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Notch signaling promotes airway mucous metaplasia and inhibits alveolar development

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The airways are conduits that transport atmospheric oxygen to the distal alveolus. Normally, airway mucous cells are rare. However, diseases of the airway are often characterized by mucous metaplasia, in which there are dramatic increases in mucous cell numbers. As the Notch pathway is known to regulate cell fate in many contexts, we misexpressed the active intracellular domain of the mouse Notch1 receptor in lung epithelium. Notch misexpression resulted in an increase in mucous cells and a decrease in ciliated cells in the airway. Similarly, mouse embryonic tracheal explants and adult human airway epithelium treated with Notch agonists displayed increased mucous cell numbers and decreased ciliated cell numbers. Notch antagonists had the opposite effect. Notably, Notch antagonists blocked IL13-induced mucous metaplasia. IL13 has a well-established role as an inflammatory mediator of mucous metaplasia and functions through Stat6-mediated gene transcription. We found that Notch ligands, however, are able to cause mucous metaplasia in *Stat6*-null cultured trachea, thus identifying a novel pathway that stimulates mucous metaplasia. Notch signaling may therefore play an important role in airway disease and, by extension, Notch antagonists may have therapeutic value. Conversely, in the distal lung, Notch misexpression prevented the differentiation of alveolar cell types. Instead, the distal lung formed cysts composed of cells that were devoid of alveolar markers but that expressed some, but not all, markers of proximal airway epithelium. Occasional distal cystic cells appeared to differentiate into normal proximal airway cells, suggesting that ectopic Notch signaling arrests the normal differentiation of distal lung progenitors before they initiate an alveolar program.

KEY WORDS: Airway epithelial cell fate, Lung disease, Notch, Mouse

INTRODUCTION

The lung consists of conducting airways and gas-exchanging alveoli. The predominant airway epithelial cells are Clara cells, which metabolize inhaled pollutants using P450 enzymes, ciliated cells, which help propel mucus out of the lungs, and basal cells, which are thought to be progenitors for the overlying epithelium (Cardoso, 2001; Rawlings and Hogan, 2006; Warburton et al., 2008). The function of rare, scattered neuroendocrine cells is unknown (Youngson et al., 1993). Mucous cells produce airway mucus, comprise a few percent of the cells in the large airways and occur only sporadically in smaller airways. A hallmark of many airway diseases is an overabundance of mucous cells, referred to as mucous metaplasia (Whitsett, 2002; Williams et al., 2006). This finding has been associated with increased or decreased expression of particular transcription factors, including E2f4, Foxa2 and Spdef (Danielian et al., 2007; Park et al., 2007; Wan et al., 2004). Asthma, bronchitis and cystic fibrosis all share the common pathology of mucous metaplasia.

In the mammalian brain, pancreas and intestines and the zebrafish kidney and *Xenopus* epidermis, Notch signaling alters the relative proportions of various cell fates (Yang et al., 2001; Murtaugh et al., 2003; Milano et al., 2004; Stanger et al., 2005; van Es et al., 2005; Liu et al., 2007; Ma and Jiang, 2007; Deblandre et al., 1999; Hayes et al., 2007). Notch is a single-pass cell-surface receptor that binds to a family of cell-surface ligands including the Delta-like and Jagged families. Upon Notch activation, a proteolytic cleavage event mediated by γ-secretase liberates the intracellular component of the Notch receptor, the Notch intracellular domain (NotchIC). NotchIC enters the nucleus, where it associates with transcription factors and activates downstream Notch genes. In the lung, the best-characterized Notch target is Hes1. Hes1 and Mash1 (Ascl1 – Mouse Genome Informatics) repress each other's expression, and the relative expression of these two factors dictates cell-fate choice (Borges et al., 1997; Ito et al., 2000).

