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Persistent induction of goblet cell differentiation in the airways: Therapeutic approaches

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Abstract

Dysregulated induction of goblet cell differentiation results in excessive production and retention of mucus and is a common feature of several chronic airways diseases. To date, therapeutic strategies to reduce mucus accumulation have focused primarily on altering the properties of the mucus itself, or have aimed to limit the production of mucus-stimulating cytokines. Here we review the current knowledge of key molecular pathways that are dysregulated during persistent goblet cell differentiation and highlights both pre-existing and novel therapeutic strategies to combat this pathology.

Keywords

Goblet cell, Asthma, COPD, Cystic Fibrosis, Mucus, Epithelium, Signaling Pathways, Therapeutic, micro-RNA, Mechanical Stress, Monoclonal Antibody Therapy

Table of Contents**1. Introduction****2. Goblet cell pathology in Chronic Airways Disease***2.1 Asthma**2.2 COPD and chronic bronchitis**2.3 Cystic Fibrosis***3. Signaling pathways associated with goblet cell differentiation (GCD)***3.1 EGFR and GCD**3.2 Role of type-2 interleukins in GCD**3.3 Critical proteins linked to persistent GCD**3.4 Role of Notch in GCD**3.5 Wnt/ β -catenin signaling and GCD**3.6 Other molecules implicated in GCD**3.6.1 Protein candidates suggested to regulate GCD**3.6.2 Micro-RNA regulation of GCD*

3.7 Mechanical stress and GCD

3.8 Viral induced GCD

4. Therapeutic strategies targeting GCD

4.1 Drug treatments

4.2 Monoclonal antibody therapy

4.3 Therapies utilizing miRNA technology

5. Conclusions

6. Reference

Abbreviations

Cyclic Adenosine Monophosphate (cAMP), Chronic Obstructive Pulmonary Disease (COPD), Club Cell Secretory Protein (CCSP[†]), Cystic Fibrosis (CF), Epidermal Growth Factor Receptor (EGFR), Epithelial to Mesenchymal Transition (EMT), Extracellular Signal-Regulated Kinase (ERK), Goblet Cell Differentiation (GCD), Heparin Binding-EGF-like Growth Factor (HB-EGF) House Dust Mite (HDM), Human Neutrophil Elastase (HNE), Mechanistic Target of Rapamycin (mTOR), Mitogen Activated Protein Kinase (MAPK), Monoclonal Antibody (mAb), Notch Intracellular Domain (NICD), Ovalbumin (OVA), Phosphatidylinositol-3 Kinase (PI3K), Protein Kinase B (AKT), Short-Hairpin RNA (shRNA), Signal Transducer and Activator of Transcription (STAT), Suppressor of Cytokine Signaling (SOCS), Transforming Growth Factor (TGF), Tumor Necrosis Factor (TNF).

[†]Naming convention: Uppercase lettering denotes protein (e.g. FOXA2). Italics denote genes and mRNA products (e.g. *FOXA2* (human) or *Foxa2* (murine)).

1. Introduction

The human airway epithelium is the principal barrier protecting the lungs from the external environment. It is comprised of three major cell types, basal cells, ciliated cells and secretory cells (Goblet cells and Club cells) (Fig. 1). Goblet cells, together with submucosal glands are the principal secretors of MUC5AC and MUC5B mucin glycoproteins respectively (Hovenberg, et al., 1996). These gel-forming mucins form the glycoprotein component of airway mucus.

Goblet cell differentiation (GCD) is believed to have evolved to combat parasitic infection and is an essential step for the clearance of inhaled pathogens from the airways (Evans, et al., 2009). In healthy individuals, transient GCD results in an increase in goblet cell number at the affected areas of the epithelium, and temporarily upregulates mucus production. In contrast, chronic airways diseases, such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), are characterized by a persistent goblet cell phenotype and continually high levels of secreted mucins (McCullagh, et al., 1995). Over time this persistent mucin production is linked to intraluminal mucus accumulation, increased rates of infection and airway obstruction (Rose & Voynow, 2006).

Numerous signaling pathways have been implicated in the shift from ‘normal’, primarily ciliated epithelium to an overtly secretory phenotype. How these pathways become altered in diseases such as asthma, COPD/chronic bronchitis and CF remains an intense area of research and promises the development of new drugs targeting GCD and mucus overproduction. To date however, conventional therapies to treat intraluminal mucus accumulation have focused on targeting specific properties of mucus itself, such as altering mucus viscoelasticity, restoring ‘normal’ mucus structure as well as improving the rates of mucociliary clearance (Beeh, et al., 2008). Expectorant medicines, designed to promote the clearance of mucus from the airways, prove inadequate in curbing underlying mucus overproduction (Malerba & Ragnoli, 2008; Poole, et al., 2015). This is because they are largely interventions that aim to suppress the disease symptoms rather than pathogenic processes. Targeting the mechanisms that drive persistent GCD and downstream overproduction of mucus provides an alternative and potentially disease modifying solution. While not the focus of this review, submucosal gland morphology is also grossly altered in chronic airways disease and impacts disease pathophysiology. During both asthma and COPD there is an enlargement of tracheobronchial submucosal glands (hypertrophy), which leads to the increased production of the MUC5B mucin (Kirkham, et al., 2002; Hovenberg, et al., 1996). Dysregulation of gland function is also an early hallmark of CF and results in increased detection of MUC5B within submucosal glands (Ostedgaard, et al., 2017). Increased MUC5B is associated with chronic airways disease, but is not sufficient for intraluminal mucus accumulation. It is only in the presence of high levels of MUC5AC that mucus

accumulation occurs (Evans, et al., 2009). Furthermore, experimentally reducing MUC5B has been shown to abolish mucocilliary clearance and greatly increase infections at the airway in animal models; whereas reducing MUC5AC does not (Roy, et al., 2014). As such, reducing the production of MUC5AC by therapeutically targeting goblet cells is a viable strategy for minimizing mucus accumulation during chronic airways disease.

In this article, we review the currently identified, as well as emerging molecular pathways that result in persistent GCD and how these overlap across a range of chronic airways diseases. Additionally, although the focus of this review is GCD in the human airway epithelium and emerging technologies that allow for more comprehensive studies *ex vivo*, reference is also made to critical experiments within the large airways of rodents. Although there is debate regarding how appropriate rodent models are when focusing on the human airway epithelium, there are models that accurately reflect key disease features of asthma and COPD (Beckett, et al., 2013; Kim, et al., 2017; Thorburn, et al., 2010), and it is clear that key mechanistic data remains applicable to both systems (Chen, et al., 2009; Laoukili, et al., 2001; Liu, Chen, et al., 2016; Rajavelu, et al., 2015; Starkey, et al., 2013; Wills-Karp, et al., 1998). Nonetheless a cautious approach must be taken when comparing the two systems and efforts be made to validate *in vivo* animal data in relevant human tissues. By providing an in-depth summary of the dysregulated pathways currently associated with aberrant GCD, we highlight emerging novel targets that may influence excess mucus production from the airway epithelium. As such, we aim to emphasize the value of therapeutic strategies targeted towards persistent GCD and provide potential ways forward in the treatment of excess mucus production across a range of airways diseases.

2. Goblet cell pathology in Chronic Airways Disease

Abnormal production of gel-forming mucin has been demonstrated in airways of asthmatic, COPD and CF patients (Kirkham, et al., 2002; Caramori et al., 2009; Robinson et al., 2002). During normal gel-layer formation, linear polymers of MUC5B, released from submucosal glands, are coated and anchored by the MUC5AC released by goblet cells (Thornton, et al., 2008; Ermund, et al., 2017).

Healthy mucus consists of approximately 3% solids (including mucins, salts, lipids), however this proportion can reach almost 15% in chronic airways disease (Fahy & Dickey, 2010). The proportionate increase in solid mass leads to many more intermolecular interactions and results in a thicker, stickier gel-layer. Although there are some morphological similarities in airways when comparing; asthma, COPD and to some extent CF, these arise through very different processes (Kaliner, et al., 1986) (Fig.2; *previously unpublished data*).

2.1 Asthma

A range of overt structural anomalies are commonly exhibited by airways of asthmatics, such as smooth muscle cell hyperplasia/hypertrophy, goblet cell hyperplasia, subepithelial fibrosis and sub-mucosal gland hyperplasia/hypertrophy (Bai & Knight, 2005; Warner & Knight, 2008; Ordonez, et al., 2001; Wilson & Li, 1997). This 'airway remodeling' is believed to occur in response to repeated epithelial injury through inhalation of allergens and numerous environmental stimuli, infection, as well as oversensitive and misperceived host inflammatory/immune responses (Fahy, et al., 1995; Jackson & Johnston, 2010; Marini, et al., 1992; Woolnough, et al., 2016). Indeed, the symptoms of asthma have been compared to infestation of the lungs by helminth worms, a downstream effect of which is the massive upregulation of goblet cells and immediate induction of mucin MUC5AC (Evans, et al., 2009). Although asthma is understood to be a heterogeneous disorder of numerous distinct endotypes, a skewed T-helper class 2 (Th2) immune response is a prominent feature in most cases (Fahy, 2015; Hansbro, et al., 2011). During cases of both mild and severe asthma there is a significant increase in the number of goblet cells lining the upper airways compared to non-asthmatics (Jackson, 2001; Ordonez, et al., 2001; Parker, et al., 2013).

