections of the biopsies found that both antigens co-localised in approximately 40% of epithelial cells. In Chapter 2, the electrophysiological activity, mRNA and protein expression of K_{Ca}3.1 were found in primary HBEC cultures, but the methods did not distinguish between individual phenotypes, and

thus did not identify whether goblet cells express $K_{Ca}3.1$. Previous studies have isolated mucin granules from primary HBEC ALI cultures using immuno-based isolation with antibodies to gob-5/mclca3, the ortholog to human Ca^{2+}-activated Cl^- channel CLCA1 present on mucin granule membranes (Raiford et al. 2011). However, while this allows isolation of granules, it does not permit purification of goblet cells for further study. In this Chapter, the immunostaining data suggest that goblet cells do express $K_{Ca}3.1$, suggesting that $K_{Ca}3.1$ might be involved in goblet cell function. Identifying the role of $K_{Ca}3.1$ in goblet cells, and whether it regulates mucin expression, production, and secretion, was the principle aim of this study.

Further analysis of immunohistochemistry data revealed that bronchial epithelial expression of $K_{Ca}3.1$ and MUC5AC is higher in severe asthma than in mild asthma and in health. Correlations found a significant association between $K_{Ca}3.1$ and MUC5AC immunostaining across all healthy and asthmatic donors, and across the different severities of asthma. Combined with patch clamp findings, this indicates a potentially interesting difference between health and disease.

To investigate whether $K_{Ca}3.1$ modulates mucin expression and secretion, the $K_{Ca}3.1$ blockers TRAM-34 and ICA-17043 were applied to H292 cells 30 min prior to stimulation with rh-AREG. However, no inhibition of rh-AREG-induced MUC5AC mRNA expression was achieved. Analysis of H292 lysates and apical washes by ELLA revealed that $K_{Ca}3.1$ blockade also had no effect on rh-AREG-induced intracellular mucin expression or mucin secretion. This was supported by MUC5AC immunostaining of H292 ALI culture cross-sections; 200 nM TRAM-34 did not inhibit the rh-AREG-induced increase in intracellular mucin expression after 24 h.

Although $K_{Ca}3.1$ blockade had no effect in qPCR, ELLA and immunohistochemistry experiments, there are potential limitations with the use of H292 cultures. H292 cells were used as an alternative to primary HBEC ALI cultures because they are straightforward and cost-effective to grow, and have been shown to be a potentially useful model for studying mucus secretion in previous studies (Kai et al. 1996, Iwashita et al. 2010, Takeyama et al. 2000, Bautista et al. 2009). However, the cells are derived from lung cancer, and are likely to demonstrate important phenotypic differences compared to normal and asthmatic goblet cells. Importantly, in this study MUC5AC mRNA expression in H292 cells was not

upregulated by rh-IL-13, as reported in previous studies (Rose et al. 2000), and this is in contrast to the response of healthy primary HBECs (Kanoh et al. 2011, Harrop et al. 2013, Thavagnanam et al. 2011). Furthermore, in Chapter 3, ELLA did not detect an increase in the mucin content of H292 lysates or apical washes following treatment with ATP. Studies with hamster primary tracheal epithelial cells have shown mucin release in response to ATP stimulation, detected by radiolabelling of mucins (Kim et al. 1991, Kim et al. 1992, Kim et al. 1993, Ko et al. 1997). In addition, ATP induced goblet cell granule release from human airway epithelial explants mounted in perfusion chambers, visualised by videomicroscopy to assess degranulation and ELISA to quantify mucins (Lethem et al. 1993). In Chapter 3, ELLA also failed to detect an increase in the mucin content of H292 lysates and apical washes following incubation with supernatants of activated HLMCs. HLMCs express and release a plethora of mediators and cytokines, among which AREG and IL-13 are known mucin secretagogues (Bradding et al. 2006, Bradding et al. 1994, Wang et al. 2005).

