Short-term Inhibition of NOTCH3 Signaling Ablates MUC5AC Production in Human Airway Epithelium

Andrew T Reid[1,2], Kristy S Nichol[2,3], Punnam Chander-Veerati[2,3], Fatemeh Moheimani[1,2], Anthony Kicic[4,5,6,7,8], Steven M Stick[4,5,6,7], Nathan W Bartlett[1,2], Chris L Grainge[2,3,9], Peter A B Wark[2,3,9], Philip M Hansbro[1,2], Darryl A Knight[1,2,10]

1. School of Biomedical Sciences and Pharmacy, University of Newcastle, Callaghan, NSW, Australia.

2. Priority Research Centre for Healthy Lungs, Hunter Medical Research Institute, The University of Newcastle, New South Wales, Australia.

3. School of Medicine and Public Health, University of Newcastle, Callaghan, NSW

4. School of Paediatrics and Child Health, University of Western Australia, Nedlands, 6009, Western Australia, Australia.

5. Telethon Kids Institute, University of Western Australia, Nedlands, 6009, Western Australia, Australia.

6. Department of Respiratory Medicine, Princess Margaret Hospital for Children, Perth, 6001, Western Australia, Australia.

7. Centre for Cell Therapy and Regenerative Medicine, School of Medicine and Pharmacology, University of Western Australia, Nedlands, 6009, Western Australia, Australia.

8. Occupation and Environment, School of Public Health, Curtin University, Bentley, 6102, Western Australia, Australia.

9. Department of Respiratory and Sleep Medicine, John Hunter Hospital, Newcastle, New South Wales, Australia.

10. Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia, Vancouver, Canada.

Funding support by the Australian National Health and Medical Research Council (NHMRC) grant APP1064405 (D.K., P.H., S.S., and A.K.). Travel funding was provided by the Thoracic Society of Australia and New Zealand (TSANZ) and the Priority Research Centre for Healthy Lungs, University of Newcastle (A.R.).

Author Contributions: A.R. designed and performed experiments, analyzed results, and wrote the manuscript. K.N. and P.V. helped with methodology, performed experiments and analyzed results. F.M., A.K., S.S. and N.B. helped design experiments and reviewed the manuscript. P.W. provided primary human bronchial epithelial cells and reviewed the manuscript. C.G. and P.H. helped conceptualize design of the project, helped interpret data and reviewed the manuscript. D.K. helped conceptualize project design, supervised the project, interpreted results and reviewed the manuscript.

Correspondence to: Andrew Reid, Hunter Medical Research Institute, New Lambton Heights 2305, Australia. Tel.: +61 2 40420108 E-mail: Andrew.reid@newcastle.edu.au

Short Running Head: Notch3 regulates MUC5AC production in airway

Word Count: 3,467 words

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Abstract

Rationale: In asthma, goblet cell numbers are increased within the airway epithelium, perpetuating the production of more viscous mucus that is more difficult to clear and results in airway mucus plugging. Previous studies have identified that either Notch1, 2 or 3, or a combination regulates goblet cell differentiation within airway epithelium. How the expression of specific Notch isoform differs in adult asthmatic epithelium and how Notch may influence mucin production in these cells is currently unknown.

Objectives: To quantify different Notch isoforms in the airway epithelium of severe asthmatics and to examine the impact of NOTCH signaling on mucin MUC5AC production within differentiated human primary bronchial epithelial cells from asthmatics and non-asthmatics.

Methods: Primary bronchial epithelial cells were differentiated at air-liquid interface for 28 days. Notch isoform expression was analyzed by real-time qPCR. Immunohistochemistry was used to localize and quantify Notch isoforms in human airway sections. Notch signaling was inhibited *in vitro* using dibenzazepine or isoform-specific small interfering RNA, followed by analysis of MUC5AC.

Measurements and Main Results: NOTCH3 expression was increased in asthmatic airway epithelium compared to non-asthmatic. Dibenzazepine treatment of differentiated primary airway epithelial cells from asthmatics and non-asthmatics significantly reduced MUC5AC concomitant with a suppression in NOTCH3 intracellular domain protein. Notch3 specific knockdown recapitulated the dibenzazepine-induced reduction in MUC5A, which was independent of goblet cell numbers.

