Notch signaling prevents mucous metaplasia in mouse conducting airways during postnatal development

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SUMMARY

Goblet cell metaplasia and mucus overproduction contribute to the pathogenesis of chronic lung diseases, including asthma and chronic obstructive pulmonary disease (COPD). Notch signaling regulates cell fate decisions and is crucial in controlling goblet cell differentiation in the gut epithelium. Little is known, however, about how endogenous Notch signaling influences the goblet cell differentiation program that takes place in the postnatal lung. Using a combination of genetic and in vitro approaches here we provide evidence of a novel role for Notch in restricting goblet cell differentiation in the airway epithelium during the postnatal period. Conditional inactivation of the essential Notch pathway component Pofut1 (protein O-fucosyltransferase1) in Tgfb3-Cre-expressing mice resulted in an aberrant postnatal airway phenotype characterized by marked goblet cell metaplasia, decreased Clara cell number and increase in ciliated cells. The presence of the same phenotype in mice in which the Notch transcriptional effector Rbpjk was deleted indicated the involvement of the canonical Notch pathway. Lineage study in vivo suggested that goblet cells originated from a subpopulation of Clara cells largely present in proximal airways in which Notch was disrupted. The phenotype was confirmed by a panel of goblet cell markers, showed no changes in cell proliferation or altered expression of proinflammatory cytokines and was associated with significant downregulation of the bHLH transcriptional repressor Hes5. Luciferase reporter analysis suggested that Notch directly repressed MUC5AC transcription in lung epithelial cells. The data suggested that during postnatal life Notch is required to prevent Clara cells from differentiating into goblet cells.

KEY WORDS: Notch, Airway, Mucus, Goblet cell metaplasia, Mouse

INTRODUCTION

The airway epithelium is in contact with the external environment, serving as a major defense barrier against inhaled toxic substances and pathogens. Proper differentiation of the airway epithelium into ciliated, neuroendocrine (NE), secretory Clara and goblet cells is crucial to maintain lung homeostasis and prevent disease (Knight and Holgate, 2003; Puchelle et al., 2006). Mucin-producing goblet cells are found in the epithelium of a variety of tissues, including the respiratory, digestive and gastrointestinal tract, where they have multiple functions, including hydration and clearance of particulates and pathogens (Davis and Dickey, 2008; Gipson and Argueso, 2003; van Es et al., 2005). A number of studies show that interleukins, such as Il-4 and Il-13, and transcription factors, such as Foxa2 and Spdef (SAM pointed domain-containing ets transcription factor), influence goblet cell differentiation (Chen et al., 2009; Grunig et al., 1998; Jain-Vora et al., 1997; Park et al., 2007; Wan et al., 2004; Wills-Karp et al., 1998). In addition, the Notch pathway has been identified as a major regulator of goblet cell fate, both in development and in disease (Crosnier et al., 2005; Guseh et al., 2009; Kang et al., 2009; Okamoto et al., 2009; Shinoda et al., 2010; Tilley et al., 2009; van Es et al., 2005). In the gastrointestinal tract, disruption of Notch signaling by γ-secretase inhibitors results in increased number of goblet cells (Milano et al., 2004; Ridgway et al., 2006; Searfoss et al., 2003; Wong et al., 2004). Likewise, genetic inactivation of the Notch pathway leads to a massive conversion of proliferative crypt cells into postmitotic goblet cells (Crosnier et al., 2005; van Es et al., 2005). Conversely, constitutive Notch activation impairs goblet cell differentiation in the intestine (Fre et al., 2005; Stanger et al., 2005).

Respiratory conditions, such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis are typically associated with goblet cell metaplasia, which is characterized by overabundance of goblet cells and mucus hypersecretion (Jackson, 2001; Rogers, 2003; Rose and Voynow, 2006). Goblet cell metaplasia has been recently reported in a transgenic mouse model in which Notch signaling is constitutively activated in distal lung progenitors (Guseh et al., 2009). Previous studies in which important Notch pathway components have been deleted in the early developing lung epithelium using a Shh-Cre driver revealed a crucial role for Notch in formation of secretory Clara cells (Morimoto et al., 2010; Tsao et al., 2009). However, information on the goblet cell program was somewhat limited, as in murine airways differentiation of these cells has been reported to occur mostly during postnatal life (Pack et al., 1980) and nearly all the mutants died at birth. Thus, whether and how endogenous Notch signaling influences goblet cell differentiation postnatally remains an open question.
In the present work, we addressed this issue by using a Tgfb3-Cre deleter mouse line and mice carrying floxed alleles of the Pofut1 gene, which encodes an O-fucosyltransferase essential for Notch-ligand binding (Shi and Stanley, 2003). A preliminary analysis of lungs from a Tgfb3-Cre;ROSAC126-lacZ(R26R) reporter mice suggested that the Tgfb3-Cre line targeted the developing airway epithelium in a mosaic fashion and at relatively late stages. Conditional inactivation of Notch using this approach resulted in an overall less severe phenotype than that reported previously (Morimoto et al., 2010; Tsao et al., 2009) and allowed survival to adulthood. Analysis of these mutants revealed a postnatal lung phenotype characterized by goblet cell metaplasia in areas of reduced number of Clara cells, without major changes in cell proliferation. The effect appears to have resulted from derepression of a Notch-mediated mechanism that restricts mucin gene expression in a subpopulation of Clara cells. Our findings reveal a novel role for Notch in restricting goblet cell differentiation in the airway epithelium during the postnatal period. Together the data suggest that Notch is crucial in maintaining lung homeostasis and preventing mucous hypersecretion, a major feature of COPD.