Upregulation of MUC5AC mRNA expression and the mucin content of lysates and apical washes of H292 cells by rh-AREG supported findings of previous studies (Okumura et al. 2005). AREG, a mediator produced and released by activated mast cells, is associated with the asthmatic phenotype and goblet cell hyperplasia. Hence, rh-AREG stimulation of H292 cells was used as a model of mucin secretion for this study. Nevertheless, $K_{Ca}3.1$ blockade was ineffective with respect to mucus expression and secretion. However, before concluding firmly that $K_{Ca}3.1$ is not involved in the regulation of mucus secretion from human airways, it was important to repeat these experiments in primary ALI cultures. This was initially undertaken by stimulating healthy HBEC ALI cultures for 12 days with 10 ng/ml rh-IL-13.

Experiments with HBEC ALI cultures demonstrated that $K_{Ca}3.1$ blockade did not affect IL-13-induced MUC5AC mRNA expression after 12 days. This supported the conclusion that $K_{Ca}3.1$ does not modulate mucin mRNA expression in the epithelium. However, due limited availability of cells, the role of $K_{Ca}3.1$ in intracellular mucin accumulation and secretion from the primary cultures was not investigated in this study. Although rh-IL-13 increased the number of MUC5AC-

expressing cells in the cultures after 12 days, identified by immunochemistry, the increase was minimal, and the number of MUC5AC-positive cells per culture was small. Hence, immunohistochemistry data for primary HBEC ALI cultures was presented as MUC5AC-positive cells per µm^2 rather than percentage of MUC5AC immunostaining. In addition, rh-IL-13 for 12 days did not induce any increase in absorbance in ELLA analysis of apical washes. The implications here are that the HBEC ALI cultures did not secrete mucins; IL-13 does not induce mucin secretion from the cultures; ELLA did not specifically detect mucins secreted from HBECs; HBECs did not produce sufficient mucins for detection by ELLA; or secreted mucins were not collected efficiently. The first two implications are unlikely; qPCR and immunohistochemistry data demonstrate that rh-IL-13 promoted mucin expression in the cultures. Consequently, the sensitivity and specificity of ELLA should be examined in future work. As mentioned in Chapter 3, additional validation of this assay, such as the development of a human mucin positive control, is required. In addition, the study was limited by the availability of cell cultures.

Chapter 7

Discussion and Future Work

7.1   *Discussion*

This thesis has examined the expression and activity of Orai and $K_{Ca}3.1$ channels in primary HBECs isolated from patients with asthma and healthy controls, the expression of MUC5AC and $K_{Ca}3.1$ in asthmatic and healthy bronchial epithelium, and the role of $K_{Ca}3.1$ in mucin expression, production and secretion from *in vitro* cultures. This thesis has also assessed different methods of quantifying mucin production from *in vitro* cultures.

The key and novel findings of this study include: i) the identification of $K_{Ca}3.1$ channels in primary HBECs and the detection of larger functional $K_{Ca}3.1$ currents in asthmatic HBECs compared to healthy HBECs, ii) the co-localisation of $K_{Ca}3.1$ and MUC5AC immunostaining in bronchial epithelial cells in both healthy and asthmatic bronchial biopsy specimens, iii) greater $K_{Ca}3.1$ and MUC5AC expression in the bronchial epithelium of patients with severe asthma compared to mild asthma and healthy controls, and iv) the observation that $K_{Ca}3.1$ activity did not modulate cytokine-dependent mucin expression, production or secretion from *in vitro* airway epithelial cell cultures. In addition, in spite of mRNA and protein expression, characteristic Orai channel currents could not be recorded from primary HBECs.