Conclusions: We demonstrate that Notch3 is a critical regulator of MUC5AC production. Increased Notch3 signaling in the asthmatic airway epithelium may therefore be an underlying driver of pathological MUC5AC production.

Word Count: 249 words

Keywords: Notch Signaling, Airway Epithelium, Mucus, MUC5AC, Pharmacological Inhibition.

At a Glance Commentary

Scientific Knowledge on the Subject:

Asphyxiation due to airway mucus plugging is the principal cause of death in asthma. Current therapeutic strategies to reduce mucus accumulation are insufficient and fail to address underlying mucin overproduction. The mechanisms that drive excess mucin production in asthma are incompletely understood.

What This Study Adds to the Field:

Notch3, a transmembrane receptor protein is expressed at high levels in the airway epithelium in asthma. MUC5AC production is significantly reduced following Notch3 inhibition in airway epithelial cells differentiated at air-liquid interface, both from asthmatic and non-asthmatic donors. Targeting epithelial Notch3 may be a viable strategy to ameliorate mucin overproduction in asthma and other chronic airways diseases.

Mucus overproduction is one of many irregularities typically associated with inflammatory airway diseases such as asthma, chronic obstructive pulmonary disease (COPD) and bronchiectasis. When coupled with increases in viscosity, it is linked to the formation of mucus plugs and is associated with morbidity driven by airflow obstruction (1, 2). Two major gel-forming mucins exist in the airway mucus layer, MUC5AC and MUC5B. In the healthy airway epithelium MUC5AC is the predominant mucin expressed by goblet cells whilst MUC5B is chiefly secreted by mucous cells within submucosal glands (3, 4). Furthermore, production of MUC5AC regularly increases in patients with asthma (5), whereas MUC5B production varies. Levels of MUC5AC have been shown to directly correlate with mucus viscoelasticity and airway mucus accumulation (6).

The NOTCH family of proteins, Notch1-4, are single-pass membrane receptor proteins responsible for regulating cell proliferation, differentiation (including cell fate determination) and cell death (7). Numerous studies have demonstrated that NOTCH signaling controls goblet cell differentiation within the airway epithelium both in humans and mice (8-11). Notch signaling is activated via ligand-induced cleavage, facilitated by gamma-secretase, and leads to the nuclear translocation of the Notch intracellular domain (NICD) (12) and subsequent gene expression. Signaling through the Notch1, 2 and 3 isoforms has been implicated in the differentiation of goblet cells and downstream mucus production in human airway epithelial cells cultured under air-liquid interface (ALI) conditions (10, 13). Indeed, pharmacological inhibition of Notch signaling prevents goblet cell differentiation and associated MUC5AC production (10, 13). To date very few studies have focused on the role of Notch isoform expression and signaling in an already differentiated airway epithelium and the impact, if any, of asthma on these parameters.

We hypothesized that Notch signaling is dysregulated in differentiated airway epithelial cells from asthmatics and this would influence airway MUC5AC production. To investigate this we used ALI cultures of primary bronchial epithelial cells (pBECs) from asthmatics and non-asthmatics (14). We show that the airway epithelium of asthmatics exhibits dysregulated Notch isoform expression, with increased Notch3 and decreased Notch1 expression. Furthermore, acute inhibition of the Notch3 intracellular domain (NICD3) results in dramatically reduced mucin production in ALI from asthmatics and non-asthmatics. We suggest that Notch3 is the key regulator of mucin production within the airway epithelium and is expression and activity is increased in asthmatics. Notch3 should be considered as a viable pharmacological target in the treatment of mucus hypersecretion during chronic airways disease.

Methods

Study approvals

All experiments were conducted in accordance with Hunter New England Area Health Service Ethics Committee and University of Newcastle Safety Committee (85/2012) approvals.

pBEC collection

Primary bronchial epithelial cells (pBECs) were obtained from individuals clinically assessed as asthmatic or non-asthmatic and all of whom were non-smokers (Table 1). Asthmatics had a documented history of bronchial hyperresponsiveness and were defined as severe asthmatic according to GINA guidelines. Asthmatics and non-asthmatics underwent fiber-optic bronchoscopy as previously described (15). Cells were cultured and passaged as previously described (16). Minimally-immortalized bronchial epithelial cells (BCi-Ns1.1) were kindly donated by Prof. RG Crystal (17).

pBEC cell culture and pharmacological inhibition

All cells were maintained in bronchial epithelial cell growth medium (BEGM; Lonza, Basel, Switzerland) as previously described (16) Cells were maintained at ALI for 25 days prior to pharmacological treatment with dibenzazepine (DBZ) (YO-01027; Selleck Chemicals, TX, USA) (online supplement). Cells were harvested for RNA, protein or fixed in 10% Formalin (Lomb Scientific, NSW, Australia) for histology.