2.2 COPD and chronic bronchitis

The term COPD defines a complex range of airways/lung diseases that tentatively fall under two non-exclusive subtypes; chronic bronchitis and emphysema, and is characterized by fixed airflow obstruction. Airflow obstruction in individuals with primarily bronchitic COPD is caused by gross

changes in airway morphology, including persistent GCD, while airflow obstruction in primarily emphysematous COPD is caused by the destruction of alveolar cells. There are similarities between the airways of individuals suffering from COPD and those with asthma, such as chronic airway inflammation, airway wall thickening, increased airway smooth muscle deposition as well as persistent mucus plugging (Hartley, et al., 2016; Jeffery, 1999; Pini, et al., 2014). Unlike asthma, the epithelial pathophysiology of COPD is extremely heterogeneous and arises from exposure to toxic compounds, most commonly within cigarette smoke (Gao, et al., 2015). Exposure to cigarette smoke has been shown to directly upregulate MUC5AC stores and increases goblet cell size and number (Innes, et al., 2006). This exposure also compromises barrier integrity by disrupting cell-cell junctions, ciliary function and mucociliary clearance and increasing permeability of the epithelium, culminating in increased rates of infection (Ganesan, et al., 2013). Additionally, the infiltration of neutrophils and CD8⁺ lymphocytes is associated with structural changes, including GCD in the airways of COPD subjects (Jeffery, 2001).

2.3 Cystic Fibrosis

In the airways of patients with CF there is distinct, thickened mucus that results from a deficiency in the epithelium's ability to hydrate surface mucus. This dehydrated mucus manifests via a mutation in the CF transmembrane conductance regulator (*CFTR*) gene, resulting in epithelial cells that are deficient in a critical cAMP-dependent anion channel (Anderson, et al., 1991). The majority of studies in CF airways have focused on the process of mucus hydration; however, there is also evidence, albeit controversial, of increased GCD. Burgel et al. (2007) found excessive goblet cell hyperplasia in the epithelium surrounding mucus plugs in CF lung sections. Additionally, in mice with airway-specific overexpression of the epithelial Na⁺ channel (β ENaC-Tg), GCD is stimulated by interactions with abnormal surface mucus (Mall, et al., 2008). Conversely, Hays et al, (2006) showed increased volume of submucosal glands, but no change in GCD in bronchial biopsies taken from subjects with CF. Although mucus plugging has been shown in the CF airway, even in the absence of GCD (Gehrig, et al., 2014), increased rates of infection within CF airways are known to trigger increases in GCD (Hao

et al., 2012). The additive pathogenic effect of excess mucin secretion from increased goblet cell numbers would undoubtedly worsen the disease (Henderson, et al., 2014).

3. Signaling pathways associated with GCD

Although very few studies have been conducted comparing the mechanisms of GCD between asthma, COPD, and CF it is likely that key signaling pathways, such as epidermal growth factor receptor (EGFR), interleukins (Th2 cytokines), Notch, signal transducer and activator of transcription 6 (STAT) 6, and WNT signaling are similar between these diseases (Fig. 3) (Takeyama, et al., 1999; Takeyama, et al., 2001). Non-biological pathways, such as mechanical (shear and compressive) stress, may also contribute to GCD (Liu, et al., 2013).

3.1 EGFR and GCD

EGFR is a multifunctional receptor protein required for the rapid transmission of growth responses. EGFR also regulates the stimulation of innate immune responses including GCD in the healthy airway. Dysregulation of EGFR-based signaling is linked to an enormous range of diseases/syndromes including cancers, as well as many pathophysiological features associated with asthma, COPD and CF (Kedzierski, et al., 2017; Allahverdian, et al., 2008; Acciani, et al., 2016; Chung, 2001; Sharma, et al., 2007; Stolarczyk, et al., 2016; Voynow, et al., 2005). EGFR immunoreactivity is enriched in regions of increased GCD in human bronchi (Takeyama, et al., 2001).

EGFR signaling involves four cell surface receptor proteins (EGFR/ErbB1, ErbB2, ErbB3 and ErbB4), and is directly activated by eight known ligand families; epidermal growth factor (EGF), transforming growth factor (TGF)- α , heparin binding-EGF like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin, epigen, and neuregulin (Cohen, 1964; Derynck, et al., 1984; Higashiyama, et al., 1991; Jones, et al., 1999; Peles, et al., 1992; Sasada, et al., 1993; Shoyab, et al., 1989; Strachan, et al., 2001). Upon ligand binding, ErbB receptor dimers (both heterodimers and homodimers) undergo a conformational change resulting in the activation of one or more downstream

protein complexes including phosphatidylinositol-3 kinase/ protein kinase B (PI3K/AKT), mitogen activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK), STAT and mechanistic (or mammalian) target of rapamycin (mTOR), controlling airway homeostasis (Ikegami, et al., 2008; Jiang, et al., 2010; Liu, et al., 2013; Xu, et al., 2015). Ligands such as TGF- α and amphiregulin are secreted by a range of inflammatory cells, including eosinophils and neutrophils as well as epithelial cells (Burgel, et al., 2001; Calafat, et al., 1997; Okumura, et al., 2005; Wong, et al., 1990). The impact of these eosinophil and neutrophil-derived ligands on EGFR signaling in asthmatic, COPD and even CF airway epithelium remains an important area of investigation. Suppressor of cytokine signaling (SOCS)5 can downregulate EGFR signaling *in vitro* and as such elicits a protective role against influenza A infection in the mouse airway, a key inducer of MUC5AC production (Kedzierski, et al., 2017; Nicholson, et al., 2005; Barbier, et al., 2012). Reduced SOCS5 expression has been observed in epithelial cells from COPD patients (Kedzierski, et al., 2017). Tyner et al, implicated EGFR activation in the trans-differentiation of ciliated cells to goblet cells within mouse airways (Tyner, et al., 2006). In this study EGFR signaling through PI3K was shown to prevent ciliated cells from undergoing apoptosis. These cells then were shown to transdifferentiate into goblet cells upon stimulation by interleukin-13 (IL-13).

Although EGFR has been clearly implicated in GCD, therapeutic approaches targeting EGFR receptors face considerable challenges. Progress is being made with regard to EGFR tyrosine kinase inhibitors but safety versus efficacy remains an issue (Ha & Rogers, 2016). Indeed, clinical trials using an inhaled EGFR inhibitor, BIBW 2948 failed to reduce epithelial mucin stores and were poorly tolerated by subjects with COPD (Woodruff et al., 2010). A number of proteins/ molecules that act downstream of EGFR signaling, or act via crosstalk may therefore offer a more effective target for pharmacological therapy.

3.2 Role of type-2 interleukins in GCD

Acting in concert with EGFR signaling and essential in the control of healthy GCD in the airway epithelium are a number of well characterized interleukins; IL-13, IL-4, IL-5, and IL-9 (Grunig, et al.,

1998; Tanabe, et al., 2014). IL-13 is arguably the most comprehensively studied molecule that can induce persistent GCD in airway epithelial cells across many species (Atherton, et al., 2003; Grunig, et al., 1998; Kanoh, et al., 2011; Kondo, et al., 2006; Kondo, et al., 2002; Shim, et al., 2001; Wills-Karp, et al., 1998; Zhu, et al., 1999).

Early studies suggested that IL-13 dependent GCD involved EGFR signaling since inhibition of a specific EGFR tyrosine kinase prior to IL-13 treatment ablated GCD in rat airway epithelium *in vivo* (Shim, et al., 2001). However, a follow up study by Atherton et al, showed that the potent EGFR-tyrosine kinase inhibitor (AG-1478) did not prevent IL-13 driven GCD (Atherton, et al., 2003). It is important to note that the study by Shim et al, was performed in rats *in vivo* and involved an intraperitoneal injection one-day prior to IL-13 exposure, whereas Atherton et al, treated primary cultures of human bronchial epithelium grown in air-liquid interface (ALI) culture with the inhibitor and cytokine simultaneously. This suggests species-based and/or temporal-based differences in IL-13/EGFR crosstalk. In the rat at least, the effect of IL-13 on GCD is leukocyte dependent as pre-treatment with an inhibitor of leukocyte cell production prevented IL-13 induced GCD (Shim, et al., 2001). Although allergic inflammation increases IL-13 production, there is evidence in mouse models that Sendai-virus induces mucous cell metaplasia via macrophage-derived IL-13 secretion (Kim, et al., 2008). Thus dysregulation of the innate immune response may also be a key stimulator of IL-13 signaling.

IL-4 and -5 have been well documented as mucus inducing agents, particularly in asthmatics largely due to their well-documented roles in regulating allergic inflammatory responses (Hansbro, et al., 2013; Lopez, et al., 1988; Wang, et al., 1989). Additionally, IL-4 exhibits a high structural similarity to IL-13, signals through the same receptor complex and both share several signaling pathways (Mueller, et al., 2002). Although IL-4 and IL-5 overexpressing mice both exhibit significant GCD (Lee, et al., 1997; Temann, et al., 1997), mice deficient in these factors show similar GCD to controls following exogenous treatment with tumor necrosis factor (TNF)- α (Cohn, et al., 1999; Cohn, et al., 1997). Thus IL-4 and IL-5 have been hypothesized to regulate GCD by recruiting TNF- α secreting cells to the epithelium (Cohn, et al., 1997; Lee, et al., 1997; Temann, et al., 1997). Whether

recruitment of TNF- α producing cells is a major function of IL-4 and IL-5 in human airways is unclear. IL-4 is becoming more recently acknowledged as a therapeutic target for chronic asthma, particularly in combination with IL-13 (Hansbro, et al., 2013; Kau & Korenblat, 2014). Presumably this would include inhibition of mucus hypersecretion and GCD.