$K_{Ca}3.1$ expression in the bronchial epithelium co-localised with MUC5AC expression. This indicated that goblet cells express $K_{Ca}3.1$, thereby implying a potential role for $K_{Ca}3.1$ in goblet cell function. Although patch clamp experiments could not distinguish between different epithelial cell phenotypes, the larger functional $K_{Ca}3.1$ currents in asthmatic HBECs indicates a potentially interesting difference in $K_{Ca}3.1$ activity in asthma compared to health. This is supported by findings of elevated $K_{Ca}3.1$ expression in the bronchial epithelium of patients with severe asthma in comparison with healthy controls. In addition, $K_{Ca}3.1$ and MUC5AC bronchial epithelial expression formed a positive correlation across the different severities of asthma. Together, these data suggest that $K_{Ca}3.1$ expression in the bronchial epithelium in asthma has functional relevance, although the role it plays in airway epithelial biology is unclear.

Although $K_{Ca}3.1$ blockade did not inhibit rh-AREG or rh-IL-13-induced MUC5AC mRNA expression in H292 cells or HBECs, respectively, this study does not exclude

the possibility that $K_{Ca}3.1$ might play a role in mucin secretion in response to other stimuli such as ATP (Bradding et al. 2006, Bradding et al. 1994, Wang et al. 2005); ATP induces mucin secretion, and activation of purinergic receptors is an important signalling mechanism in the respiratory epithelium (Okumura et al. 2005). Additionally, activation of $P2Y_2$ receptors of primary HBEC cultures by ATP and UTP induces mucin secretion accompanied by Ca^{2+} entry (Kreda et al. 2007, Roger et al. 2000, Kellerman 2002).

Ca^{2+} signalling represents an important pathway in mucin secretion, and in other cell types $K_{Ca}3.1$ contributes to regulation of $[Ca^{2+}]_i$ (Kreda et al. 2007, Rossi et al. 2007, Kouzaki et al. 2011). In addition, Ca^{2+} signalling has been associated with targeted gene expression (Bahra et al. 2004). It might therefore appear surprising that this study could not detect a role for $K_{Ca}3.1$ in mucus expression or secretion. However, there is increasing evidence for the regulation of Ca^{2+}-dependent responses by Ca^{2+} microdomains driven by spatially and temporally restricted Ca^{2+} signals (Duffy et al. 2004, Rossi et al. 2007, Tuvim et al. 2009, Jacquot et al. 1995, Maizieres et al. 1998, Takahashi et al. 1998, Duffy et al. 2007). Thus $K_{Ca}3.1$ might well be excluded from these Ca^{2+}-dependent pathways, and other K^+ channels involved. Further work is required to identify the Ca^{2+} channels regulating mucus expression in goblet cells and any associated K^+ channels. For instance, inwardly-rectifying K^+ channels (Kir) regulate changes in $[Ca^{2+}]_i$ in microglial cells; Kir currents modulate Ca^{2+} influx via Ca^{2+} permeable membrane channels by controlling the membrane potential (Di Capite et al. 2009a, Parekh et al. 2011, Samanta et al. 2014).

Orai channel expression and activity in HBECs must also be examined further. Previous studies have reported characteristic Orai channel activity in airway epithelial cells isolated from CF patients and healthy controls (Di Capite et al. 2009a, Parekh 2008a, Parekh et al. 2011), and in the SV-40 transformed human bronchial epithelial cell line 16HBE (Franchini et al. 2004). Consequently, the absence of Orai channel activity in this study requires additional investigation. Orai channels represent a major pathway for store-operated Ca^{2+} entry and thus contribute to many Ca^{2+} signalling mechanisms (Balghi et al. 2011). Samanta K, et al. demonstrated functional Orai channel expression in 16HBEs, and found that

Ca^{2+} entry through Orai channels stimulated EGF gene expression, via activation of c-fos and NFAT transcription factors, and modulated 16HBE cell migration (Samanta et al. 2014). However, there are many other channels which can mediate Ca^{2+} entry including TRP channels, voltage-gated Ca^{2+} channels, and the P2X family of ion channels (Parekh et al. 2005, Di Capite et al. 2009b). It is known that P2X receptors and TRP channels are expressed in epithelial cells, and these would be worthy of future study with respect to mucus production and secretion (Samanta et al. 2014). Gene expression microarray analysis of bronchial brushings and immunohistochemistry staining of bronchial epithelium samples have found that TRPV1 channels are overexpressed in the airway epithelium in severe asthma (Zhu et al. 1996, Boulay et al. 1999, Visentin et al. 1999, Suzuki et al. 2010).