BCI-Ns1.1 cell culture and siRNA transfection

BCI-Ns1.1 cells were maintained in BEGM containing penicillin-streptomycin (100U/mL-100µg/mL final) and Amphotericin (2.5µg/mL final) until confluent. Cells were transfected as per manufacturer's instructions using predesigned S*ilencer select* Notch3 siRNA (s9640; Thermo Fisher Scientific, MA, USA), Allstars negative control siRNA (SI03650318; Qiagen, Hilden, Germany), or media control (online supplement) in the presence of 4 or 25ng/mL rhEGF for 96 hours.

Human lung tissue immunohistochemistry and colorimetry

Human lung sections were obtained from 6 asthmatics and 6 non-asthmatics that died as a result of asthma or non-respiratory causes respectively. Sections were obtained from the UBC James Hogg Research Centre (University of British Columbia, BC, Canada). Sections were subjected to immunohistochemical staining (IHC) (online supplement). A minimum of 5 images were taken of small and medium sized airways per section (between 1 and 5mm inner diameter). Epithelial regions were subjected to colour deconvolution as previously described (18) (online supplement).

Air-liquid interface protein extraction and Immunoblotting

Following endpoints for each ALI culture, protein was extracted in ice-cold lysis buffer and subjected to immunoblotting as previously described (19). Membranes were blocked and incubated with primary antibodies; anti-NOTCH3 (0.2μg/mL), anti-NOTCH1 (0.2μg/mL), anti-CLCA1 (MABS444; Merck Millipore) (0.5μg/mL) or anti-βactin (ab8227; Abcam) (0.05μg/mL) (online supplement).

IHC, immunofluorescence and PAS/alcian blue staining in ALI cultures

Following fixation, ALI membranes were stained with periodic acid Schiff (PAS) and Alcian blue or IHC using anti-MUC5AC (2μg/mL; ab3649). For immunofluorescence (IF), ALI sections were labelled with anti-MUC5AC and counterstained with DAPI (online supplement).

Real-time quantitative RT-qPCR

Total RNA was extracted from cells using the RNeasy kit (Qiagen, Hilden, Germany) and cDNA prepared as previously described (20). Quantitative PCR (qPCR) targeting *NOTCH1* (Hs01062014_m1), *NOTCH2* (Hs01050702_m1), *NOTCH3* (Hs01128537_m1), *NOTCH4* (Hs00965889_m1), *SPDEF* (Hs00171942_m1) and *MUC5AC* (Hs00873651_m1) was normalized to 18S ribosomal RNA (*18S rRNA*) (4318839; Thermo Fisher Scientific) as previously described (21). All qPCR results are presented as mean $\log_2$ fold change relative to control with statistics performed on ΔΔCt from independent biological replicates (22) (online supplement).

MUC5AC ELISA

As purified standards of MUC5AC are commercially unavailable, MUC5AC-rich protein was obtained from cultures of Calu-3 human lung adenocarcinoma epithelial cells grown to ALI (23). Serial dilutions were made of Calu-3 apical surface fluid and linear MUC5AC standard curve was confirmed (data not shown). Mucin-containing ALI apical surface fluid was incubated with 500µL warm PBS and lightly agitated before aspiration. Aspirates and standards were agitated at 1000rpm at 42°C to homogenize consistency, coated into multi-well plates (Thermo Fisher Scientific) and dried overnight in 37°C oven (Benchmark Scientific, NJ, USA). After washing, plates were blocked (5% w/v BSA in PBS) and incubated with biotinylated mouse monoclonal anti-MUC5AC (ab79082; Abcam) (0.2µg/mL) for 1 hour. Plates were incubated with Streptavidin-HRP conjugate (Thermo-Fisher Scientific) and developed using 3,3',5,5'-tetramethylbenzidine (TMB; Thermo-Fisher Scientific) (online supplement).