IL-9 appears to upregulate GCD in cells that are undergoing differentiation, such as would occur during injury repair or before a fully differentiated epithelium has developed (Vermeer, et al., 2003). The induction of IL-9 has been shown to occur in the presence of IL-4 and TGF β , which act in concert to differentiate Th2 cells into a Th9 subtype (Veldhoen, et al., 2008). Th9 cells have been shown to elicit allergic airway inflammation and GCD when transferred to mice previously challenged with OVA to induce experimental asthma (Staudt, et al., 2010). Furthermore, blocking IL-9 with monoclonal antibodies ameliorated the inflammation and GCD in treated mice (Staudt, et al., 2010). Human airway epithelial cells grown in ALI and transdifferentiated into goblet cells via IL-13 treatment have recently been shown to release IL-4, -5, -9 and -13 at higher levels than ciliated cells (Tanabe & Rubin, 2016). Low level release of IL-4, -5 and -13 also occurs following epithelial damage (Hodge, et al., 2002; Wu, et al., 2010; Allahverdian, et al., 2008). This release is hypothesized to enhance the inflammatory response in asthma to produce an increased level of GCD.

3.3 Critical proteins linked to persistent GCD

A number of proteins have been shown to act downstream of these initial receptor-activated signaling events in the control of GCD, including STAT6, forkhead box protein A2 (FOXA2), SAM Pointed Domain Containing ETS Transcription Factor (SPDEF) and γ -aminobutyric acid $_A$ receptor (GABA $_A$ R).

STAT6 is a key transcription factor selectively activated by IL-13 and IL-4 that influences the expression of multiple target genes, including those involved in GCD upon activation, and as such is a central mediator of cytokine signaling (Goenka & Kaplan, 2011). STAT6 deficient mice do not exhibit epithelial GCD following IL-13 exposure, but it is restored following epithelial reconstitution of STAT6, implicating a crucial role for the protein during IL-13 based signaling (Kuperman, et al.,

2002). *STAT6* has also been shown to exhibit aberrant histone acetylation patterns at the transcriptional start site within the asthmatic airway epithelium (Stefanowicz, et al., 2009). *STAT6* upregulates GATA binding protein 3 (*GATA3*) expression, which is intimately linked to the production of Th2 cytokines and airway remodeling. Interestingly, although *STAT6* deficient mice are insensitive to IL-13 stimulation, they are still able to exhibit GCD upon activation of the Notch signaling pathway (Guseh, et al., 2009), suggesting that although IL-13-mediated induction of GCD is dependent on *STAT6*, IL-13/*STAT6* independent pathways are also involved.

FOXA2 is a transcription factor required for maintaining surfactant homeostasis as well as host defense and has been shown to repress GCD in the healthy airway epithelium (Wan, Kaestner, et al., 2004; Wan, Xu, et al., 2004). *FOXA2* deficient mice show increased goblet cell hyperplasia as well as increased numbers of neutrophils and macrophages in bronchiolar lavage fluid (Wan, et al., 2004). Furthermore, deletion of *Foxa2* also leads to upregulation of IL-4 and IL-13 (Chen, et al., 2010). A significant reduction in *FOXA2* levels has been shown in airways from asthmatics, whilst aberrant methylation patterns have recently been observed in the *FOXA2* promoter in COPD epithelium (Park, et al., 2009; Song, et al., 2017). Downstream events following *FOXA2* activation are currently unknown; however evidence suggests that *FOXA2* is a negative regulator of myeloid dendritic cell recruitment and activation (Chen, et al., 2010).

SPDEF is a transcription factor that controls GCD and a network of genes associated with airway epithelial mucin production. *SPDEF* plays a critical role during development of the airway epithelium as well as epithelial remodeling following exposure to allergen (Rajavelu et al., 2015). Chen et al (2009) used transgenic mice with inducible *Spdef* expression combined with cell-lineage tracing analysis to demonstrate that increased *SPDEF* leads to goblet cell metaplasia (Chen, et al., 2009). Microarray analysis has revealed upregulation of genes associated with allergen exposure following *SPDEF* induction, such as *FOXA3*, anterior gradient 2 (*AGR2*) and chloride channel accessory 1 (*CLCA1*) (Chen, et al., 2014; Nakanishi, et al., 2001; Schroeder, et al., 2012; Zhen, et al., 2007). The induction of *SPDEF* also resulted in downregulation of genes associated with differentiation of healthy airway epithelia, including *FOXA2* and thyroid transcription factor 1 (*TTFI*) (Chen, et al.,

2009; Maeda, et al., 2011; Park, et al., 2009). Additionally, forkhead box M1 (FOXM1) is required for induction of *Spdef* transcription and in mice conditional deletion of FOXM1 from Club cells has been shown to inhibit expression of SPDEF and as a consequence, drastically reduces goblet cell number (Ren, et al., 2013; Sun, et al., 2017). Recently, Song et al (2017), used a novel technique known as ‘targeted epigenetic editing’ to modify the *Spdef* promoter to increase binding of transcriptional repressors within human lung epithelial cells (Song, et al., 2017). This downregulated SPDEF levels and resulted in a reduction of MUC5AC expression (Song, et al., 2017). Dysregulated SPDEF and FOXA3 activity may be a hallmark of all persistent GCD associated diseases, as epithelial expression of each of these proteins is increased in asthma, COPD and CF (Chen, et al., 2009). Targeting SPDEF and/or FOXA3 may therefore represent alternative strategies to alleviate mucus accumulation in chronic airways disease.

GABA_A receptors are pentameric Cl⁻ channels that are upregulated in mice following sensitization and challenge with OVA. Treatment of cultured human small airway epithelial cells with IL-13 results in a dramatic increase in GABA_A β2 subunit protein concomitant with GCD and blocking of GABA signaling reduced GCD associated with IL-13 stimulation (Xiang, et al., 2007). However this did not affect IL-13 production itself, suggesting a role for GABA_A downstream of IL-13 signaling. As GABA_A appears to regulate GCD in epithelial cells following IL-13 exposure it may prove an effective point of therapeutic manipulation to reverse GCD in a high IL-13 environment.

3.4 Role of Notch in GCD

The Notch family consists of four (NOTCH1-4) transmembrane receptor proteins and is most well-known for its roles in cell fate determination during development. Upon ligand binding, the Notch intracellular domain (NICD) is released via two proteolytic cleavage events and translocates to the nucleus to switch on/off key transcriptional profiles (Schroeter, et al., 1998). The key transcriptional effector and binding partner to NICD during Notch signaling is recombination signal binding protein for immunoglobulin kappa J region (RBPJ) (Castel, et al., 2013; Tamura, et al., 1995). Binding of the NICD to RBPJ is well documented to activate the transcriptional repressors hairy and enhancer-of-

split (*HES*) and hairy and enhancer-of-split related with YRPW motif (*HEY*) (Fischer & Gessler, 2007), which repress airway ciliogenesis (Gerovac, et al., 2014; Tsao, et al., 2009). Notch signaling also represses expression of multicilin (MCIDAS) and forkhead box J1 (FOXJ1) in airway epithelium, which in turn inhibits ciliated cell differentiation (Gerovac & Fregien, 2016; Gerovac, et al., 2014). Additionally, recent cell lineage tracing studies have highlighted Notch as a key regulator of epithelial trans-differentiation in the adult lung (Lafkas, et al., 2015). Ablation of Notch signaling using antibodies against the Notch-activating ligands Jagged-1 (JAG1) and Jagged-2 (JAG2) results in a direct conversion of Club cells into ciliated cells without increased cell division or cell death (Lafkas, et al., 2015).

Although Notch is clearly implicated in the decisions of epithelial cell fate, one important caveat is that different Notch isoforms elicit different transcriptional responses. For example, Notch1 and to a lesser extent Notch2 activate the lymphoid enhancer binding factor 1 (LEF-1) transcription factor whilst Notch3 does not (Ross & Kadesch, 2001). Beatus et al (1999), also showed that Notch1 upregulates transcription of HES1 approximately 80-fold compared to a 3-fold induction by Notch3.

Recently, Notch2 has been identified as key for inflammatory cytokine-driven goblet cell metaplasia in murine lung in vivo and importantly, this effect was also seen in differentiated human bronchial epithelial cells in vitro (Danahay, et al., 2015). In contrast to this finding, the overexpression of either Notch1 or Notch3 has been shown to drive goblet cell metaplasia in a system devoid of inflammatory cytokines such as IL-13 (Gomi, et al., 2015). This indicates that different Notch isoforms impact on GCD both within IL-13-high and IL-13-low environments (Gerovac & Fregien, 2016; Guseh, et al., 2009; Gomi, et al., 2015).

In addition to its role within the epithelium, Notch has also been shown to control cell fate during T-cell development and as such influences the production of Th2 associated cytokines, including IL-13 (Kang, et al., 2009). Notch regulation of Th2 cytokine release by T-cells may then further regulate GCD at the airway epithelium. As such, Notch signaling is predicted to control mucus homeostasis through these epithelial and T-cell based mechanisms, and dysregulation of one or both will likely

result in GCD (Zong, et al., 2016). An improved understanding of the functional role and interactions of specific Notch isoforms in the control of ciliogenesis/GCD may identify therapeutically exploitable targets to reduce persistent GCD regardless of inflammatory cytokine profiles.