7.2   *Study limitations*

7.2.1   *Primary HBEC culture*

Primary HBECs were isolated from asthmatic and healthy volunteers by bronchoscopy. This provided a small quantity of cells per brushing, and hence limited the number of cells available for culture per donor. A significant observation during this study was the rapid deterioration of the cultures with each passage; after 3 passages cell morphology degenerated and the cultures often failed to reach confluence. This limited the number of cells available for experiments. ALI cultures required a seeding density of at least 2×10^5 cells per Transwell, and thus contributed to the limited availability of cells per donor and restricted the number of ALI cultures that could be produced.

In this study, ALI cultures were only used if ciliogenesis and mucus production were visualised. Despite optimising cell isolation, culture and passaging, a substantial proportion of cultures failed to differentiate and could not be used. In Chapter 6, the role of $K_{Ca}3.1$ in rh-IL-13-induced mucin expression, production and secretion from primary HBECs could not be fully examined due to a lack of HBEC donors; for the final experiments HBEC ALI cultures were provided by Dr Rob Hirst. Future work will depend upon the availability of primary HBECs, and will

require sufficient quantities of cells to allow further optimisation of the development of high quality ALI cultures.

7.2.2 *Bronchial biopsies*

In Chapter 6, $K_{Ca}3.1$ and MUC5AC expression were examined by immunohistochemistry in biopsies from 10 healthy controls, and 3 mild, 6 moderate and 10 severe asthmatics. Ideally, a larger number of biopsies from mild and moderate asthmatic donors would have been used for this study to allow a comparison between the different severities of asthma and the healthy controls. The availability of biopsies was not only limited by the low numbers of mild and moderate asthmatic donors, but also by the presence of epithelium within the biopsy specimens. Future work could continue to evaluate the expression of $K_{Ca}3.1$ and MUC5AC in bronchial biopsies to add to this data, thus providing further insight into the expression of $K_{Ca}3.1$ in the bronchial epithelium in different severities of asthma.

7.2.3 *Validation of mucin quantification assays*

In Chapter 6, the role of $K_{Ca}3.1$ in mucin production and secretion was determined by quantifying the mucin content of H292 lysates and apical washes by ELLA. These findings were supported by immunohistochemistry. MUC5AC immunostaining is a well-characterised method of visualising intracellular mucins, and its reliability and specificity is supported by the use of an isotype control antibody. In contrast, ELLA is not so well validated. Although mucin from bovine submaxillary glands produced a concentration-dependent response in ELLA, the mucins secreted by H292 cells might not be comparable to bovine mucin. Structural analysis of bovine mucin and H292 cell mucin should be conducted to establish the validity of this positive control.

In Chapter 5, ELLA did not detect upregulation of mucin production from H292 cells by either ATP or HLMC supernatants. In addition, in Chapter 6, ELLA did not reveal an increase in mucin secretion from primary HBEC ALI cultures after 12 days with rh-IL-13. These findings are different to those reported in previous

studies (McGarvey et al. 2014, Zsembery et al. 2004). Whether this was due to the suitability of H292 cells as a model of mucin secretion, the specificity of ELLA, or the ability of H292 and HBEC cultures to secrete mucus, could not be established in this study.