Immunofluorescence and In-cell ELISA

Following fixation, BCi-NS1.1 cells were blocked and permeabilized in 3% w/v BSA containing 0.5% v/v Triton X-100 (Sigma-Aldrich) in PBS. For immunofluorescence, cells were washed and incubated overnight with anti-MUC5AC (2µg/mL) followed by Alexa-fluor 594-conjugated donkey anti-mouse secondary antibody. Cells were counterstained with Hoechst 33342 (Sigma-Aldrich) (0.6µg/mL) and visualized immediately on Axio-Imager M2. For in-cell ELISA, blocked cells were incubated with biotinylated anti-MUC5AC, streptavidin and TMB as above. Experiment was repeated in 3 separate passages of BCi-NS1.1 cells. Absorbance was measured across 200 points per well and mean absorbance was calculated using 6 technical replicates for each treatment. Mean values were normalized to media control (4ng/mL rhEGF).

Statistics

Students *t* test (two-tailed unpaired) and non-parametric Mann-Whitney U test (two-tailed, unpaired) (JMP, SAS Institute, Cary, NC) was used to determine statistical significance between two groups. One-way analysis of variance (ANOVA) using ranks or Kruskal-Wallis test was used for all non-parametric data sets containing more than 2 groups. Statistical significance was determined when $P < 0.05$.

Results:

NOTCH3 Expression is Increased in Differentiated pBECs from Asthmatics

pBEC cultures, from 5 severe asthmatic and 5 non-asthmatic donors (Table 1 for demographics), were differentiated at ALI for 29 days. Cultures all consisted of basal cells, ciliated cells, and mucus secreting goblet cells (Figure 1A). Both non-asthmatic and asthmatic pBECs expressed baseline levels of *NOTCH1-3* but not *NOTCH4* mRNA (Figure 1B). Expression of *NOTCH3* was significantly greater (2.5-3.5 fold) in both cohorts compared to *NOTCH1 and 2*. Furthermore, *NOTCH3* was significantly elevated in ALI cultures from asthmatics ($p < 0.05$) compared to non-asthmatics. In contrast, *NOTCH1* and *NOTCH2* expression levels were similar between cohorts.

NOTCH3 Protein Levels are Increased and NOTCH1 Levels Decreased in Asthmatic Airway Epithelium

Based on *NOTCH3* expression profile and the role of Notch1 during cytokine independent differentiation (10), Notch1 and 3 were evaluated further.

Expression of both NOTCH1 and 3 proteins was analyzed in human airways using IHC and color segmentation (Figure 2A). NOTCH3 was highly expressed in the airway epithelium of asthmatics, exhibiting diffuse localization throughout the cells. In agreement with mRNA data,

color segmentation analysis demonstrated a significant increase ($P < 0.001$) in the level of NOTCH3 protein in the epithelium of asthmatic airways (Figure 2B). Interestingly, NOTCH1 was expressed at a much lower level ($P < 0.001$) in the epithelium of asthmatic airways compared to non-asthmatic. Furthermore, examination of sequential sections confirmed that the same cells that exhibited high NOTCH3 expression exhibited low NOTCH1 expression. Finally in order to determine whether differences in NOTCH3 and NOTCH1 protein were mimicked within ALI pBECs we conducted immunoblotting on protein extracted from ALI cultures and observed the same expression pattern (Figure 2C).

DBZ treatment Potently Reduces NOTCH3 mRNA and Protein Levels

In order to evaluate the functional significance of Notch in differentiated pBECs we next inhibited Notch signaling in ALI cultures using the gamma-secretase inhibitor DBZ. Cells were treated with 0.1, 1 or 10μM DBZ (basal media) for 96 hours after which mRNA and protein were extracted. *NOTCH1-3* mRNAs were quantified using Taqman qPCR (Figure 3A). Curiously, in ALI's from non-asthmatics, *NOTCH1* and *2* showed the same pattern of expression in which the lowest concentration of DBZ (0.1μM) reduced each isoform to approximately half its baseline value ($P < 0.05$) and higher concentrations of DBZ failed to reduce these mRNAs any further. In contrast, expression of *NOTCH3* in ALI cultures from both asthmatics and non-asthmatics was reduced by ~85% following treatment with all concentrations of DBZ ($P < 0.001$). Immunoblot analysis also revealed a dramatic reduction in NICD3 in both non-asthmatic and asthmatic cells (Figure 3B). In contrast, NICD1 protein levels did not differ from baseline for any concentration of DBZ in either cohort.