3.5 *Wnt/ β -catenin signaling and GCD*

The WNT family of extracellular secreted ligands is crucial to lung morphogenesis and tissue maintenance in the adult lung. Wnt-signaling is generally characterized as either canonical (stabilization and translocation of β -catenin to the nucleus) or non-canonical (β -catenin independent) (Corda & Sala, 2017). In the canonical pathway, WNT ligands act to stabilize the cytosolic and nuclear expression of the transcriptional co-activator β -catenin, which can bind to and activate numerous downstream transcription factors, including the well-known LEF-1 and TCF-1/3/4 (Baarsma, et al., 2013). This is regulated by WNT ligand binding to Frizzled (FZD) receptors, which in the presence of the LRP5/6 co-receptors inactivates the so-called destruction complex normally responsible for β -catenin degradation. This destruction complex, consisting of APC, Axin, casein kinase 1 α and GSK-3 β (Kimelman & Xu, 2006), targets β -catenin for phosphorylation and subsequent ubiquitination in the absence of WNT ligands, but is functionally repressed upon WNT activation. β -Catenin signaling in airway epithelial cells is key to differentiation and proliferation in response to WNT ligands and other extracellular ligands.

Okubo and Hogan initially discovered that surfactant protein C promoter (SpC)-driven expression of a constitutively active β -catenin/LEF-1 fusion protein promotes a gene expression signature in the lung reminiscent of GCD (Okubo & Hogan, 2004). Transgenic expression of a constitutively active mutant form of β -catenin, lacking exon 3 (Catnb ^{Δ (ex3)}), into club cell secretory protein (CCSP) rich airway epithelial cells yielded a similar phenotype with massive goblet cell hyperplasia (Mucenski, et al., 2005). Furthermore, in a toluene diisocyanate model of chemically-induced asthma, treatment with a tankyrase inhibitor (which stabilizes the β -catenin destruction complex) or with the β -catenin/CBP inhibitor ICG-001, sharply reduced GCD in both cases (Yao, et al., 2017). In contrast though, ICG-

001 treatment in OVA challenged mice had no such effect on GCD (Koopmans, et al., 2016), indicating that the role of β -catenin in GCD may be stimulus-specific. A better understanding of this specificity and the signaling effectors controlling β -catenin mediated GCD may open therapeutic avenues for GCD treatment.

3.6 Other molecules implicated in GCD

In addition to the signaling pathways and proteins listed above, there are a number of emerging candidate proteins and miRNAs that also influence GCD within the airway epithelium. These molecules play roles downstream of selected signaling pathways listed above and may prove to be useful candidates when targeting persistent GCD.

3.6.1 Protein candidates suggested to regulate GCD

Kinesin family member 3A (*KIF3A*) is a known asthma susceptibility gene (Kim, et al., 2011; Kovacic, et al., 2011), regulating aeroallergen response through a Th2 mediated pathway in airway epithelial cells (Vummidi Giridhar, et al., 2016). Mice with selective deletion of *Kif3a* from airway epithelial cells exhibited GCD and an increased level of eosinophilic inflammation following exposure to aeroallergen. These mice also showed increased expression of *Il-4*, *-13* and *17a* as well as *Ccl-11* (Vummidi Giridhar, et al., 2016). Interestingly, KIF3A may also play a role in Notch signaling as primary epidermal keratinocytes extracted from *Kif3a* deficient mouse embryos exhibit a deficiency in NICD-3 translocation to the nucleus *in vitro* (Ezratty, et al., 2011).

Neuregulin 1 β 1 (NRG1 β 1) treatment has recently been shown to increase MUC5AC and MUC5B as well as GCD via EGFR signaling in human bronchial epithelial cells grown under ALI conditions (Kettle, et al., 2010). As inhibition of p38 MAPK, ERK and PI3K/AKT kinases blocked GCD *in vitro*, it appears that NRG1 β 1 operates upstream of EGFR signaling (Kettle, et al., 2010). Whether NRG1 β 1 plays a primary role in GCD in chronic airways disease requires further investigation. If so it may provide a novel target for persistent GCD.

A transcription factor that has recently been implicated in the progression of GCD is Runt-related transcription factor 2 (Runx2). Following shRNA-induced knockdown of *RUNX2*, *SPDEF* expression was abolished in bronchial epithelial cell cultures (Shi, et al., 2017). Mice administered with *Runx2* shRNA also exhibit significantly reduced airway mucus production and improved pulmonary function (Shi, et al., 2017). Chromatin immunoprecipitation assays using antibodies against *RUNX2* showed enrichment of the protein at the human *SPDEF* promoter following IL-13 stimulation indicating a direct role for *RUNX2* in *SPDEF* mediated GCD (Shi, et al., 2017). Additionally, increased expression of *RUNX2* is seen in airways from asthmatics proximal to areas enriched with goblet cells (Shi, et al., 2017). Although further testing of the effects of *RUNX2* inhibition is required, it may also be a viable candidate in the treatment of persistent GCD.

TNF- α converting enzyme (TACE), also known as a disintegrin and metalloprotease 17 (ADAM17), is a membrane-tethered enzyme that cleaves numerous cell-surface proteins. A number of the proteins/ signaling molecules that TACE cleaves have been shown to play a major role in GCD. These TACE substrates include EGFR ligands, Notch ligands and the NOTCH1 receptor (Brou, et al., 2000). TACE has also been shown to be a mediator of human neutrophil elastase (HNE) induced GCD as mice deficient in TACE do not develop a goblet cell rich phenotype following exposure to HNE when compared to controls (Park, et al., 2013). Due to its pivotal role in the regulation of neutrophilic inflammation TACE has been suggested as an ideal target to prevent GCD in severe asthma, COPD and CF (Park, et al., 2013).

3.6.2 miRNA regulation of GCD

miRNAs are becoming increasingly well recognized in their ability to influence epithelial cell differentiation (Martinez-Anton, et al., 2013; Moheimani, et al., 2016). Understanding the contribution made by miRNAs in persistent GCD is necessary for the development of novel miRNA-based therapies. Here we provide a brief overview of miRNA species associated with GCD. miR125b is significantly reduced in the sputum of asthmatic children, especially those with eosinophilic asthma (Liu, Chen, et al., 2016). Intranasal delivery of a miR-125b mimic in a house dust mite (HDM)

induced mouse model of allergic airway inflammation significantly reduced GCD and mucus production (Liu, Chen, et al., 2016). miR-125a and miR-125b have been shown to directly target TNF alpha induced protein 3 (TNFAIP3), which plays a role during inflammatory cytokine (e.g. TNF- α) signaling by negatively regulating nuclear factor kappa B (NF- κ B) activity (Hsu, et al., 2017; Kim, et al., 2012). Expression of miR-449a is significantly downregulated in the airway epithelium of asthmatics (Solberg, et al., 2012). Mir-449a influences the NOTCH1 based repression of FOXJ1 and reduces GCD both within the airway and intestinal epithelium when overexpressed (Capuano, et al., 2011; Marcet, et al., 2011). Furthermore, miR-449a has been shown to target *NOTCH1* mRNA, turning off the ‘high-Notch’ secretory cell differentiation pathway.

miR-106a has previously been found to inhibit expression of the anti-inflammatory cytokine IL-10 in human B-cells (Sharma, et al., 2009). Knockdown of mouse miR106a in OVA-induced models significantly reduced features associated with allergic airway inflammation, including goblet cell metaplasia (Sharma, et al., 2012). Finally, two other miRNAs, miR-124a2 and miR-205 have been shown to regulate GCD but not within airway epithelium directly. In mammary and kidney epithelium, miR-205 regulates the epithelial to mesenchymal transition (EMT) by targeting *ZEB1* and *ZEB2* and has recently been shown to upregulate GCD when induced in intestinal epithelial cells (Eyking, et al., 2016; Suojalehto, et al., 2013). Similarly, miR124a2 has been shown to regulate GCD within pancreatic B cells (Baroukh, et al., 2007). Interestingly, a key target of miR-124a2 is *FOXA2* and as such is likely to play a role in pulmonary inflammation and GCD at the airway epithelium (Baroukh, et al., 2007). It is clear that certain miRNAs regulate the induction of GCD in the epithelium. Supplementation of miR-125b reduces GCD and shows promise as an inhaled therapeutic component (Liu, Chen, et al., 2016). Targeting miRNAs to regulate GCD in the future may produce novel, efficient and safe alternatives to small molecule therapies.

3.7 Mechanical stress and GCD

In addition to the dysregulation of protein/miRNA pathways listed above, mechanical stress has also been shown to contribute to GCD in chronic airways disease. Bronchoconstriction is the main

physiological event in asthma and is also common in COPD and CF. Bronchoconstriction generates significant mechanical force within the airways during chronic airways disease (Tschumperlin & Drazen, 2001; Wiggs, et al., 1997). These forces include compression and shear stress which act perpendicular and parallel to the epithelial cell surface, respectively (Tarran, et al., 2005; Tschumperlin, et al., 2003). It has been shown *in vivo* that repeated bronchoconstriction is associated with goblet cell hyperplasia at the human airway epithelium in the absence of inflammation and this can be mimicked by apical compression models *in vitro* (Grainge, et al., 2011; Park & Tschumperlin, 2009).