Little is known, however, about the role of Notch signaling in regulating mammalian lung cell types, in part because null mutations in Notch receptors and ligands often result in early embryonic lethal phenotypes (Swiatek et al., 1994; Conlon et al., 1995; Hamada et al., 1999; Xue et al., 1999). Transgenic studies in which NotchIC is expressed throughout the lung epithelium suggest that constitutive Notch signaling arrests the differentiation of distal progenitor cells into mature alveolar type 1 and type 2 cells (Dang et al., 2003). Recent complementary evidence shows that antagonizing Notch signaling in the embryonic lung results in an expansion of distal lung progenitors at the expense of their proximal airway counterparts (Tsao et al., 2008). In addition, null mutations in Notch target genes have previously been associated with abnormal airway epithelial cell differentiation. *Mash1*-null mice lack neuroendocrine cells (Borges et al., 1997; Ito et al., 2000), whereas Hes1-deficient mice display precocious neuroendocrine differentiation and have fewer Clara cells (Ito et al., 2000).

The embryonic *Xenopus* mucociliary epidermis, like the mammalian airway, is composed of scattered goblet and ciliated cells. Interestingly, epidermal misexpression of NotchIC in this surface epithelium eliminates ciliated cells (Deblandre et al., 1999; Hayes et al., 2007). In the present study, we similarly misexpress the active intracellular domain of the mouse Notch1 receptor (NotchIC)

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(Murtaugh et al., 2003) in the embryonic lung epithelium. We confirm that Notch activation inhibits the differentiation of distal lung progenitors into alveolar cells (Dang et al., 2003). We also demonstrate that activated Notch signaling increases the number of airway mucous cells and decreases the number of ciliated cells, consistent with the result in *Xenopus* mucociliary epidermis (Deblandre et al., 1999; Hayes et al., 2007) and the zebrafish pronephros (Liu et al., 2007; Ma and Jiang, 2007). In vitro experiments using agonists and antagonists of Notch signaling confirm this result in mouse tracheal explants and human airway epithelial cultures.

MATERIALS AND METHODS

Animals

SPC-Cre mice were previously described (Okubo et al., 2005). Rosa-NotchIC-IRES-GFP mice were previously described (Murtaugh et al., 2003) and maintained on a BL6/C57 genetic background. *Stat6*-null mice (stock number 002828) were acquired from Jaxx Laboratories. In timed pregnancies, the day of a vaginal plug was considered embryonic day 0.5 (E0.5). Progeny of described matings were genotyped using the following primers: *Cre* Fwd, 5′-CCAGGTTACGGATATAGTTCATG-3′; Rev, 5′-TGCCACGACCAAGTGACAGC-3′ (600 bp).

Preparation of tissue

Lungs and tracheal explants for immunohistochemistry were fixed in 4% paraformaldehyde for 1 hour at 4°C and embedded in OCT or paraffin.

Immunohistochemistry

Primary antibodies used were: rabbit anti-Hes1 (1:50; raised using a KLH-conjugated peptide sequence as described by Ito et al. (Ito et al., 2000); chicken anti-green fluorescent protein (1:500; Aves Labs); rabbit anti-TTF1/Nkx2.1 (1:200; Zymed); rabbit anti-CC10 (1:50; Santa Cruz); mouse IgG_1 anti-human Ki67 (1:10; BD Pharmingen); rabbit anti-prosurfactant protein C (SP-C) (1:200; Upstate); rabbit anti-EphA7 (1:50; Santa Cruz); mouse IgG_1 anti-Muc5AC (1:250; Neomarkers); rabbit anti-β-tubulin (1:200; Fitzgerald).); rat anti-E-cadherin (1:1000; Zymed); rat anti-Keratin 5 (1:500; Abcam); mouse IgG2a anti-p63 (1:50; Santa Cruz); rabbit anti-Sox2 (1:100; Abcam); rabbit anti-HNF 3β (1:1000; Abcam); goat anti-Muc1 (1:50; Santa Cruz).