MATERIALS AND METHODS

Mouse strains

Tgfb3-Cre mice were generated as previously described (Yang et al., 2008). Floxed Pofut1 (Pofut1loxP) and RbpjkloxP mice were kindly provided by Pamela Stanley (Shi et al., 2005) and Tasuku Honjo (Han et al., 2002), respectively. Both Tgfb3-Cre and RbpjkloxP mice were on a C57BL/6 background, and Pofut1loxP mice were maintained on a mixed 129/C57BL/6 background. Rosa26 reporter mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA).

G-Red mice were generated using a transgenic vector consisting of the cytomegalovirus (CMV) enhancer, the rabbit β-actin promoter, a loxP cassette containing eGFP cDNA and SV40 polyA, and the downstream DsRed1 cDNA with SV40 polyA sequences (Lin et al., 2011). Tgfb3-Cre mice were mated to Pofut1loxP mice to generate Tgfb3-Cre;Pofut1loxP offspring, which were then crossed to Pofut1loxP to create Tgfb3-Cre;Pofut1loxP (Pofut1crib) conditional knockout mice. For the Rbpjk model, the same approach was taken to generate conditional deletion of Rbpjk as above. Pofut1loxP and G-Red mice were crossed to obtain mice homozygous for the floxed Pofut1 allele and G-Red transgene (Pofut1loxP;G-Red+/−), which were mated to Tgfb3-Cre;Pofut1loxP to generate Pofut1loxP;G-RedloxP;G-RedloxP;Tgfb3-Cre;Pofut1loxP conditional mutant reporter mice, respectively. Genotyping was performed on tail biopsies by PCR. The primers for genotyping the floxed Pofut1 allele, Cre, and G-Red have been described previously (Lin et al., 2011; Tsao et al., 2009; Yang et al., 2008). All mice were maintained on mixed genetic backgrounds.

Lung tissues of Hex5 null mice were kindly provided by Dr Ryoichiro Kageyama (Cau et al., 2000). All protocols were approved by the Animal Care and Use Committee of the National Taiwan University Hospital and National Health Research Institutes.

X-gal staining

To detect β-galactosidase expression, lungs were inflated with 4% PFA for 30 minutes at 4°C and then processed for frozen sectioning. Sections were incubated in 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/ml X-gal solution for 4-8 hours at 37°C. Sections were counterstained with Eosin.

Immunohistochemistry

Lungs were inflated with 4% PFA for 1 hour to overnight at 4°C and embedded in OCT or paraffin. Periodic acid Schiff (PAS) and Alcian Blue (AB) staining and immunohistochemical staining for Ki67, Foxj1, β-tubulin, Tα1, and proSP-C followed previously described methods (Tsao et al., 2009). The other primary antibodies used were: rabbit anti-Tfsl (1:100, Leica); rabbit anti-Ccsp (1:1000, Upstate); goat anti-Ccsp (1:100, Santa Cruz); mouse anti-Mucin5ac (1:100, Abcam); rabbit anti-FoxA2 (1:400, Abcam); rabbit anti-HNF-3y (Foxa3) (1:100, Santa Cruz); rabbit anti-GFP (1:500, Invitrogen); rabbit anti-Hes5 (1:200, Chemicon) (Batts et al., 2009; El-Hashash et al., 2011; Li et al., 2009; Wang et al., 2010). Immunofluorescence was performed using secondary antibodies conjugated to Alexa Fluor 488 or 596 (1:200, Invitrogen) and analyzed using a Zeiss confocal laser-scanning microscope (LSM510 META) as previously described (Tsao et al., 2008).