Although compression and shear stress occur simultaneously during bronchoconstriction, the majority of work to date has focused on compressive forces in the airway (Ressler, et al., 2000; Swartz, et al., 2001; Tschumperlin & Drazen, 2001; Wiggs, et al., 1997). These forces increase the concentration of secretory cell regulators into the lateral intercellular space, which impact the adjacent airway epithelial cells (Tschumperlin, et al., 2004). These regulators include $\text{TGF}\beta_2$, endothelin (Tschumperlin, et al., 2003), EGFR ligands such as HB-EGF, epiregulin and amphiregulin (Chu, et al., 2005), plasminogen activator inhibitor-1 (PAI-1) (Chu, et al., 2006), YKL-40 and chitinase-like protein (Park, et al., 2010). Compression-induced increases in some of these mediators have been shown to increase goblet cell number and mucin production at the airway epithelium (Park & Tschumperlin, 2009; Zhu, et al., 2015). Increased mucus production and goblet cell number has also been demonstrated *in vitro* via apical compression of human primary bronchial epithelial cells. This increase was significantly attenuated with an EGFR kinase inhibitor (AG1478) and abolished using anti-TGF- β_2 monoclonal antibody treatment (Park & Tschumperlin, 2009). To date, it is unknown if treatment with anti-TGF- β_2 would be effective in preventing compression induced GCD *in vivo*. Mechanical stress may therefore act to compound the problem of persistent GCD, particularly during episodes of bronchoconstriction. Whether compression and/or shear stress stimulate GCD-regulating pathways outside of EGFR is currently unknown. Future studies describing the role of these mechanical forces on GCD and mucus accumulation are clearly required.

3.8 Viral induced GCD

Virus infection, and particularly respiratory virus infection, has also been shown to upregulate GCD (Kim, et al., 2008; Shibata, et al., 2014; Villenave, et al., 2012; Walter, et al., 2002). Respiratory syncytial virus (RSV) induces GCD in bronchial epithelial cells isolated from children (Villenave, et al., 2012) and this correlates with excessive mucus production which is known to occur in RSV bronchiolitis (Hall 2001). Infection with Sendai virus has been shown to cause chronic goblet cell metaplasia in the airway epithelium of mice. This persistence of goblet cells occurs in wild-type mice and even in ICAM-1 deficient mice, which are protected from infection-stimulated airway inflammation and hyperreactivity (Walter, et al., 2002). Rhinovirus (RV), which is responsible for more than 50% of cases of the common cold (Leigh & Proud, 2015), is known induce GCD through various pathways. Neonatal mice, infected with RV1B demonstrate mucous cell metaplasia at the airway epithelium which persists for up to 60 days post infection (Schneider, et al., 2012). Disruption of IL-13 signaling using either anti-IL-13 neutralizing antibody or IL-4R deficient mice prevents RV1B-induced mucous cell metaplasia, indicating a critical role for IL-13 signaling. RV infection has also been found to induce *Foxa3* expression in mice *in vivo* and human airway epithelial cells *in vitro* (Chen, et al., 2014), leading to downstream increases in GCD.

4. Therapeutic strategies targeting GCD

To date, a number of different therapeutic approaches have been trailed for the treatment chronic airways disease including persistent GCD, albeit with some limitations (Table 1). As outlined above, numerous signaling pathways act in concert to regulate GCD within the airway epithelium. Most studies that have identified the pathways regulating GCD have been primarily conducted using *in vivo* animal models or *in vitro* culture of human bronchial epithelial cells. While no model can perfectly emulate what occurs in the human lung, the value of using such models cannot be overemphasized. *In vivo* animal models allow for variable mimicking of asthma, COPD and CF. This allows the assessment of GCD in the presence of a functional immune system, during development, following drug treatment or as a result of genetic modification. Conversely, *in vitro* studies in human tissues and

cells from human donors enables the direct study of GCD in cells from asthmatic, COPD, CF and healthy cohorts. This has allowed for extremely high-throughput data collection and the ability to screen many potential therapeutic agents on human samples with minimal risk. Using such models to assess the efficacy of selected therapeutic compounds not only helps us better understand the processes involved in dysregulated GCD, but is also critical in the identification of new targets for clinical application.

4.1 Drug treatments

For years, the symptoms of GCD (i.e. mucus hypersecretion), rather than GCD itself have constituted the major pharmacological target. This has led to a vast increase in the number of compounds with mucolytic/ mucoregulatory activity being tested (Amaral, et al., 2016). Ambroxol, N-acetylcysteine and carbocysteine are common expectorants used to treat diseases with a dysregulated mucus hypersecretion phenotype (Zhang & Zhou, 2014). Although such agents have been shown to improve mucociliary clearance with some effectiveness, they do not target the cause of mucus dysregulation and generally offer late term amelioration and symptom control only. A number of alternative therapeutic agents that instead target the upregulation of GCD may therefore offer a more effective preventative solution. Some progress has been made targeting key aspects of the pathways involved in GCD and has resulted in a number of therapeutic agents.

Corticosteroids are the most commonly prescribed medications for the treatment of asthma and are currently the most effective way to moderate airway inflammation in allergic asthmatics. They are often ineffective in patients with more severe asthma and COPD (Hansbro, et al., 2017). As part of a larger clinical trial, budesonide has been shown to significantly reduce persistent GCD in patients exposed to both low and high dose HDM (De Kluijver, et al., 2005). Additionally, corticosteroids such as dexamethasone have been shown to reduce MUC5AC production and GCD in animal models of asthma (Lundgren, et al., 1988). However, separate studies have revealed that corticosteroid withdrawal can stimulate a rebound increase in GCD to levels above that of allergen induction alone (Southam, et al., 2008). In human bronchial epithelium grown at ALI, dexamethasone does not reduce

GCD when cultures are co-stimulated with IL-13 (Kano, et al., 2011). Recent studies by Leigh, et al., (2016) present an exhaustive list of genes upregulated in human bronchial brushings and endobronchial biopsies following budesonide treatment. Numerous genes associated with GCD such as *ErbB4*, *RUNX2*, as well as JAK/STAT and PI3K/AKT signaling pathways, were upregulated in response to budesonide (Leigh, et al., 2016). Thus, while it is clear that corticosteroids offer effective reduction in airway inflammation, the potential long-term induction of genes involved in GCD may increase the likelihood of persistent GCD outside of inflammation.

Target	Effector molecule (s)	Major treatment outcomes	Effect of treatment on GCD	Reference
IgE	Anti-IgE mAb	Significantly reduced; OVA/HDM-stimulated airway hyper-responsiveness, neutrophil/eosinophil infiltration, IgE and IgG1 production ^A	Significantly attenuated RSV and OVA/HDM-stimulated GCD during aerosol sensitization but not systemicsensitization ^A	(Mizutani, et al., 2015; Tumas, et al., 2001)
IgE	Omalizumab (Anti-IgE mAb*)	Significantly reduced asthma exacerbations ^C	Goblet cell number not assessed ^C	(Busse, et al., 2001)
IL-13	Anti-IL13 mAb	Significantly reduced; transpulmonary resistance, airway hyperresponsiveness, eosinophil infiltration, proinflammatory cytokine production, serum IgE and airway remodeling during OVA-stimulation ^A Prevents subsequent allergic airways disease when exposed to Chlamydia infection during early life ^A	Significantly attenuated GCD ^A	(Starkey, et al., 2013; Yang, et al., 2004)
IL-13	Tralokinumab (Anti-IL13 mAb*)	Significantly reduced IL-13 induced airway hyperresponsiveness, airway	No significant effect on IL-13 induced GCD ^A	(Blanchard, et al., 2005)

		eosinophilia and oesophageal eosinophilia ^A		
		Potentially associated with increased lung function, increased peripheral blood eosinophils ^C	Goblet cell number not assessed ^C	(Piper, et al., 2013)
IL-13	Lebrikizumab (Anti-IL13 mAb*)	Significant increase in FEV ₁ in individuals with poorly controlled asthma ^C	Goblet cell number not assessed ^C	(Corren, et al., 2011)
IL-4Ra	Dupilumab (Anti-IL4R mAb)	Significant reduction in asthma exacerbations. Significant increase in FEV1 when added to inhaled corticosteroids and LABAs Clinically significant improvement in ACQ5 score ^C	Goblet cell number not assessed ^C	(Wenzel, et al., 2013)
IL-5Ra	Benralizumab (Anti-CD125 mAb)	Significantly reduced annual asthma exacerbation rate. Improved prebronchodilator FEV ₁ ^C	No current data on GCD ^C	(Bleecker, et al., 2016)
mTOR	Rapamycin	Significantly reduced airway hyperreactivity, inflammatory cell number	Significantly attenuated IL-13 stimulated GCD for	(Mushaben, et al., 2011)

and IgE when administered both treatment
together with HDM regimes^A
sensitization/ challenge.
Significantly reduced airway
hyperreactivity, IgE and T-
cell activation but not
inflammatory cell counts
when administered between
sensitization and challenge
phases^A