Secondary antibodies included rhodamine-conjugated donkey anti-rabbit (1:250; Jackson ImmunoResearch); FITC-conjugated donkey anti-chicken antibody (1:250; Jackson Immunoresearch); Alexa fluor 568-conjugated goat anti-mouse IgG_1 (1:500; Molecular Probes); Alexa fluor 568-conjugated goat anti-rabbit (1:500; Molecular Probes).

BrdU incorporation was detected using Amersham Cell Proliferation Kit (GE Healthcare; RPN20). Cell death was detected using DeadEnd Fluorometric TUNEL System (Promega; #G3250).

Cell counting

Representative images from multiple tissue samples were counted ($n\geq3$). In airways, 627 epithelial cells were counted in controls, and 684 were counted in Notch-activated lungs. Five hundred and eighty post-BADJ cells were assayed for ectopic CC10 expression, and 736 embryonic airway cells were counted. In adult human airway explants, at least 200 cells were counted for each unique culture condition. A *P*-value less than 0.05 in the Student's *t*-test was deemed significant.

Tracheal explant culture

Whole tracheas were dissected at E14.5 in PBS and opened longitudinally. The explants were grown in 50% DMEM (Gibco), 50% Ham's F12 (CellGrow), with penicillin, streptomycin and glutamine (Gibco) at 37°C. Media was changed every 24 hours with repeated addition of relevant agonists. IL13 was used at 100 ng/ml (RD Systems). Recombinant mouse (40 ng/ml) and human Dll4 (400 ng/ml) (R&D Systems) were used in combination. DBZ (Calbiochem) was used at indicated concentrations.

RESULTS

Constitutive Notch signaling in embryonic lung prevents the differentiation of alveolar epithelium

To study the role of Notch in the developing lung epithelium, we used a genetic system that permits expression of NotchIC in tissues expressing Cre-recombinase (Cre) (Murtaugh et al., 2003). We crossed mice heterozygous for a transgene in which the human *surfactant protein C* (*SPC*) promoter drives Cre (Okubo et al., 2005) to mice bearing homozygous alleles that permit inducible constitutive expression of both NotchIC and green fluorescent protein (GFP) (Fig. 1A). In the mice bearing homozygous alleles that permit inducible Notch expression, *loxP* sites surround a strong upstream transcriptional STOP sequence to prevent downstream transcription of NotchIC and GFP, which are both expressed from the Rosa26 locus. In the presence of Cre, the STOP sequence is excised, resulting in expression of both NotchIC and GFP. The SPC transgene is expressed exclusively in the lung epithelium, starting at E10.5, and persists throughout development (Okubo et al., 2005) (see Fig. S1A,B in the supplementary material). We observed robust GFP expression throughout the endoderm as early as E11.5 (see Fig. S1C in the supplementary material), confirming early and ubiquitous activity of Cre throughout the lung epithelium.

Doubly transgenic SPC-Cre; NotchIC mice possessed grossly normal lungs with normal branching, size and lobulation (Fig. 1B,B′). However, on closer inspection, transgenic lungs contained dilated cysts instead of normal saccules (Fig. 1C,C′) in agreement with a prior transgenic model (Dang et al., 2003). Cysts occurred solely in regions of lung expressing GFP and thus NotchIC (see Fig. S1B in the supplementary material). By contrast, lung tissue lacking transgene expression demonstrated normal histology (see Fig. S1B in the supplementary material). Despite mosaic NotchIC expression, all transgenic pups died at birth.

The Notch target, Hes1, is normally present in E18.5 trachea, bronchi, lobar bronchi and distal bronchiolar airways (Fig. 2A). By contrast, the distally located saccules display greatly reduced Hes1 expression in the post-bronchiolar lung epithelium (Fig. 2A). The bronchioalveolar duct junction (BADJ) is defined as the portion of the distal airway that is characterized morphologically by an abrupt increase in luminal diameter. In E18.5 transgenic lungs, robust *Hes1* expression extended beyond the BADJ to include all of the abnormally dilated cystic epithelium (Fig. 2A′). In regions of lung with mosaic transgene expression, the absence of Hes1 correlated with normal morphology.