Morphometric analysis

Digital images of airways were acquired with a Leica DM750 LED microscope and imported into MetaMorph imaging software version 7.1 (Universal Imaging Corp.). At PN30, large (hilar airways with associated pulmonary artery and vein), medium and small (bronchioles down to terminal bronchioles) airways were analyzed (Park et al., 2007; Tsao et al., 2009). AB staining was quantified by tracing the area of epithelial AB positive staining in airways. The area measurements of AB staining were normalized to total longitudinal length along the basement membrane of epithelial surveyed to give a density value and presented as μm²/mm. The percentage of labeled epithelial cells immunostained for Ccsp (Scgb1a1 – Mouse Genome Informatics) and Foxj1 was performed as previously described (Tsao et al., 2009). For our analysis of Pofut1loxP;G-RedloxP;Tgfb3CreloxP conditional mutant reporter mice, we performed Ccsp-eGFP double staining in mutant lungs. The proportion of Ccsp+ cells within eGFP– cell population was calculated as the number of Ccsp+/eGFP– cells divided by the total number of eGFP– cells. The same was done to determine the proportion of Ccsp+ cells in the eGFP+ cell population. We then determined the proportion of eGFP+/eGFP– cells in either Ccsp+ or Muc5ac+ cell populations using a similar method. A total of five sections was analyzed per animal (n=3) at 200 times magnification.

Real-time PCR

Total RNA from lung tissues or cell lines was isolated using the Trizol method. For reverse transcription, 2 μg of total RNA were transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time RT-PCR was performed in a DNA Engine Opticon 2 (Bio-Rad, CA, USA) using iQ SYBR Green supermix (Bio-Rad, CA, USA). Briefly, 100 ng of the reversed transcribed cDNA were used for each PCR reaction with 250 nM of forward and reverse primers. The primer sequences for real-time PCR are presented in supplementary Table S1. The threshold cycle (Ct) values were obtained for the reactions, reflecting the quantity of the product in the sample. The relative concentration of RNA for each gene to GAPDH mRNA was determined using the equation: 2−ΔΔCt, where ΔΔCt=(CtRNA – CTGAPDHmRNA).

Bronchoalveolar lavage and cell counts

Bronchoalveolar lavage (BAL) was performed as previously described (Hsieh et al., 2008). Cell pellets were resuspended in PBS. Cell numbers were counted with a hemocytometer, and inflammatory cell differentials were determined on cytopsin smears (Tsao et al., 2007).

Luciferase activity and transfection in vitro

The MUC5AC promoter-luciferase (MUC5AC-Luc) construct consisted of a 3.7 kb segment of the 5′ flanking region of the human MUC5AC gene (Li et al., 1998) and was prepared as previously described (Wang et al., 2007). L44 cells, a murine adenocarcinoma cell line that expresses Muc5ac endogenously, were transfected with 500 ng of MUC5AC-Luc and 10 ng renilla luciferase control vector (Promega, Madison, WI) by the MicroPorator (Digital Bio Technology, Seoul, Korea). The transfected cells were treated with DAPT {N-[N-(3, 5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester} (10 μM, Sigma) or DMSO (control) for 48 hours. Alternatively, L44 cells were co-transfected with MUC5AC-Luc and 1.0 μg of constitutive active Notch1 intracellular domain (caN1) or dominant negative (dnN1) constructs to constitutively activate or inhibit Notch1 signaling, respectively (Small et al., 2001), or Hex5 cDNA (Hojo et al., 2000) for 48 hours. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), in a BD monolight 3010 luminometer (BD Biosciences, San Diego, CA) in accordance with the manufacturer’s instructions and normalized to renilla luciferase activity.
To compare gene expression in LA4 cells with a primary respiratory tract epithelial cell, we isolated adult mouse tracheal epithelium as previously described and processed for quantitative RT-PCR (qRT-PCR) analysis (You et al., 2002).

**Statistics**

Data were expressed as mean±s.e.m. Statistical analysis was performed using Student’s t-test; differences were considered significant at \( P<0.05 \).