Bacterial	Clarithromycin	Significantly reduced IL-13	Significantly	(Nagashima,
50S		stimulated increases in	attenuated IL-13	et al., 2016;
Ribosome		MUC5AC, CLCA1, pSTAT6	stimulated GCD [†]	Tanabe, et
<i>(primary)</i>		and pERK1/2 protein as well		al., 2011)
Various		as <i>Muc5ac</i> , <i>Clca1</i> and <i>Spdef</i>		
immunomo		mRNA in human bronchial		
dulatory		epithelial cells grown in ALI [†]		
targets				
<i>(secondary)</i>				
GCR	Ciclesonide	Significantly prevented and	Significantly	(Leung, et
		reversed OVA-induced	attenuated OVA-	al., 2005)
		airway eosinophilia, T cell	stimulated GCD ^A	
		activation and bronchial		
		hyperresponsiveness ^A		
GCR	Fluticasone	Significantly prevented and	Significantly	(Leung, et
	propionate	reversed OVA-induced	attenuated OVA-	al., 2005)

		airway eosinophilia and T cell activation, but was only effective at reversing bronchial hyperresponsiveness	stimulated GCD ^A	
GCR	Budesonide	Significantly reduced mucosal eosinophils, neutrophils and T lymphocytes ^C	Significantly reduced goblet cell number in asthmatics compared to placebo ^C	(De Kluijver, et al., 2005)
TGF-β_2	Anti-TGF- β_2 mAb	Completely abolished compression based increases in MUC5AC production [†]	Abolished GCD [†]	(Park & Tschumperlin, 2009)
EGFR Kinase	Gefitinib	Significant reduction in airway inflammation, goblet cell and airway smooth muscle cell hyperplasia ^A . Chronic exposure significantly reduced MUC5AC and IL-13 in BALF	Significantly reduced OVA-stimulated GCD ^A	(Song, et al., 2016)
PDE4	Rolipram	Significantly prevented OVA-induced increases in airway resistance, eosinophil, lymphocyte, and neutrophil accumulation in the BALF ^A	Significantly attenuated OVA-stimulated goblet cell hyperplasia ^A	(Kanehiro, et al., 2001)

M₃	Tiotropium	Increased FEV ₁ at 24 months	Goblet cell number not	(Zhou, et
Muscarinic	Bromide	in COPD patients of GOLD	assessed ^C	al., 2017)
receptor		stage 1 or 2. Also		
		ameliorated annual		
		FEV ₁ decline after		
		bronchodilator ^C		
		Prevented allergen-induced	Significantly reduced	(Bos, et al.,
		mucous gland hypertrophy.	MUC5AC labelled	2007)
		Partially reduced the increase	goblet cell numbers in	
		in MUC5AC-positive goblet	OVA-stimulated	
		cell numbers and eosinophil	animals. Non-	
		infiltration ^A	significantly ($p = 0.06$)	
			reduced PAS labelled	
			in OVA-stimulated	
			animals ^A	
				(Kistemaker
		Both attenuated and reversed	Significantly	, et al.,
		IL-13 induced GCD.	attenuated and	2015)
		Restored aberrant FOXA2	reversed IL-13	
		and FOXA3 expression	stimulated GCD [†] .	
		following IL-13 treatment [†]		

^A Animal model; ^C Clinical trial; [†] In vitro model; *mAb variants; RSV respiratory syncytial virus;

LABA long acting β -adrenoceptor agonist; ACQ5 asthma control questionnaire (5-question variant);;

GCR glucocorticoid receptor; BALF bronchoalveolar lavage fluid; PDE4 phosphodiesterase 4.

Anticholinergics, specifically tiotropium bromide, have shown promise in combatting COPD.

Recently, clinical trials assessing tiotropium in patients with GOLD stage 1 or 2 COPD have shown significant improvements in lung function as measured by FEV1 (Zhou, et al., 2017).

Furthermore, anticholinergics have been approved as add-on treatments for difficult-to-treat asthma (GINA guidelines 2017). Although acetylcholine is best known for its role as neurotransmitter regulating bronchoconstriction and secretion of mucus from submucosal glands, more recent findings also support a role for non-neuronal acetylcholine secreted by airway epithelial cells (Kistemaker & Gosens, 2015). GCD induced by IL-13 in human airway epithelial cells in ALI culture can be prevented and reversed by tiotropium treatment, suggesting a direct role for such non-neuronal acetylcholine (Kistemaker, et al., 2015). Animal models confirm this contention and show reduced GCD after treatment with anticholinergics in both COPD and asthma models (Bos, et al., 2007; Kistemaker, et al., 2014; Pera, et al., 2011).

Kaempferol is a common flavonoid that acts as an antioxidant and is known to block endoplasmic reticulum (ER) stress response. Kaempferol reduces eosinophil infiltration and inflammation as well as downstream mucous cell differentiation in OVA sensitized/challenged mice (Gong, et al., 2012; Park, et al., 2015). In addition to inhibiting lipopolysaccharide induced CCL11 expression, kaempferol dose-dependently reduces TNF- α stimulated epithelial expression of ICAM-1 and eosinophil expression of integrin β 2, which attenuates the eosinophil-airway interaction (Gong, et al., 2012). Kaempferol is believed to exert these effects by moderating Janus activated kinase/ signal transducers and activators of transcription (JAK/STAT) signaling, culminating in the disruption of NF- κ B activation (Gong, et al., 2012).

Macrolide antibiotics including clarithromycin and rapamycin have been used on the basis of their immunosuppressive activity, to attenuate the symptoms of chronic airways disease (Essilfie, et al., 2015; Gibson, et al., 2017; Mushaben, et al., 2011; Shinkai, et al., 2008). In addition to reductions in airway hyperreactivity, cytokine secretion and airway inflammation, a decrease in goblet cell number is consistently observed following macrolide antibiotic treatment following allergen exposure (Mushaben, et al., 2011; Nagashima, et al., 2016). Clarithromycin treatment of human bronchial epithelial cells stimulated with IL-13 drastically reduces the levels of SPDEF, goblet cell specific CLCA1, ERK1/2 phosphorylation and overall goblet cell number (Nagashima, et al., 2016; Tanabe, et al., 2011). Likewise, rapamycin has been shown to suppress mucous cell metaplasia in mice

challenged with HDM in an acute asthma model; however no changes in GCD were evident in a chronic/established asthma model (Fredriksson, et al., 2012; Mushaben, et al., 2013; Mushaben, et al., 2011). Similar to clarithromycin, rapamycin induces concomitant reductions in mouse CLCA3 (equivalent to human CLCA1) and SPDEF (Mushaben, et al., 2013). This attenuation is likely due to the inhibition of mTOR (Mushaben, et al., 2013). Indeed, although the widespread and consistent use of antibiotics such as rapamycin and clarithromycin may raise concerns in the treatment of persistent GCD, it is clear that understanding their mechanisms of action may provide us with a novel way forward in tackling this aspect of chronic airways disease.

Another drug that may prove beneficial is the EGFR tyrosine kinase inhibitor, gefitinib (IRESSA). To date, gefitinib has been identified as a potential inhibitor of goblet cell hyperplasia (Song, et al., 2016). Pre-treatment of mice with gefitinib for 7 days alleviated OVA-induced allergic airway symptoms and pathologies including GCD. This alleviation appears to be time dependent as a 3 day intervention with gefitinib at the end of a 7 day OVA challenge failed to exert these effects (Song, et al., 2016). Thus gefitinib may be better suited as a preventative drug to treat excessive GCD associated with allergic asthma. Gefitinib has also been shown to reverse the smoke-induced loss of ciliated cells in human bronchial epithelial cells grown under ALI culture (Valencia-Gattas, et al., 2016). Furthermore, cells subjected to cigarette smoke recover a ciliated phenotype more quickly in the presence of gefitinib. This may offer an effective treatment for defective mucociliary clearance which is associated with the pathogenesis of COPD/ chronic bronchitis (Sethi, 2000).

Preventative amiloride therapy has been shown disrupt GCD in mouse models of CF (Zhou, et al., 2008). Amiloride directly blocks the epithelial sodium channel (ENaC), which improves mucus hydration, and when utilized as a preventative therapy in β ENaC-overexpressing mice, reduces airway inflammation along with IL-13 production, goblet cell metaplasia and mucus hypersecretion (Zhou, et al., 2008). Together with a reduction in morphological characteristics associated with mucus obstruction, preventative amiloride treatment has also been shown to lower mortality and reduce the number of necrotic airway epithelial cells often seen in CF lungs (Zhou, et al., 2008). Pilot studies have shown benefits from aerosolized amiloride treatment within the airways of CF patients slowing

the loss of forced vital capacity (FVC) and reducing mucus viscoelasticity (Knowles, et al., 1990). Nonetheless, follow up clinical trials revealed no significant benefit on forced expiratory volume in 1 second (FEV1) or FVC or sputum volume (Bowler, et al., 1995; Graham, et al., 1993; Pons, et al., 2000). The effect of preventative amiloride treatment on GCD is not well documented in the human airway however and requires further investigation.

4.2 Monoclonal antibody therapy

A number of studies have utilized monoclonal antibody (mAb) treatment to attenuate signaling pathways involved in numerous airways diseases, including asthma and COPD (Arora, et al., 2006; Kondo, et al., 2006; Kumar, et al., 2004; Syk, et al., 2016). Monoclonal antibodies are thought to exhibit a number of advantages over small molecule based therapies; 1) mAbs are considered to exert greater specificity for the target molecule, 2) they do not form toxic metabolites following breakdown, and 3) they generally have longer half-lives (Chames, et al., 2009). The most well recognized of these is the chimeric monoclonal antibody that binds and neutralizes IgE, omalizumab. Omalizumab is the only mAb approved for treatment of early onset severe IgE dependent asthma and works by targeting the high-affinity domain of IgE, preventing its interaction with mast cells (Omalizumab, 2002). Omalizumab also significantly reduces circulating IL-13 and peripheral eosinophils in asthmatics underlying its significant impact on airway remodeling including GCD (Noga, et al., 2003). Other emerging mAbs may also hold promise as effective pharmaceutical agents against persistent GCD and these include lebrikizumab (IL-13), tralokinumab (IL-13), benralizumab (IL-5R α) and dupilumab (IL-4R α) (Hansbro, et al., 2011; Hansbro, et al., 2013; Wenzel, et al., 2013). To date however, in depth clinical analyses on how such mAb therapies may affect GCD are yet to be carried out (Table 1).