DBZ treatment Potently Reduces Mucin Content in Differentiated pBECs

Notch3 was the only isoform significantly reduced following 10μM DBZ for 96 hours. Thus, we performed histochemical staining of ALI cultures exposed to this concentration of DBZ to identify potential Notch3-driven changes in mucin production. PAS/alcian blue stains were used to label neutral/acidic mucin glycoproteins respectively (Figure 4A) (24). Unstimulated cells from both non-asthmatics and asthmatics showed typical PAS/Alcian blue overlap, appearing as a heavy dark blue/magenta stain primarily associated with goblet cell-derived mucus. It was these PAS/Alcian blue overlapping regions that were most heavily reduced in both asthmatic and non-asthmatic cohorts following treatment with DBZ. This was reinforced by subsequent IHC data targeting MUC5AC (Figure 4B). Control ALI cultures showed distinct labelling of MUC5AC within goblet cells in both non-asthmatics and asthmatics. Following 96 hour treatment with DBZ, MUC5AC staining was completely abolished in all replicates. Gene expression of the key secretory cell regulator *SPDEF* was significantly reduced within cells from the same cultures following DBZ treatment (Figure 4C).

Inhibition of Notch Signaling Significantly Reduces MUC5AC Expression and Secretion

We next aimed to determine whether the changes in NICD3 influence the expression of *MUC5AC*. Exposure to DBZ for 96 hours, dramatically reduced *MUC5AC* mRNA ($P < 0.01$) in cells from non-asthmatics and asthmatics (Figure 5A). In order to determine whether the reduction in MUC5AC protein translated to reduced mucin secretion we next performed ELISA targeting MUC5AC obtained from apical surface washes taken from ALI cultures treated with DBZ for 24h, 48h, 72h, and 96h. At the earlier time points up to 72h there was no significant reduction in MUC5AC for any concentration of DBZ (Supplementary Figure E1). However, at 96h there was a dramatic reduction in soluble MUC5AC observed in ALI cultures from both asthmatics and non-asthmatics (Figure 5B).

Differentiated pBECs from asthmatics produced a thicker mucus layer compared to cells from non-asthmatics. We washed, fixed, embedded and sectioned cells from each cohort and performed immunofluorescent localization targeting MUC5AC to observe differences in secreted mucin architecture. MUC5AC glycoprotein adopted a filamentous appearance (Figure 5C; arrow) above the apical surface (Figure 5C; dashed line) of pBECs from asthmatics. This mucin was also tethered to the apical surface at numerous points, likely areas of cellular secretion. This was in contrast to minimal MUC5AC above the apical surface of pBECs from non-asthmatics. To identify whether Notch signaling inhibition resulted in dysregulation of goblet cell number we performed immunoblots targeting the goblet cell marker CLCA1. Somewhat surprisingly, we observed no effect of DBZ on CLCA1 expression in ALI cultures from either cohort (Figure 5D).

NOTCH3 siRNA treatment significantly reduces MUC5AC production

Finally, in order to confirm the results with our pharmacological agent and to specifically target Notch3 we used siRNA inhibition. To do this we modified a previously established model (25) in which MUC5AC production could be stimulated and analyzed within a monolayer cultures. Notch3 knockdown was confirmed by RT-qPCR and immunoblot. *NOTCH1* and *NOTCH2* mRNA was unchanged following Notch3-siRNA treatment whereas as *NOTCH3* expression was significantly reduced by ~65% (Figure 6A). Full-length and NICD protein levels were also significantly reduced following transfection (Figure 6B). Non-transfected and scrambled-siRNA transfected cells showed upregulated MUC5AC production when treated with 25µg/mL EGF for 96 hours (Figure 6C and 6D) and this effect was significantly reduced by Notch3 knockdown.