**RESULTS**

**Inactivation of Pofut1 in Tgfb3-expressing sites in the lung**

Previous analysis of disruption of \( Pofut1 \) in the lung using \( Pofut1^{F/F} \) and \( Shh-Cre \) deleter mouse mutants (Harfe et al., 2004) showed that loss of Notch signaling in lung epithelial progenitors resulted in dramatic changes in the balance of differentiated cell types in the airway epithelium (Tsao et al., 2009). This model, however, did not allow inferring about how Notch influences postnatal differentiation of the lung, because of postnatal lethality. Whether lethality resulted from the deletion of Notch in all Shh-expressing cells in the lung or other organs has not been determined. We argued that using a different Cre-deleter mouse line that did not target all epithelial precursors at once and allowed survival could provide information about postnatal functions of Notch in the lung. Previous studies have shown that Tgfb3 is expressed in the developing airway epithelium and that Tgfb3 regulatory sequences could drive Cre expression to the lung in transgenic mice (Coker et al., 1996; Pelton et al., 1990; Yang et al., 2008). In a preliminary analysis of Tgfb3-Cre:ROSA26-lacZ(R26R) reporter mice, we observed lacZ reporter expression in a mosaic-like pattern and at a relatively later stage in the developing airway epithelium, compared with Shh-Cre:ROSA26-lacZ (see Fig. S1A-C in the supplementary material). As represented in embryonic day 14.5 (E14.5) lungs, signals could be seen in only few epithelial cells of some airways, although it was strongly detected in the cartilage primordia, smooth muscle layer and pleura (see Fig. S1D-F in the supplementary material). Increasing labeling was observed postnatally, so that by postnatal day 30 (PN30, adult) most of the airway epithelium expressed LacZ (see Fig. S1G,H in the supplementary material). The data suggested that the Tgfb3-Cre deleter could target efficiently the developing airway epithelium, particularly postnatally at later stages. Thus, to investigate postnatal functions of Notch in the lung we used mice carrying a Tgfb3-Cre allele and a \( Pofut1^{F/F} \) mouse line.

Male mice heterozygous for the Tgfb3-Cre allele and the floxed \( Pofut1 \) allele (\( Pofut1^{F/F};Tgfb3^{Cre/+} \)) were crossed with female mice homozygous for the floxed \( Pofut1 \) allele (\( Pofut1^{F/F} \)) (see Fig. S1I in the supplementary material). Genotyping of litters derived from this breeding scheme after birth (\( n>500 \)) revealed \( Pofut1^{F/F};Tgfb3^{Cre/+} \) (conditional deletion of both \( Pofut1 \) alleles; termed \( Pofut1^{Thb} \) onwards), \( Pofut1^{F/F};Tgfb3^{Cre/+} \) (conditional deletion of a single \( Pofut1 \) allele), \( Pofut1^{F/F} \) and \( Pofut1^{F/+} \) (no deletion of \( Pofut1 \), hereby termed ‘control’) offspring at an expected Mendelian distribution. The \( Pofut1^{Thb} \) mice were viable, although they showed progressive hair loss after 3 weeks

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**Fig. 1. Goblet cell metaplasia in Pofut1cTb3 mutant lungs at PN30.** Tracheal (tr) (A,B,E,F,I,J) and bronchial (br) (C,D,G,H,K,L) epithelia stained with Hematoxilin and Eosin (HE), AB and PAS. Abundant AB or PAS staining (arrow) in cells with goblet cell morphology in Pofut1cTb3 mutant lungs (F,H,J,L). Scale bar: 100 μm.
Notch restricts goblet cell differentiation in the airway epithelium postnatally

We analyzed the lungs of Pofut1<sup>cTb3</sup> at PN30 and found no obvious differences in gross morphology compared with control lungs. Distal lung differentiation, including formation of alveolar sacs and type I and II cells, was not affected by Pofut1 deletion, as suggested by immunohistochemical assessment of markers, such as Ttf1 (thyroid transcription factor 1), T1alpha (podoplanin, Pdpn) and surfactant-associated protein C (Sftpc, SP-C) (see Fig. S2 in the supplementary material). Surprisingly, we found that the airway epithelium of mutant trachea and lungs was lined by a large number of goblet-like cells (Fig. 1A-D). We performed AB and PAS staining to identify acidic and neutral mucins characteristic of goblet cells in the airways of these animals. Staining was rarely observed in the trachea and bronchi of controls but was found in a large number of cells in Pofut1<sup>cTb3</sup> mutants (Fig. 1E-L).

Real-time PCR for genes known to be associated with goblet cell differentiation, such as Muc5ac, Spdef, anterior gradient 2 (Agr2) and Foxa3 showed marked upregulation in lungs from Pofut1<sup>cTb3</sup> mutant compared with controls (Fig. 2A) (Chen et al., 2009; Gregorieff et al., 2009; Noah et al., 2010; Park et al., 2007). Immunohistochemistry confirmed the strong expression of these gene products in goblet cells and revealed the extensive mucous metaplasia in airways of mutants (Fig. 2B-E). Consistently with this, Foxa2, which is normally present in the airway epithelium and required to inhibit goblet cell differentiation (Wan et al., 2004), was absent in the goblet cells of mutants (Fig. 2F,G). The presence of Ttf1 in the goblet cells of mutants suggested that the respiratory cell identity was maintained in the airway epithelium and that transdifferentiation to an intestinal-like epithelium was unlikely (Fig. 2H,I).