4.3 Therapies utilizing miRNA technology

A tantalizing approach to target the expression of specific proteins involved in persistent GCD is the administration of exogenous miRNAs and/or inhibitors of them, termed antagomirs. Antagomirs are small oligonucleotides that are perfectly complementary to a specific miRNA and have been shown to lead to the effective degradation of miRNA *in vivo* (Krutzfeldt, et al., 2005). Indeed a number of antagomir based studies conducted in mice have yielded promising results. For example, inhibition of the miR-126 and the let-7 miRNA family in a mouse model of asthma reduces total number of inflammatory cells, cytokine production as well as levels of Muc5ac (Mattes, et al., 2009; Polikepahad, et al., 2010). Inhibition of miR-106a or miR-221 is shown to reduce inflammation in mouse models of asthma (Qin, et al., 2012; Sharma, et al., 2012). Likewise, deletion of *miR-21* or *miR-155* leads to significantly lower numbers of eosinophils in OVA-challenged mice (Lu, et al., 2011; Malmhall, et al., 2014). There is significant potential in the use of miRNA antagomirs and mimics to ameliorate not only persistent GCD, but other pathophysiological features of chronic airways disease.

One advantage in treating airway-based diseases in humans is the ability to deliver small molecule therapeutics via inhalation. Aerosolization, as well as dry powder inhalation, have been shown to be effective, low-risk methods of administering compounds to treat numerous airways diseases (Doukas, et al., 2009; Guilleminault, et al., 2014; Rubin & Williams, 2014). This is because aerosol-delivery increases compound bioavailability at the airway epithelium and reduces potential adverse effects typically associated with systemic delivery. Unfortunately, in chronic airways disease, the thicker and less hydrated mucus layer is a significant obstacle for aerosol-delivery. A potential solution to this problem is combining bioactive components, such as antagomir molecules, with a mucolytic aerosol performance enhancer, such as D-mannitol, to increase airway epithelial interactions and allow for transport into the cells (Li, et al., 2014; Muralidharan, et al., 2016). In addition to standard aerosol delivery has also been the advent of nanoparticle inhalation technology (Kuzmov & Minko, 2015). Nanoparticulate dry powder inhalation in combination with D-mannitol has been shown to effectively reach smaller airways in an *in vitro* predictive lung deposition model (Muralidharan, et al., 2016).

For persistent GCD in the airway epithelium, antagomir therapy may prove to be an important novel approach to restore the expression of numerous proteins that are downregulated during chronic airways disease; one example being FOXA2 which is negatively regulated by miR-124a. Thioredoxin-interacting protein has been shown to downregulate the expression of miR-124a, and increases expression of the goblet cell regulator FOXA2 (Jing, et al., 2014; Wan, et al., 2004). Antagomir based therapies against miR-124a would further increase effectiveness in elevating FOXA2 expression. Furthermore, the addition of miRNA based therapies to inhaler technology would allow target delivery of antagomirs directly to the airway epithelium.

5. Conclusions

Accumulation of mucus in the airways is a key feature of chronic airways disease. Resolving persistent GCD during chronic airways disease is necessary to abolish the downstream pathogenic effects of intraluminal mucus accumulation. Currently, it is well recognized that dysregulation of several critical signaling pathways can lead to persistent GCD during chronic airways disease. These can include anomalies in EGFR signaling, interleukin production (IL-13, IL-4, IL-5, and IL-9), SPDEF, NOTCH, WNT/ β -Catenin, GABA_AR and KIF3A. A number of miRNAs also control the expression of several proteins within these pathways and have been shown to directly affect GCD. Gene-based approaches targeting the molecules regulating these pathways have been shown to limit GCD in mouse models of chronic airways disease. Recent advancements in laboratory based technologies, including novel cell culture/ co-culture methods, such as bronchospheres and organ-on-chip microfluidics, will allow *ex vivo* studies to more accurately recapitulate *in vivo* events (Danahay, et al., 2015; Esch, et al., 2015).

Therapeutic regulation of the above pathways at the human airway epithelium is the logical next step to combating persistent GCD. Although targeting GCD through inhibition of such master regulators as EGFR requires a cautious approach, one advantage of the airway epithelium is the ability to deliver drugs to the affected cells via aerosol. Monoclonal antibody therapy, such as the use of omalizumab,

has been shown to significantly reduce Th2 cytokines, including IL-13, and is expected to have a dramatic effect on GCD. However, in depth studies of the response of the human airway epithelium to omalizumab are yet to be carried out. Dupilumab treatment, targeting IL-4 and IL-13 signaling also holds promise against persistent GCD during chronic airways disease and is currently undergoing clinical trials. With the pathways regulating persistent GCD becoming increasingly well understood and advancements in therapeutic design, *in vivo* mimicking technologies and drug delivery we are making clear strides toward resolving mucus associated pathogenesis during chronic airways disease.

6. References

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Figure 1: Normal vs diseased airway epithelium. In the normal airway epithelium (A) basal cells undergo regulated differentiation to form two major cell types, ciliated cells or secretory (Club and Goblet) cells. Goblet cells and submucosal glands are responsible for regulated mucus secretion into the airway which is normally present as a mucous bilayer. Club cells act to protect the airways through detoxification of harmful compounds and secretion of glycoproteins and lipids which physically protect surfactant and small airways. Mucociliary clearance which is dependent on ciliary function actively moves mucus to remove foreign particles. In chronic airways disease (B) persistent goblet cell differentiation (GCD) results in goblet cell hyperplasia. In addition to GCD, is the enlargement or hypertrophy of submucosal glands. These two events dramatically upregulate the production and secretion of mucin glycoproteins and result in a highly viscous and thickened mucus layer that is difficult to clear. This persistent GCD is directly linked to increased mucus accumulation in airways. MCVs: Mucin containing vesicles.

Figure 2: (A) Representative micrograph of a non-asthmatic human airway section. (B) Comparative section from an individual with fatal asthma, showing excessive intraluminal mucus accumulation (*M*), goblet cell hyperplasia (*arrowhead*) and basal lamina thickening (*arrow*). Tissue sections were obtained with consent from the UBC James Hogg Research Centre. Scale: 50µm

Figure 3: Key signaling events controlling the induction of goblet cell differentiation (GCD) at the airway epithelium. Critical regulators are highlighted (bold) that are known to be dysregulated in chronic airways disease. Inhibitors of selected signaling pathways are shown in red. *Unpublished data from our group suggests Notch based regulation of SPDEF.

Table 1: Effectiveness and limitations of selected therapeutic compounds in alleviating goblet cell hyperplasia at the airway epithelium.

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Conflict of Interest statement: The Authors declare that there are no conflicts of interest.

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Target	Effector molecule (s)	Major treatment outcomes	Effect of treatment on GCD	Reference
IgE	Anti-IgE mAb	Significantly reduced; OVA/HDM-stimulated airway hyper-responsiveness, neutrophil/eosinophil infiltration, IgE and IgG1 production ^A	Significantly attenuated RSV and OVA/HDM-stimulated GCD during aerosol sensitization but not systemic sensitization ^A	(Mizutani, et al., 2015; Tumas, et al., 2001)
IgE	Omalizumab (Anti-IgE mAb*)	Significantly reduced asthma exacerbations ^C	Goblet cell number not assessed ^C	(Busse, et al., 2001)
IL-13	Anti-IL13 mAb	Significantly reduced; transpulmonary resistance, airway hyperresponsiveness, eosinophil infiltration, proinflammatory cytokine production, serum IgE and airway remodeling during OVA-stimulation ^A Prevents subsequent allergic airways disease when exposed to Chlamydia infection during early life ^A	Significantly attenuated GCD ^A	(Starkey, et al., 2013; Yang, et al., 2004)
IL-13	Tralokinumab (Anti-IL13 mAb*)	Significantly reduced IL-13 induced airway hyperresponsiveness, airway	No significant effect on IL-13 induced GCD ^A	(Blanchard, et al., 2005)

		eosinophilia and oesophageal eosinophilia ^A Potentially associated with increased lung function, increased peripheral blood eosinophils ^C	Goblet cell number not assessed ^C	(Piper, et al., 2013)
IL-13	Lebrikizumab (Anti-IL13 mAb*)	Significant increase in FEV ₁ in individuals with poorly controlled asthma ^C	Goblet cell number not assessed ^C	(Corren, et al., 2011)
IL-4Rα	Dupilumab (Anti-IL4R mAb)	Significant reduction in asthma exacerbations. Significant increase in FEV ₁ when added to inhaled corticosteroids and LABAs Clinically significant improvement in ACQ5 score ^C	Goblet cell number not assessed ^C	(Wenzel, et al., 2013)
IL-5Rα	Benralizumab (Anti-CD125 mAb)	Significantly reduced annual asthma exacerbation rate. Improved prebronchodilator FEV ₁ ^C	No current data on GCD ^C	(Bleecker, et al., 2016)
mTOR	Rapamycin	Significantly reduced airway hyperreactivity, inflammatory cell number and IgE when administered together with HDM	Significantly attenuated IL-13 stimulated GCD for both treatment	(Mushaben, et al., 2011)