A significant limitation encountered during optimising different assays for mucin quantification was the lack of positive control. No recombinant human MUC5AC is commercially available; previous studies relied on pooled apical washings of primary HBEC ALI cultures and purified human mucin standards as positive controls (McGarvey et al. 2014). Additionally, a negative control would provide extra validation for the assays. For instance, a MUC5AC knockdown cell line could be produced with adenoviruses targeting MUC5AC in well-characterised mucin-expressing cell lines such as HT-29 cells or H292 cells (Jones et al. 2012, Kanoh et al. 2011, Harrop et al. 2013, Thavagnanam et al. 2011, Davis 2002). Knockdown efficiency could be confirmed by qPCR and mass spectrometry, thus providing a specific control for MUC5AC assays.

7.3 Future work

7.3.1 Orai channel expression

Examining the expression of cell surface Orai1, Orai2 and Orai3 protein in primary HBECs should be conducted to investigate further the absence of characteristic Orai channel activity in primary HBECs in this study. The expression and activity of STIM1 should also be assessed (Kemp et al. 2004, Hewson et al. 2004, Goswami et al. 1994). To begin with, Orai1 cell surface expression detected by flow cytometry should be repeated in more HBEC donors. In this study this was not done due to the limited availability of the cells.

Furthermore, the presence of TRP channels should be determined; Orai1 and TRP proteins interact and mediate Ca^{2+} entry (Okumura et al. 2005, Takami et al. 2012, Iwashita et al. 2010, Sheehan et al. 2004). Some TRP channels are Ca^{2+}-permeable, and others are monovalent-selective cation channels that can be activated by store depletion (Wu et al. 2006, Luik et al. 2008). Orai protein in primary HBECs could

contribute to other SOC currents rather than forming characteristic Orai channels, and this should be investigated further.

7.3.2 $K_{Ca}3.1$ channel expression in different epithelial cell phenotypes

Patch clamp experiments could not distinguish between different epithelial cell phenotypes. To identify whether goblet cells exhibit functional $K_{Ca}3.1$ currents and whether these currents are larger in asthma than in health, methods of identifying goblet cells during patch clamp experiments should be explored. For instance, labelling MUC5AC with green fluorescence protein (GFP) could allow visualisation of goblet cells in the experimental dish.

To support immunohistochemistry data demonstrating $K_{Ca}3.1$ and MUC5AC co-localisation in bronchial epithelium biopsy specimens, $K_{Ca}3.1$ and MUC5AC expression could be examined in ALI cultures by immunostaining or dual labelling and confocal microscopy.

7.3.3 Ca^{2+} signalling in primary HBECs

Examining Ca^{2+} signalling in goblet cells could help identify mechanisms of mucin secretion. Thus, the role of K^+ channels in Ca^{2+}-mediated mucin secretion could be established. Ca^{2+} imaging using the fluorescent dye, Fura-2, which labels free intracellular Ca^{2+}, could be used to assess the effect of different secretagogues such as AREG and IL-13 on Ca^{2+} signalling, and the role of K^+ channels in HBEC activity in response to different stimuli.

7.3.4   Assessing goblet cell exocytosis

To supplement mucin quantification assays, fluorescence-labelling of intracellular granules could provide insight into mechanisms of mucin release, and the effects of $K_{Ca}3.1$ blockade. FM1-43 is a fluorescent styryl dye used for visualising exocytosis and movement of secretory granules, most commonly within motor nerve terminals (Jardin et al. 2009, Jardin et al. 2008, Ong et al. 2007). In addition, quinacrine-based confocal microscopy could be used to identify intracellular

granules. However, these methods should be used with caution for investigating the role of K_{Ca}3.1 and Ca^{2+} signalling; FM dyes reportedly induce store-operated Ca^{2+} entry (Perez et al. 2002, Hofmann et al. 2003). In addition, quinacrine localisation is associated with ATP content of granules, and consequently may not provide a specific method of identifying mucin granules (Amaral et al. 2011).