To investigate the onset of the goblet cell metaplasia, we extended our analysis of Pofut1<sup>cTb3</sup> lungs to different post- and prenatal stages, as a recent study suggests an early appearance of this cell type (Roy et al., 2011). Analyses of E14.5 and E18.5 showed no AB staining in airways of either controls or mutants (n=3 or 4 per group), suggesting that the goblet cell phenotype in Pofut1<sup>cTb3</sup> mice did not develop before birth (not shown). We also had no evidence of this abnormality within the immediate days of postnatal life, as confirmed by morphometry and by similar levels of Muc5ac, Foxa3 and Spdef expression in controls and mutants at P4, by real-time PCR (Fig. 3A and see Fig. S3 in the supplementary material). However, goblet cell metaplasia was found consistently at PN8 (not shown), PN17 and PN30 (Fig. 3).

Interestingly, the aberrant formation of goblet cells followed a proximodistal (PD) pattern of distribution with increasing age, even though Tgfb3-Cre;ROSA26-lacZ(R26R) signals could be seen already throughout the airway epithelium (see below and Fig. S1G-H in the supplementary material). Initially, the abundant AB-positive goblet cells of mutants were restricted almost exclusively to the trachea, later being found in both trachea and bronchi (large airways) at PN17. By PN30, morphometric analysis confirmed the aberrant goblet cell differentiation in smaller bronchioles, which normally lack goblet cells (Fig. 3).

To provide additional evidence that the Pofut1<sup>cTb3</sup> phenotype was a function of disrupted Notch signaling, we used the same Tgfb3-Cre mouse deleter approach to inactivate expression of the crucial Notch canonical transcriptional effector gene Rbpjk (Rbpj – Mouse Genome Informatics) (previously known as Rhpsuh) in the airway epithelium. We found that Rbpjk<sup>−/−</sup>;Tgfb3<sup>Cre/+</sup> (Rbpjk<sup>cTb3</sup>) mutant mice at postnatal day 7 (PN7) recapitulated the...
The goblet cell metaplasia phenotype of the Pofut1cTb3 mouse (see Fig. S4 in the supplementary material). We concluded that Notch signaling postnatally restricts goblet cell differentiation in airways.

**Pofut1cTb3 mutants show an imbalance in differentiated cellular phenotypes in airways**

To start investigating the origin of the Pofut1cTb3 lung defect, we asked whether the phenotype could be a function of aberrant proliferation of goblet cells or the transformation of another resident airway epithelial cell into a goblet cell. Thus, first we assessed cell proliferation by Ki67 immunohistochemistry in lung sections from control and mutant mice at PN30. Sections were then counterstained with AB (Fig. 4A,B) and the number of putative proliferating goblet cells in proximal airways was quantified in both groups and compared. This analysis showed that, in spite of the increase in number of AB-labeled cells in mutants, Ki67 labeling in these cells relative to total goblet cells in the airway increased in number of AB-labeled cells in mutants, Ki67 

The effect of Tgfb3-Cre deletion of Pofut1 in airway epithelial differentiation was consistent with previous findings of the Notch requirement to balance secretory and ciliated cells (Morimoto et al., 2010; Tsao et al., 2009). The relatively unchanged levels of Ccsp and Foxj1 mRNA between control and mutants at PN4 further supported the late onset of the Pofut1cTb3 phenotype postnatally (see Fig. S3 in the supplementary material).