To characterize the differentiation of the cyst cells, we analyzed the expression of a number of markers known to be expressed in the distal embryonic lung epithelium. Cyst cells expressed Nkx2.1, a pan-lung epithelial marker (Fig. 2B,B′), but failed to express SPC, a marker of both distal type 2 pneumocytes and pulmonary progenitor cells (Fig. 2C,C′). Therefore, cyst cells remain specified as lung epithelium but are not type 2 pneumocytes or normal distal progenitor cells. Lungs from mice doubly transgenic for a Cre-dependent GFP and the previously used SPC Cre transgene did not display altered morphology or SP-C differentiation (see Fig. S1 in the supplementary material). The basal cell markers keratin 5 and p63 (Tcp1 – Mouse Genome Informatics) were both absent from cysts (data not shown); however, other proximal markers of the airway, including E-cadherin (cadherin 1 – Mouse Genome Informatics) (Fig. 2D,D′) and Foxa2 (data not shown), were present in the distal cysts. Interestingly, cysts were surrounded by a layer of ectopic smooth muscle (Fig. 2E,E′). Ordinarily, smooth muscle is present exclusively around the proximal airway epithelium (Fig.

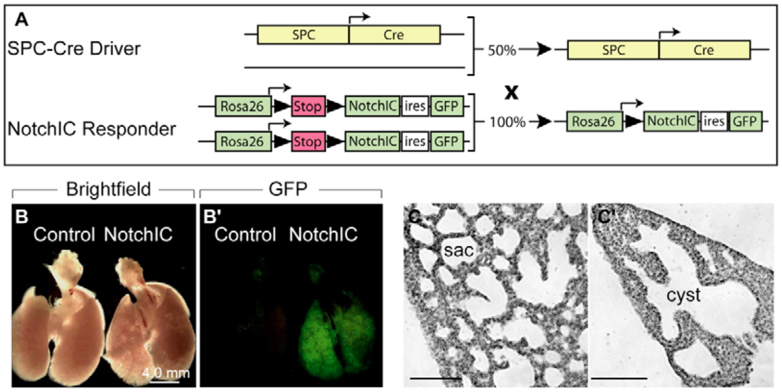

Fig. 1. Constitutive Notch expression in embryonic lung results in distal cyst formation. (**A**) Strategy to express activated Notch intracellular domain (NotchIC) in developing lung epithelium. The triangles represent *loxP* sites. (**B**,**B′**) Lungs from E18.5 NotchIC transgenic pups and control littermates (B). GFP transgene activation is evident in NotchIC transgenic lungs and absent in control littermates (B′). (**C**,**C′**) H&E staining of E18.5 control littermate (C) and NotchIC transgenic (C′) lungs reveals dilated cysts in place of alveolar saccules. Scale bars: 100 μm in C,C′.

2E). The NotchIC-expressing cystic epithelial cells might, therefore, induce surrounding mesenchyme to form smooth muscle. Alternatively, distal lung progenitors may normally inhibit smooth muscle differentiation, and this inhibitory effect may be lost in NotchIC-expressing cystic epithelial cells.

Clara cells are non-ciliated airway cells that use P450 enzymes to metabolize toxins inhaled into the lung. Normally, Clara cells are completely absent distal to the BADJ (Fig. 2F, inset). In NotchIC transgenic lungs, Clara cells occurred normally in the proximal airway epithelium (Fig. 2F). Surprisingly, however, scattered ectopic CC10^{+} cells were present in post-BADJ transgenic cysts (Fig. 2F′). In the 7±4% of cyst epithelial cells that stained for CC10, GFP was expressed, indicating NotchIC expression (*n*=8) (see Fig. S2J in the supplementary material; arrows indicate CC10 staining).