Discussion

Goblet cell differentiation and mucus production are dysregulated in severe asthma (1, 5, 6). The Notch signaling pathway is a key driver of goblet vs ciliated cell differentiation at the epithelium during development as well as in response to injury (8-10, 26). Here we have demonstrated that Notch signaling is dysregulated in differentiated airway epithelial cells from donors with asthma and that this has a direct impact on airway mucin release and MUC5AC production. Our study has shown that Notch3 is overexpressed in the airway epithelium of severe asthmatics. Furthermore, Notch1 was significantly reduced compared to expression in airway epithelium of non-asthmatics. Treatment of ALI cultures with DBZ for 96 hours preferentially prevented cleavage of NICD3 over NICD1 and resulted in the dramatic loss of the gel-forming mucin MUC5AC. Additionally, transfection of pBECs with Notch3-specific siRNA significantly attenuated MUC5AC production. Thus we have shown that Notch3 is a critical regulator of MUC5AC and highlights the importance of Notch3 in directing the airway epithelium toward a mucus producing phenotype.

Various Notch isoforms have been investigated in relation to chronic airway diseases and mucus dysregulation with the majority of these studies focusing on Notch1 (8, 27-29) and Notch2 (8, 13, 30). We have shown that Notch3 is significantly increased in asthmatic pBECs, both grown under ALI and in human lung sections. Previously, Notch3 has been reported to control the proliferation of airway basal cells through Jagged-ligand activation (31). During the establishment of a differentiated epithelium, Notch3 is believed to prime a pool of basal cells that can then undergo differentiation into secretory or cilliated phenotypes through downstream Notch1/Notch2-based signaling (31). In this study, we have shown that Notch3 also regulates MUC5AC production and secretion within secretory cells of the fully differentiated airway

epithelium. Furthermore, this regulation was consistently observed in cells from asthmatics and non-asthmatics. Given that 96 hour exposure to DBZ does not change the levels of goblet cell marker CLCA1, out data indicates that Notch3 is exerting its effect directly on MUC5AC production within goblet cells themselves. This finding also suggests that once fully differentiated (at least in ALI culture), goblet cells numbers do not change.

Notch3 has a shorter EGF-like domain and an incomplete transactivation domain compared to Notch1 and Notch2 (32). These domains regulate NICD activation and transcriptional activity explaining the weak transcriptional-activation potential described for NICD3. Indeed, compared to NICD1, NICD3 is a poor activator of the canonical Notch target HES1 which controls proliferation of stem cells (33, 34). We have identified within human lung sections that NOTCH3 protein appears enriched in regions of the epithelium when NOTCH1 is low, and vice versa. Whether competition between NICD1 and NICD3 exists that activates or represses target genes in the airway epithelium needs further investigation. Interestingly, Ou-Yang et al, (2013) revealed that overexpression of NICD1 leads to a dramatic reduction in MUC5AC production and is due to HES1 repression at the *MUC5AC* promoter in intestinal epithelial cells (35). In contrast, we have shown Notch3 signaling is associated with increased MUC5AC expression, however this is likely not due to *HES1* de-repression as *HES1* mRNA is reduced following DBZ (Supplementary Figure E2).

DBZ is a potent gamma-secretase inhibitor and therefore should inhibit the cleavage of all Notch isoforms equivalently. Nonetheless, we consistently observed that the expression of the NICD3 domain was significantly reduced following DBZ treatment compared to NICD1. This observation is likely due to non-canonical ligand-independent Notch activation previously described for the Notch1 isoform in human cells (36). This process involves ADAM

metallopeptidase domain 17 (ADAM17)-stimulated cleavage which is hypothesized to preferentially release NICD1 from endosomal compartments independent of gamma-secretase activity (36, 37). Alternatively, as the NICD3 domain has a decreased half-life compared to NICD1 in airway epithelial cells it may not persist following DBZ treatment (38). Indeed, of all Notch isoforms only Notch3 has been shown to directly interact with E3-ubiquitin ligase in HEK293T cells, resulting in an increase in lysosomal degradation of the protein that can explain its reduced persistence (38). To date however, there have been no investigations of the stability of NICDs within airway epithelium or how stability may change according to disease status.