**Goblet cells originate from a subpopulation of Clara cells in proximal airways of Pofut1cTb3 mutant lungs**

To further investigate the origin of the goblet cell metaplasia we performed lineage-tracing analysis of the cells undergoing Tgfb3-Cre-mediated deletion of Pofut1. We used a double fluorescent reporter mouse line (herein, G-Red reporter mice), which harbors a transgene comprised of a ubiquitous active chicken actin promoter that drives transcription of the eGFP gene (Lin et al., 2011). Cre-mediated recombination induces expression of the second reporter, DsRed, by deletion of loxP-flanked eGFP-Stop cassette. Thus, in Tgfb3Cre+/;G-Red double transgenic mice, cells that have undergone Cre-mediated excision could be distinguished by the absence of eGFP or the expression of DsRed (Fig. 5A). We analyzed Pofut1F/F;G-Red+/WT,Tgfb3Cre+ at PN30 by double immunofluorescence staining using anti-Muc5ac (visualized in red) and anti-eGFP (in green) antibodies. Fig. 5B shows extensive areas of recombination in proximal epithelium (eGFP negative) interspersed with only few positive for eGFP. Numerous Muc5ac-positive goblet cells lined the proximal airway epithelium of Pofut1F/F;G-Red+/WT,Tgfb3Cre+ mice but almost no overlap between eGFP and Muc5ac staining was found (Fig. 5B-D).
Morphometric analysis showed that nearly all Muc5ac+ cells (95.8%) were eGFP− (the remaining 4.2% non-targeted likely to be normally occurring goblet cells) (Fig. 5E). The data suggested that the majority of Muc5ac-positive goblet cells were derived or differentiated from Tgfb3-Cre-labeled cells in which Pofut1 expression was disrupted. However, analysis of the relative proportion of targeted versus non-targeted cells in the Ccsp+ population by Ccsp-eGFP double staining revealed that most of these cells (89.2% of all Ccsp+) underwent Cre-recombination and thus Notch inactivation, but still remained Clara cells (Fig. 5E). This suggests that Notch is a crucial component of a mechanism that restricts the goblet cell program in a relatively small but important subpopulation of Clara cells postnatally. This subpopulation is likely to be functionally distinct, as suggested by previous studies in an antigen-challenged mouse model (Evans et al., 2004).

**Loss of Notch signaling does not induce expression of proinflammatory cytokines**

An increased number of goblet cells is often a manifestation of pulmonary inflammation, which is typically accompanied by increased expression of Th2-cytokines, such as Il-4 and Il-13 (Grunig et al., 1998; Jain-Vora et al., 1997; Temann et al., 1997; Wills-Karp et al., 1998). Here we tested whether the goblet cell metaplasia in Pofut1cTb3 mutants was associated with an underlying inflammatory process in the lung, altering levels of Th2 or proinflammatory cytokines. Histological sections did not reveal noticeable inflammatory infiltrates in mutants. Thus, we performed qRT-PCR of adult lung homogenates from control and Pofut1cTb3 mutants (n=4, per group) looking for changes in expression of genes coding for selected Th2 cytokines. Fig. 6A shows no significant differences in Il-4, Il-6 or Il-13 mRNA expression between groups, suggesting that goblet cell differentiation occurred without obvious induction of these proinflammatory mediators. These results were further corroborated by the analysis of the total and differential cell counts in the BAL, which did not show differences between controls and Pofut1cTb3 mutants (data not shown).

**Notch regulates Muc5ac gene expression in lung epithelial cell line**

The Pofut1cTb3 phenotype strongly suggested that Notch signaling could regulate one or more aspects of the program of goblet cell differentiation, including mucin gene expression. To test whether Notch directly influenced transcription of a gene typically associated with this program, we assessed activity of a MUC5AC-luciferase reporter construct in transient transfection assays in LA4 cells, a murine lung adenocarcinoma cell line (Huang et al., 2009; Wang et al., 2009; Wang et al., 2007; Ye et al., 2011). PCR analysis showed that L4 cells express endogenous Notch1, Hes5, Muc5ac
and Ccsp genes typically found in freshly isolated primary airway epithelial cells (Fig. 6B). Thus, although not primary, LA4 cells provide a suitable model to study Notch activity in a cell committed to a Clara-like secretory lineage.

We cultured LA4 cells for 48 hours in medium containing DMSO (control) or DAPT (10 μM) to inhibit gamma-secretase activity and thus prevent endogenous Notch activation (Tsao et al., 2008). Luciferase analysis showed that the gamma-secretase inhibitor DAPT significantly increased the activity of the MUC5AC promoter (Fig. 6C). To further support this observation, we modulated Notch signaling in LA4 cells using a Notch1 constitutively active (caN1) construct or a dominant-negative (dnN1) construct (Small et al., 2001) in similar experiments. MUC5AC-Luc or pGL3 were co-transfected with caN1 or dnN1 constructs and luciferase activity was assessed at 48 hours. Consistent with the observations with DAPT treatment, we found a marked increase in MUC5AC promoter activity by downregulation of Notch signaling in dnN1-transfected cells. Conversely, Notch gain of function resulted in inhibited MUC5AC-luciferase expression in caN1-transfected cells (Fig. 6D).