During late development (E16.5-18.5), the distal cells of the branching endoderm divide rapidly to create the gas-exchanging alveoli. We injected pregnant mice with BrdU 2 hours before sacrifice at both E16.5 and E18.5 to assay for proliferation in transgenic cyst cells. At both stages, we observed a marked decrease in BRDU incorporation in NotchIC transgenics compared with wild-type littermates. In E18.5 littermate lungs (Fig. 2G), 18.5% of alveolar epithelial cells incorporated BrdU. Only 7.7% of cystic epithelial cells were BrdU-positive in NotchIC transgenic lungs (*P*=0.0003, *n*=13) (Fig. 2G′). In control mice doubly transgenic for the SPC Cre driver and an inducible GFP reporter, 20.8% of alveolar epithelial cells were BrdU-positive. This demonstrates that GFP expression alone is not responsible for a decrease in proliferation (*P*=0.28, *n*=13). Consistent findings were obtained using Ki67 immunohistochemistry (data not shown). TUNEL staining was also performed to assess whether the absence of alveolar differentiation correlated with an increase or decrease in apoptosis (Fig. 2H,H′). There was a 2% statistically significant increase in the number of apoptotic cells in mutant lungs, but in absolute terms this change was negligible in comparison to the changes noted in the replication rate of epithelial cells (*P*=0.001, *n*=24) (Fig. 2G-H′). Interestingly, recent studies have demonstrated that blocking early embryonic Notch signaling results in an expansion and proliferation of distal progenitor cells (Tsao et al., 2008). Our results complement this finding by demonstrating that Notch activation conversely prevents the replication of distal epithelial cells.

Notch activation in vivo results in increased airway mucous cells and fewer ciliated cells

We next examined the distribution of cell types in NotchIC transgenic airway epithelium compared to the distribution of cell types from control transgenic mice carrying only an inducible GFP reporter. In the large airways of transgenic E18.5 embryos, we found dramatic increases (40±12%) in mucus-producing cells compared with control littermates (11±3%) (*P*=0.006, *n*=4) (Fig. 3A-D,M). Mucus production occurred in cells that expressed the NotchIC transgene. The mucous cells were characterized by elevated levels of Muc5AC, Muc1 and Alcian Blue staining (see Fig. S3A-E in the supplementary material). Interestingly, a majority of these cells co-stained for CC10 (see Fig. S2J in the supplementary material). Wan et al. (Wan et al., 2004) previously demonstrated that Foxa2 downregulation results in mucous cell metaplasia. After Notch activation, however, Foxa2 staining was unchanged, despite the robust mucous metaplasia (see Fig. S2K,L in the supplementary material). Furthermore, mucous metaplasia was observed only in proximal airway epithelium and never occurred in distal cysts. Control lungs displayed GFP expression throughout the airway epithelium and in a subset of alveolar cells (see Fig. S1 in the supplementary material). GFP expression alone did not induce mucous metaplasia (see Fig. S1 in the supplementary material). We next counted ciliated cells by enumerating the number of EphA7^{+} cells. (EphA7 staining identifies ciliated cells and is a cytoplasmic stain that permits unambiguous cell identification.) The epithelium of control animals contained 40±3% ciliated cells, whereas transgenic littermates possessed only 15±9% ciliated cells (*P*=0.003, *n*=4) (Fig. 3E-L,N). Of the GFP-negative airway cells in transgenic lungs, 31±10% (*n*=3) stained for ciliated cell markers, not significantly different from the percentage in control airway (*P*=0.17). Of 1085 GFP^{+} cells counted, we found only three that stained for EphA7 (0.3%). This suggests that NotchIC expression cell-autonomously inhibits ciliated cell differentiation. Loss of E2f4 throughout the airway and nasal epithelium has been shown to inhibit the differentiation of ciliated cells and promote mucous cell metaplasia (Danielian et al., 2007), but E2f4 expression was unchanged in the airway epithelium of NotchIC transgenic lungs (data not shown). GFP expression alone did not alter ciliated cell differentiation or distribution (see Fig. S1 in the supplementary material). Clara cells were found in normal proportions in large airways (see Fig. S2A-D in the supplementary material) and small airways (see Fig. S2E-H in the supplementary material), irrespective of Notch expression. In Notch mutants, CC10 labeled 50±6% of airway cells, whereas in control lungs 47±5% of airway cells were CC10-positive (see Fig. S2I in the supplementary material). GFP expression alone did not alter Clara cell differentiation or distribution (see Fig. S1 in the supplementary material). Normally, one rare neuroendocrine cell is present on average per high-power field of the airway epithelium, but they were absent in the transgenic airways (data not shown), consistent with prior observations