		sensitization/ challenge. Significantly reduced airway hyperreactivity, IgE and T-cell activation but not inflammatory cell counts when administered between sensitization and challenge phases ^A	regimes ^A	
Bacterial 50S Ribosome <i>(primary)</i> Various immunomodulatory targets <i>(secondary)</i>	Clarithromycin	Significantly reduced IL-13 stimulated increases in MUC5AC, CLCA1, pSTAT6 and pERK1/2 protein as well as <i>Muc5ac</i> , <i>Clca1</i> and <i>Spdef</i> mRNA in human bronchial epithelial cells grown in ALI [†]	Significantly attenuated IL-13 stimulated GCD [†]	(Nagashima, et al., 2016; Tanabe, et al., 2011)
GCR	Ciclesonide	Significantly prevented and reversed OVA-induced airway eosinophilia, T cell activation and bronchial hyperresponsiveness ^A	Significantly attenuated OVA-stimulated GCD ^A	(Leung, et al., 2005)
GCR	Fluticasone propionate	Significantly prevented and reversed OVA-induced airway eosinophilia and T cell activation, but was only	Significantly attenuated OVA-stimulated GCD ^A	(Leung, et al., 2005)

		effective at reversing bronchial hyperresponsiveness		
GCR	Budesonide	Significantly reduced mucosal eosinophils, neutrophils and T lymphocytes ^C	Significantly reduced goblet cell number in asthmatics compared to placebo ^C	(De Kluijver, et al., 2005)
TGF-β_2	Anti-TGF- β_2 mAb	Completely abolished compression based increases in MUC5AC production [†]	Abolished GCD [†]	(Park & Tschumperli n, 2009)
EGFR Kinase	Gefitinib	Significant reduction in airway inflammation, goblet cell and airway smooth muscle cell hyperplasia ^A . Chronic exposure significantly reduced MUC5AC and IL-13 in BALF	Significantly reduced OVA-stimulated GCD ^A	(Song, et al., 2016)
PDE4	Rolipram	Significantly prevented OVA-induced increases in airway resistance, eosinophil, lymphocyte, and neutrophil accumulation in the BALF ^A	Significantly attenuated OVA- stimulated goblet cell hyperplasia ^A	(Kanehiro, et al., 2001)
M₃ Muscarinic receptor	Tiotropium Bromide	Increased FEV ₁ at 24 months in COPD patients of GOLD stage 1 or 2. Also	Goblet cell number not assessed ^C	(Zhou, et al., 2017)

[illegible]

Table 1

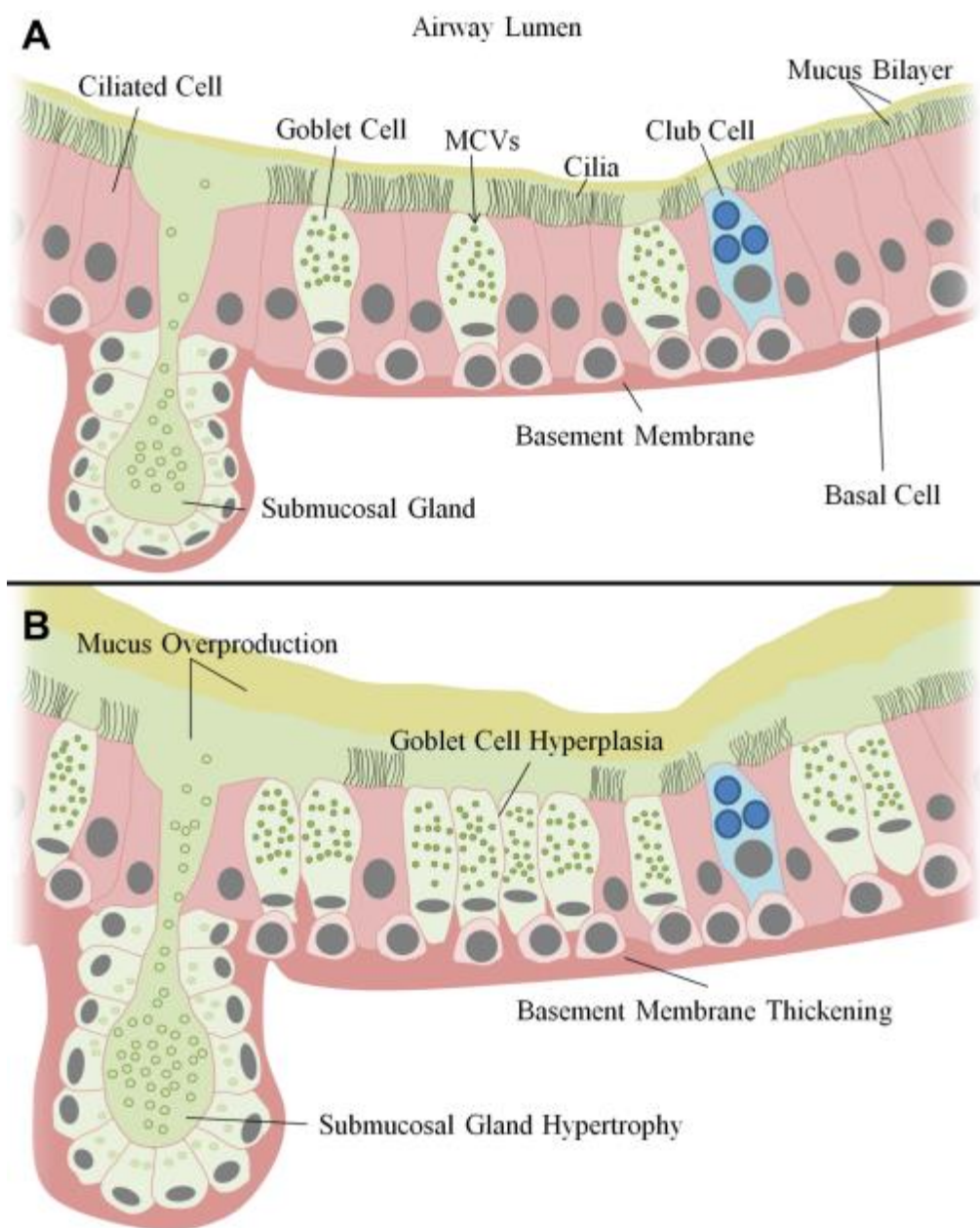


Figure 1

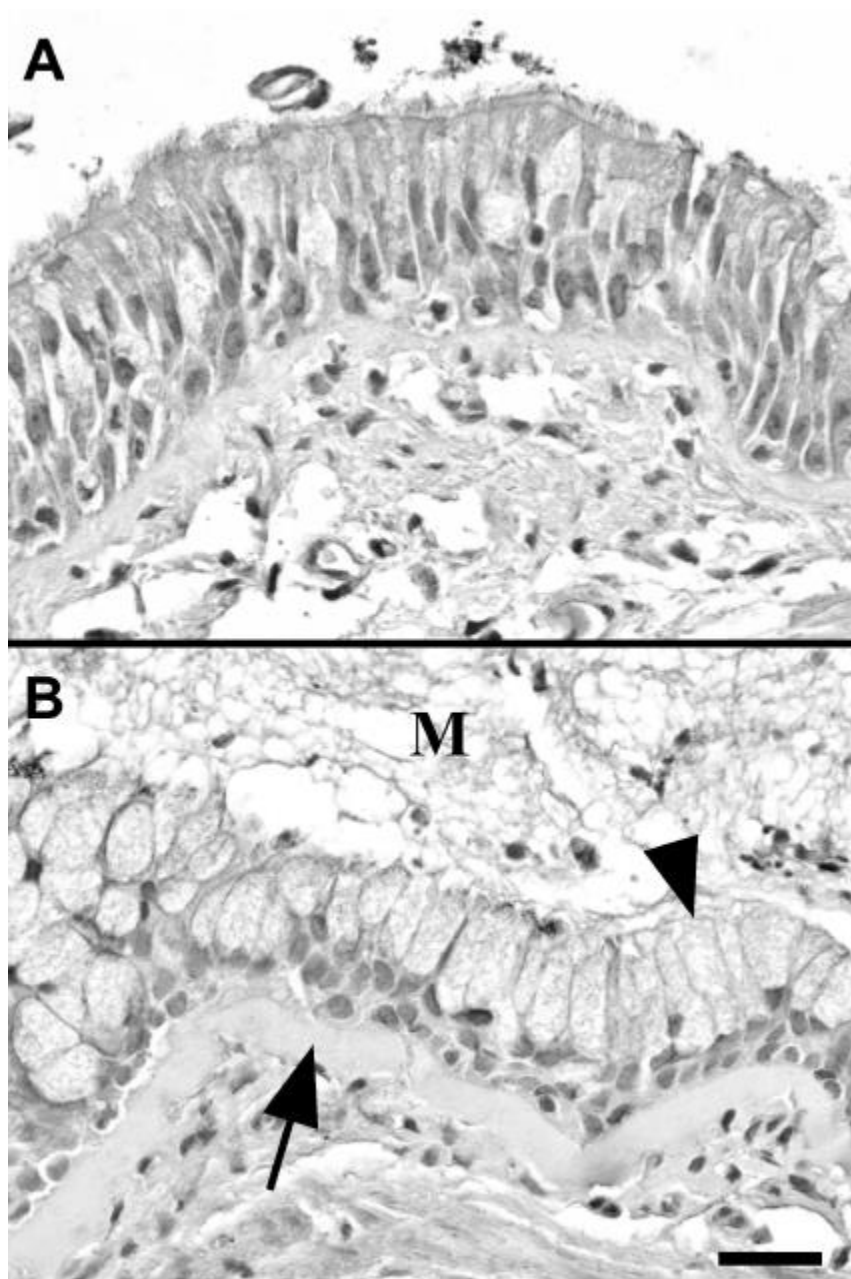


Figure 2