**Goblet cell metaplasia is associated with disruption of Notch signaling predominantly by Hes5**

To gain insights into the Notch components and downstream targets potentially involved in the regulation of goblet cell fate in the airways, we compared levels of expression of key Notch-related genes in lung homogenates of adult Pofut1cTb3 and control mice. Real-time PCR showed a marked increase in Jag1 expression and decreased Notch1 expression (Fig. 7A), consistent with the major imbalance in the number of Jag1-expressing cells compared with Notch-expressing cells we previously reported in a ShhCre-Pofut1 mouse (Tsao et al., 2009). With the exception of Dll4 mRNA, which was also downregulated, expression of the other ligands and receptors was not significantly changed. We found no changes in expression of the Notch targets Hes1 and Hey1 and significant but only marginal changes in Hey2 mRNAs. By contrast, levels of Hes5 mRNA were nearly fivefold reduced in mutants, paralleling the changes in Pofut1 mRNA (Fig. 7B). To determine whether local changes in Hes5 protein expression correlated with the aberrant airway epithelial phenotype of mutants, we performed double Hes5-Ccsp immunostaining at PN30. This allowed us to infer cell type-selective expression of Hes5 in the airway and showed that in control lungs Hes5 signals were
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restricted to Clara cells (Fig. 7C-E). Consistent with our RT-PCR findings, Hes5 staining was significantly reduced in the airway epithelium of Pofut1<sup>−/−</sup> mutants (Fig. 7F,G). Hes5 labeling was absent in goblet cells and only few Hes5-positive Clara cells could be detected (Fig. 7F).

**Hes5 as a candidate mediator of the Notch effects in restricting mucin gene expression**

Based on the observations above we hypothesized that Hes5-mediated Notch signaling could play a role in the regulation of mucin gene expression. To explore this possibility, first we tested the ability of Hes5 to directly regulate the transcriptional activity of MUC5AC. Thus we co-transfected a Hes5 full-length cDNA construct [CLIG-Hes5 (Hojo et al., 2000)] and the same MUC5AC-Luc construct previously used in LA4 epithelial cells and assessed luciferase activity after 48 hours. This resulted in a significant decrease in MUC5AC-Luc expression compared with controls in which no Hes5 cDNA was transfected. As expected, disrupting Notch signaling with a dominant-negative Notch1 construct (PcDNA3.1-dnN1) increased MUC5AC transcriptional activity (Fig. 7G, and Fig. 6D above). Remarkably, expression of a full-length Hes5 cDNA prevented the increase in MUC5AC activity induced by PcDNA3.1-dnN1 in LA4 cells (Fig. 7G). This suggested that Hes5 could play an important role in regulating the transcriptional activity of a mucin gene that is highly expressed in the airways of Pofut1<sup>−/−</sup> mutants and is typically found in goblet cell metaplasia.

To further investigate this issue, we analyzed lung differentiation in Hes5 null mice. Hes5 null mutants are fertile and have no obvious developmental defects; however, the lung phenotype has not been previously described (Cau et al., 2000). Analysis of E18.5 and PN30 lungs showed no significant differences in the profile of differentiation of Hes5 mutants compared with controls, including number or timing of Clara or goblet cell differentiation as seen by expression of key markers (see Fig. S5 in the supplementary material and data not shown). We concluded that although Hes5 is likely to restrict goblet cell differentiation by inhibiting transcription of mucin genes, this effect may be shared in vivo with other basic helix-loop-helix (bHLH) factors downstream targets of Notch. Alternatively, an additional stimulus is required to trigger a goblet cell metaplasia response in these mice.

**DISCUSSION**

Here we provide novel evidence that disruption of Notch signaling in the murine airway epithelium leads to goblet cell metaplasia during postnatal life. This phenotype is consistent with observations reported in the intestine in which Notch plays a prominent role in controlling goblet cell differentiation (Crosnier et al., 2005; Milano et al., 2004; Ridgway et al., 2006; Searfoss et al., 2003; van Es et al., 2005; Wong et al., 2004).

Previous reports using a Shh-Cre genetic approach to disrupt Pofut1 or Rbpjκ genes early in development show that Notch deficiency prenatally has minimal effect in goblet cell differentiation in the airway epithelium. Instead, it has a dramatic effect in preventing formation of Clara cells (Morimoto et al., 2010; Tsao et al., 2009). Goblet cells constitute a very small population of the normal murine adult airway, although there is recent evidence of underestimation, largely owing to sensitivity of the standard labeling techniques (Roy et al., 2011). These cells can be greatly expanded upon exposure to environmental agents, such as allergens or cigarette smoke. Although the origin of the goblet cells is still little understood, there is increasing evidence that they arise from Clara cells, basal cells or an undifferentiated progenitor cell (Alessandrini et al., 2010; Chen et al., 2009; Evans et al., 2004; Hayashi et al., 2004; Kouznetsova et al., 2007; Morrissey and Hogan, 2010; Reader et al., 2003; Roy et al., 2011; Turner and Jones, 2009) A previous study shows that antigen challenge in ovalbumin-sensitized adult mouse elicits mucin production in Clara cells in the intrapulmonary airways (Evans et al., 2004). Another study shows that in the same model the increase in number of...
Moreover, our lineage study confirmed that a large number of the goblet cells in that Notch represses the goblet cell program in a subpopulation of Clara cells during the postnatal period. Notch could be also maintaining the balance of ciliated and secretory cells in adult airways.