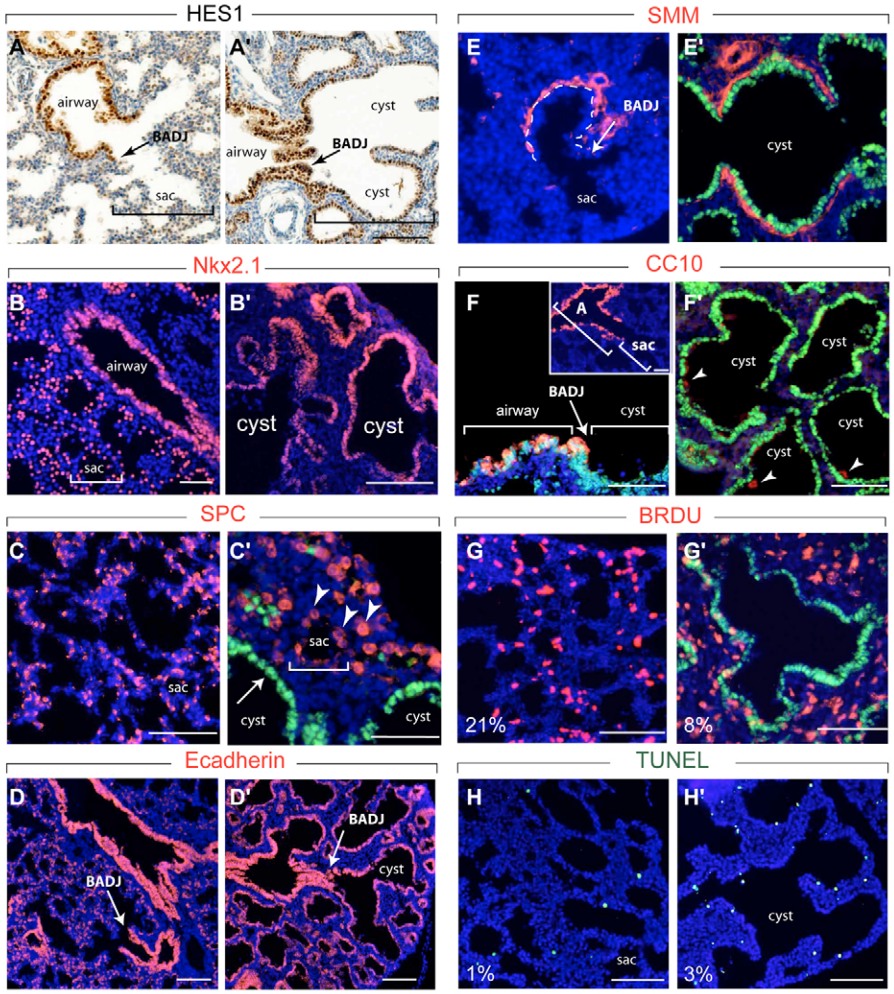

Fig. 2. Constitutive Notch expression inhibits differentiation of distal alveolar saccules. (**A**,**A′**) E18.5 Hes1 immunohistochemistry in control (A) and NotchIC transgenic (A′) lungs. The brackets indicate control saccules (A) and corresponding cysts in transgenic animals (A′). BADJ, bronchioalveolar duct junction. (**B**,**B′**) E18.5 Nkx2.1 immunohistochemistry (red) in control (B) and NotchIC transgenic (B′) lungs. (**C**,**C′**) E18.5 surfactant protein C (SPC) immunohistochemistry (red) in control (C) and transgenic NotchIC lungs (C′). GFP-positive (green) cells do not express SPC (arrow). However, SPC-positive cells persist when GFP is absent (arrowheads). The bracket indicates a GFP-negative saccule. (**D**,**D′**) E18.5 E-cadherin immunohistochemistry (red) in control (D) and NotchIC-gfp transgenic lungs (D′). E-cadherin expression stops at the BADJ in control lungs but persists throughout the cystic epithelium in transgenic lungs. (**E**,**E′**) E18.5 smooth muscle-myosin (SMM) immunohistochemistry (red) in control (E) and NotchIC-gfp (green) transgenic lungs (E′). SMM expression is restricted to proximal airways (dashed line) in control lung but is ectopically expressed surrounding distal cysts in transgenic lungs. (**F**,**F′**) E18.5 CC10 immunohistochemistry (red) demonstrates normal CC10 patterning in transgenic animals compared with controls (inset) (F). A, airway. (F′) Scattered CC10-positive cells (arrowheads) are found in GFP$^+$ transgenic cysts. (**G**,**G′**) E18.5 BrdU immunohistochemistry (red) of control (G) and NotchIC-gfp (green) transgenic (G′) lungs after a 2 hour BrdU pulse. BrdU incorporation (percentage shown) is reduced in cystic epithelial cells compared with control alveolar epithelial cells. (**H**,**H′**) TUNEL staining (red, percentage shown) of E18.5 control (H) and NotchIC transgenic (H′) lungs reveals an increase in apoptosis in transgenic lungs compared with control. Scale bars: 100 μm.

showing decreased neuroendocrine differentiation in the setting of elevated Hes1 expression (Borges et al., 1997; Ito et al., 2000). In summary, the predominant effect of Notch activation in the mouse airway was to increase the frequency of mucous-producing cells and decrease the number of ciliated cells.

Notch signaling regulates the abundance of ciliated and mucous cells in tracheal explants

To confirm the effects of Notch signaling on airway epithelial cells, we developed an embryonic tracheal explant culture assay. We harvested trachea at E14.5 when airway cells do not express differentiation markers of mucous, ciliated or Clara cells (data not shown). We cultured these explants for 10 days and observed that epithelial differentiation readily occurred in vitro (Fig. 4A-D) and that differentiated cells were present in their normal proportions. Specifically, ciliated cells comprised 27±16% of airway epithelial cells, whereas mucous cells comprised 8±2% of these cells (Fig. 4D,G).

Notch activation with non-immobilized Delta in culture has been reported (Fitzgerald and Greenwald, 1995; Qi et al., 1999; Han et al., 2000; Fung et al., 2007). Therefore, we added the Notch agonist Delta-like4 (Dll4) to explants (*n*=3). This resulted in an increased percentage of Muc5AC-positive cells (47±11%; *P*=0.02) (Fig. 4D,E,G). Conversely when a Notch signaling antagonist, the γ-