Tsao et al (2009), reported that inhibition of the Notch signaling pathway using *Rbpjk* and *Pofut* deficient mice as well as the gamma secretase inhibitor DAPT, resulted in the significant reduction of Scgb3a2 (secretory cell marker) in lungs during early development (11). Subsequent studies by this author (Tsao et al 2011), using post-natal mice showed that conditional inactivation of Notch signaling resulted in goblet cell metaplasia and mucin overproduction; thus highlighting that Notch-based control of secretory cell differentiation depends on differentiation status of the epithelium (29). Surprisingly, in this study we show that the inhibition of Notch signaling in differentiated human airway epithelial cells leads to a dramatic reduction of MUC5AC as well as *SPDEF* mRNA. It is possible that these conflicting results are brought about by species specific differences in lung/epithelial morphology between human and mouse airways especially during the manipulation of key developmental pathways, or differences in the differentiation potential of cells in different studies of different age groups. Certain studies have shown that blocking Notch signaling in human airway epithelial cells leads to reduced numbers of secretory cells (8, 10).

The increased secretory status of the asthmatic epithelium is most often linked to increases in inflammatory cytokines which can trigger remodeling of epithelial cells. Interestingly however, whether asthmatic epithelial cells respond in a different manner compared to non-asthmatic epithelial cells under conditions in which inflammatory cytokines are not present remains unclear. Here we have shown that pBECs from severe asthmatics exhibit differential expression of Notch3 compared to non-asthmatics when cultured under identical conditions. Furthermore, MUC5AC was found to adopt a filamentous appearance above the apical cell surface and this tethering of mucin is analogous to what has been reported in cases of fatal asthma *in vivo* (39). In the current study it was necessary to treat the differentiated cells basally so as to maintain cells at ALI for the full 96 hours. It is unclear how treatment of the basal surface has resulted in such a dramatic reduction of MUC5AC within cells at the apical surface, however there are likely two major factors. Firstly, DBZ is a small molecule inhibitor that can readily cross the cell plasma membrane and may diffuse readily across the small distance and into these apical cells. Secondly, the basolateral surfaces of many differentiated cells remain proximal to trans-well membrane pores and therefore would be permissive to DBZ diffusion.

Finally, we showed that siRNA-mediated knockdown of Notch3 results in significantly reduced MUC5AC production. Perrais et al (2002), reported that when NCI-H292 lung cancer cells were stimulated with 25µg/mL EGF, MUC5AC apomucin becomes detectable by immunolocalisation (25). We adopted this technique using minimally immortalized epithelial cells and combined it with immunofluorescent localization as well as in-cell ELISA. This definitively showed that the specific reduction of Notch3 prevents the stimulatory effect of EGF on MUC5AC production. Notch1 has been shown previously to play a role during EGFR signaling within NCI-H292 cells

(9), suggesting that MUC5AC production may be under the control of more than one Notch isoform, and potentially involves fine alterations in the signaling pathways involved.

Future investigations using monoclonal antibodies against individual Notch isoforms will allow for the specific targeting and inhibition/activation of distinct Notch receptors in differentiated epithelial cell culture systems (40). This study highlights Notch-based regulation of EGF stimulated epithelial mucin production and reveals a potentially detrimental role for excess Notch3 in asthmatic airway epithelium. Therefore Notch3 may be a viable target to alleviate mucin production during asthma and other chronic respiratory diseases characterized by chronic overproduction of mucus.

Author disclosures are available with the text of this article at www.atsjournals.org.

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Table and Figure captions:

Table 1: Demographics of asthmatic and non-asthmatic pBEC donors.

Figure 1: Cell morphology and relative Notch gene expression in pBECs at ALI from asthmatic and non-asthmatic donors. (*A*) Representative images of pBECs from non-asthmatic and asthmatic donors showing positive PAS/alcian blue staining of mucins at day 29 of ALI culture. PAS/alcian blue overlap (dark blue) was more prevalent in pBECs from asthmatics. (*B*) *NOTCH3* mRNA expression was significantly increased in pBECs at day 29 of ALI culture. *NOTCH3* is further increased in pBECs from asthmatic donors. Scale bar: 8µm. Images and graphs representative of n=5 donor replicates, mean ± S.D. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 2: NOTCH3 is substantially increased in asthmatic airway epithelium sections. (*A*) IHC staining of NOTCH3 was increased in asthmatic airway epithelium compared to non-asthmatic. NOTCH1 staining was decreased in asthmatic airway epithelium compared to non-asthmatic. (*B*) NOTCH3 color segmentation showed a substantial and significant increase in NOTCH3 in asthmatic epithelium compared to non-asthmatic and a highly significant decrease in NOTCH1 when normalized to nonasthmatic NOTCH1. (*C*) Increased NICD3 and decreased NICD1 protein bands were shown for pBEC ALIs from asthmatics (As) compared to non-asthmatics (Non-As) by immunoblot. Scale bar: 20µm. Images representative of a minimum of 6 fields of view from n=6 donor replicates. Color segmentation box-plots represent mean stain/μm^2/nuclei for all analysed images per donor. Immunoblotting representative of n=5 donor replicates. $***P < 0.001$.