7.3.5   *MUC5B expression*

In this study, the effect of K_{Ca}3.1 blockade on MUC5AC mRNA expression and MUC5AC intracellular immunostaining in H292 cultures was examined. Furthermore, MUC5AC expression in the epithelium of bronchial biopsies was co-localised and correlated with K_{Ca}3.1 expression. However, MUC5B is also a predominant mucin secreted into the human airways and is consistently detected in airway mucus and sputum alongside MUC5AC (Li et al. 2009). Similarly to MUC5AC, MUC5B is present in higher quantities in asthmatic sputum and during exacerbations compared to normal healthy mucus (Akopova et al. 2012, Pangrsic et al. 2007), and is hypothesised to be associated with airway inflammation and infection (Thornton et al. 2000, Thornton et al. 1997, Thornton et al. 1996a). Studying MUC5B expression in mice has found that MUC5B plays a role in mucosal immune function, mucociliary clearance, bacterial infection and inflammatory defence (Kirkham et al. 2002, Henke et al. 2007). In addition, MUC5B$^{-/-}$ mice exhibit impaired growth and survival (Thornton et al. 2008). Consequently, future work should examine whether K_{Ca}3.1 modulates MUC5B expression and secretion, and whether K_{Ca}3.1 is associated with MUC5B expression within the bronchial epithelium in asthma.

7.3.6   *The role of K_{Ca}3.1 in epithelial cell differentiation*

Primary HBEC ALI cultures exhibiting ciliogenesis and mucus secretion are terminally differentiated cultures. Consequently, the effects of secretagogues on goblet cell hyperplasia and mucus expression and secretion might be limited. Epithelial damage is implicated in asthma and can expose underlying basal cells of the epithelial layer (Roy et al. 2014). Basal cells act as resident tissue stem cells

capable of differentiating into specialised epithelial cells, and in this way aid epithelial repair (Roy et al. 2014). The prominence of goblet cell hyperplasia in asthma could suggest an imbalance of epithelial cell differentiation into a mucous-cell phenotype.

Basal cell differentiation is modulated by Notch and SPDEF pathways (Fedorov et al. 2005, Laitinen et al. 1985). Down-regulation of Notch signalling, when combined with SPDEF activation and IL-13 signalling, promotes goblet cell differentiation (Hong et al. 2004, Rock et al. 2009, Hackett et al. 2008, Hajj et al. 2007, Puchelle et al. 2006). Investigating the role of $K_{Ca}3.1$ in basal cell differentiation and in Notch-mediated development of a mucous-cell phenotype could be an interesting future experiment. For instance, supplementing or inhibiting Notch signalling in HBECs during their transformation from submerged culture to ALI culture in the presence of $K_{Ca}3.1$ blockers could provide insight into the role of $K_{Ca}3.1$ in goblet cell differentiation and hyperplasia.

7.3.7   *The role of $K_{Ca}3.1$ in epithelial mesenchymal transition*

Previous studies have found that $K_{Ca}3.1$-dependent regulation of $[Ca^{2+}]_i$ is associated with TGF-β1-induced pro-fibrotic myofibroblast function (Chen et al. 2009, Gras et al. 2013). TGF-β1 is a key growth factor driving fibrosis and epithelial mesenchymal transition (EMT) in diseases such as idiopathic pulmonary fibrosis (IPF) and COPD (Gras et al. 2013). Findings of $K_{Ca}3.1$ expression in HBECs suggest that $K_{Ca}3.1$ plays a significant role in TGF-β1-induced EMT. The effects of $K_{Ca}3.1$ blockade on TGF-β1-induced EMT in HBECs should be examined in future work.

7.4   *Summary*

In summary, this study demonstrates that $K_{Ca}3.1$ does not modulate rh-AREG-induced mucin production and secretion from H292 cells, or rh-AREG or rh-IL-13-induced MUC5AC mRNA expression. However, further investigations are needed to fully examine the role of $K_{Ca}3.1$ in mucus hypersecretion in asthma.

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