Aberrant formation of goblet cells from a subpopulation of Clara cells. Interestingly, the aberrant formation of goblet cells followed mutants probably resulted from conversion of Clara into goblet cells (Chen et al., 2009). Our findings from resident Ccsp-expressing secretory cells and that Clara cells serve as progenitors to goblet cells (Reader et al., 2003). These studies suggest that goblet cells derive from resident Ccsp-expressing secretory cells and that Clara cells serve as progenitors to goblet cells (Chen et al., 2009). Our findings from the Pofut1cTb3 Tgfb3-Cre mouse model and the association of excessive goblet cells with decreased Clara cell abundance without significant changes in proliferation in Pofut1cTb3 mutants suggested that the goblet cell metaplasia in Pofut1cTb3 mutants probably resulted from conversion of Clara into goblet cells. Interestingly, the aberrant formation of goblet cells followed a PD pattern of distribution with increasing age in spite of the presence of Tgfβ3-Cre activity in both proximal and distal regions. Moreover, our lineage study confirmed that a large number of the Pofut1cTb3 targeted cells remained Ccsp+ even in the adult PN30 lung. The observations above suggested intrinsic regional differences in susceptibility to Notch deletion in developing airways. This is in agreement with studies showing that the ability to induce a goblet cell metaplasia program in allergen-induced asthma is regionally limited to a population of Clara cells in large and medium-sized airways (Evans et al., 2004). This further supports the idea of a significant diversity of Clara cells in airways.

What could be the possible mechanism for goblet cell metaplasia in Pofut1cTb3 mutants? Disruption of Notch signaling in these mutants was consistently associated with enhanced expression of genes regulating goblet cell differentiation, including Spdef, Foxa3 and Agr2. Moreover, we showed that Notch directly downregulates Muc5ac transcription. Thus, our data are consistent with the idea that Notch represses the goblet cell program in a subpopulation of Clara cells by preventing expression of goblet cell-associated genes. Our finding of the altered balance in ciliated versus Clara cells in Pofut1cTb3 lungs is in agreement with the lateral inhibition model proposed in previous reports (Morimoto et al., 2010; Tsao et al., 2009). This model, however, is unlikely to explain how Notch prevents goblet cell differentiation. Rather, we propose that during the postnatal period, Notch signaling may be modulated by intrinsic (part of the normal developmental program of goblet cells) or extrinsic (environmental insults: disease) stimuli to control formation of goblet cells from a subpopulation of Clara cells. Presumably this could be achieved by fine-tuning levels of expression of ligands, receptors or by modulating Notch activity, thus altering the secretory cell phenotype.

Paradoxically, increased goblet cell differentiation was observed in both our Notch loss-of-function model (postnatal) and the gain-of-function model (prenatal) reported in Guseh et al. (Guseh et al., 2009). Although we could not precisely determine the molecular basis for these differences, the observation is consistent with the known opposing role of Notch in controlling cell fate even within the same organ, depending on developmental timing and the cell population it acts upon. Indeed, Notch activation has been reported to increase goblet cell number by driving differentiation of postmitotic cells into mature goblet cells in the intestine; however, it can also inhibit the intestine goblet cell program if targeted to crypt cells, being highly context-dependent (Crosnier et al., 2005; Fre et al., 2005; Milano et al., 2004; Ridgway et al., 2006; Searfoss et al., 2003; Stanger et al., 2005; van Es et al., 2005; Wong et al., 2004).

Our model predicts that airway epithelial cell fate is strongly influenced by different thresholds of Notch activation. Prenatally, in undifferentiated airway progenitors, the absence of Notch fosters the ciliated and NE cell fate, whereas endogenous (physiologic) or high (supraphysiologic) levels of Notch signaling results in differentiation towards the Clara cell or goblet cell fates, respectively. However, once Clara cell fate is established, Notch signaling is required to restrict a goblet cell differentiation program in a subpopulation of Clara cells during the postnatal period. Notch could be also maintaining the balance of ciliated and secretory cells in adult airways.
smokers with COPD, compared with control subjects. Interestingly, in the COPD group, HES5 and HEY2 were the most significantly downregulated of the Notch targets, with no change in HES1, consistent with our findings in the PufalT model. Future experiments will further examine the potential cooperative contribution of Hes and Hey genes in regulating the goblet cell program in vivo under normal or exposure to environmental injuries.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at Development online.

References


Notch in goblet cell metaplasia

**DEVELOPMENT**