Figure 3: DBZ treatment potently reduced Notch3 at 0.1, 1 and 10μM in ALI pBECs from non-asthmatics (Non-As) and asthmatics (As). (*A*) DBZ treatment for 96 hours dramatically reduced *NOTCH3* mRNA in pBECs compared to DMSO vehicle controls (D) differentiated at ALI for 29 days. *NOTCH1* and *NOTCH2* mRNA levels were significantly reduced by 0.1μM DBZ in the non-asthmatic group only. (*B*) NICD3 protein was abolished following DBZ treatment at all concentrations in both donor cohorts. NICD1 levels were unchanged following DBZ treatment when normalized to β-actin. Graphs and immunoblots are representative of n=5 donor replicates, mean ± S.D. $^{*}P < 0.05$, $^{***}P < 0.001$.

Figure 4: Notch signaling inhibition abolished MUC5AC production in differentiated pBECs. (*A*) Following 96 hour DBZ (10μM) treatment corresponding to NICD3 ablation, overlapping PAS/alcian blue mucin staining (dark blue) was severely reduced in pBEC sections from asthmatics (As) and non-asthmatics (Non-As) compared to vehicle-treated controls. (*B*) Mucin MUC5AC staining was abolished following DBZ treament. (*C*) Expression of *SPDEF* mRNA was significantly reduced in pBECs treated with 0.1, 1 and 10μM DBZ compared to vehicle-treated controls. Images are representative of 5 fields of view from n=5 donor replicates for each treatment. Scale bar: 25μm Graphs representative of n=5 donor replicates, mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$.

Figure 5: Inhibition of Notch signaling reduced *Muc5ac* expression and secretion in differentiated pBECs. (*A*) *MUC5AC* mRNA was dramatically reduced following DBZ treatment at 0.1, 1 and 10μM concentrations, in asthmatic (As) and non-asthmatic (Non-As) pBECs compared to vehicle-treated controls. (*B*) MUC5AC protein was quantified in fluid taken from the apical surface of ALI pBECs using ELISA. Secreted MUC5AC was

potently reduced following 96 hours DBZ at all concentrations in cells from asthmatic and non-asthmatic cohorts. (*C*) MUC5AC (red) routinely adopted a filamentous tethered appearance (arrow) above the cell surface (dashed line) in differentiated pBECs from asthmatics when investigated. (*D*) Goblet cell marker CLCA1 protein was unchanged following all treatments with DBZ in asthma and non-asthma cohorts. Graphs are representative of n=5 donor replicates, mean ± S.D. $^*P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$. Immunofluorescence images are representative of 5 fields of view from n=5 donor replicates. Scale bar: 32µm. Immunoblots are representative of n=5 donor replicates.

Figure 6: Specific Notch3 siRNA knockdown significantly reduces MUC5AC production in monolayers of airway epithelial cells. (*A*) Cells transfected with Notch3 siRNA had significantly reduced *NOTCH3* mRNA expression compared to media- and scrambled siRNA-treated controls. *NOTCH1* and *NOTCH2* mRNA levels were unchanged. (*B*) Knockdown of NOTCH3 full-length protein and NICD3 protein were confirmed. (*C*) MUC5AC levels (red) were potently reduced in Notch3 siRNA-transfected cells compared to scrambled control cells following treatment with 25ng/mL rhEGF. (*D*) MUC5AC expression was quantified using in-cell ELISA. Knockdown of Notch3 by siRNA transfection significantly attenuated MUC5AC production in the presence of 25ng/mL rhEGF. Graphs are representative of n=3 independent experiments, mean ± S.D. $^*P < 0.05$, $^{**}P < 0.01$. Immunofluorescence and immunoblot images are representative of 3 independent experiments. Scale bar: 30µm.