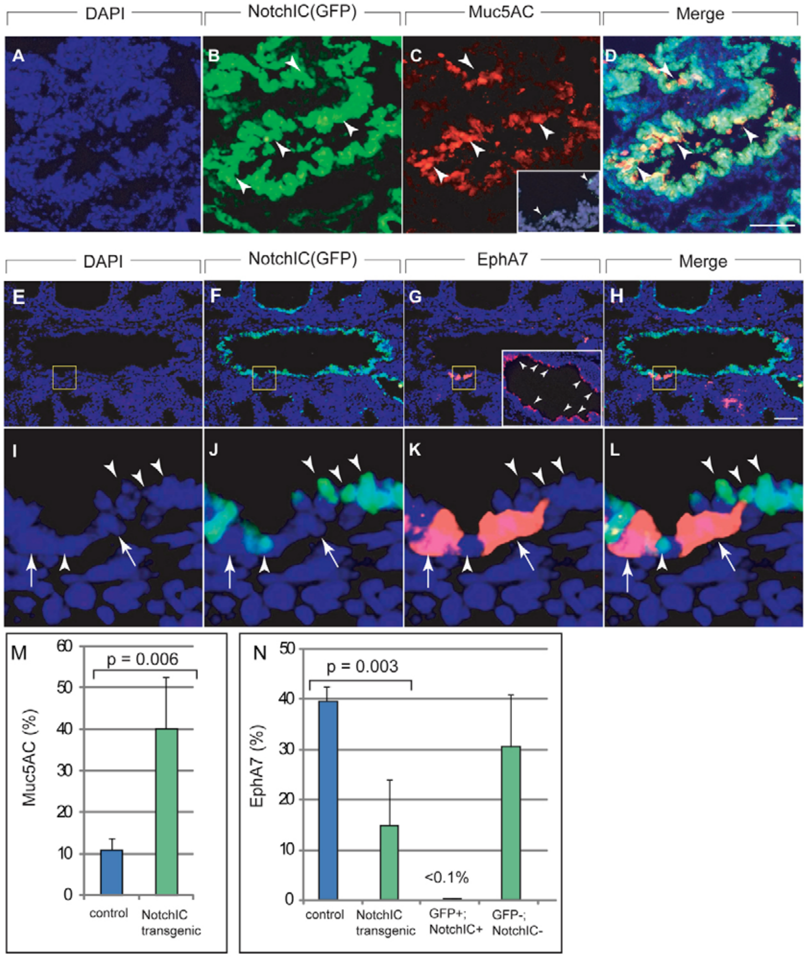

Fig. 3. Constitutive Notch activation leads to more mucous cells and fewer ciliated cells in E18.5 airway epithelium. (**A-D**) E18.5 immunohistochemistry of NotchIC airways. Transgene expression (green, B) is correlated with increased numbers of Muc5AC-positive cells (red, C). (C) Inset demonstrates the rare presence of mucous cells in control airway epithelium. (D) Color merge reveals that mucous cells co-label with GFP (yellow). Arrowheads indicate mucin-positive cells. (**E-H**) E18.5 immunohistochemistry of transgenic Notch airway epithelium shows few ciliated cells. Notch-transgene-expressing cells that are GFP-positive (green, F) lack EphA7 (red, G). (G) The inset demonstrates normal ciliated cell numbers in control epithelium as marked by EphA7 (arrowheads point to ciliated cells in red). (H) Rare residual ciliated cells marked by EphA7 (red) lack GFP (green) transgene expression. (**I-L**) The yellow-boxed regions from E-H are shown at high magnification in I-L, respectively. Merge reveals that GFP-positive NotchIC transgenic cells (arrowheads, green) are distinct from EphA7-positive (arrows, red) cells. (**M**,**N**) Quantification of Muc5AC$^+$ mucus cells and EphA7$^+$ ciliated cells in E18.5 airway epithelium demonstrates that Notch activation is associated with increased mucous cell differentiation (M) (P=0.006) but fewer ciliated cells (N) (P=0.003). GFP-negative cells in transgenic lungs showed normal ciliated cell differentiation, whereas GFP$^+$ cells showed virtually no ciliated cell differentiation. Scale bars: 100 μm.

secretase inhibitor DBZ (Milano et al., 2004; Tsao et al., 2008), was added to explants, the fraction of ciliated cells in explants increased and the number of mucous cells decreased (P=0.009) (Fig. 4D,F,G). As before, we detected no changes in Clara cell numbers with the addition of Dll4 or DBZ. These results are all consistent with findings from our in vivo genetic model of Notch activation.

γ-secretase inhibitors prevent mucous cell differentiation in human airway cultures

To test whether Notch signaling promotes mucous cell differentiation in human airway epithelium, we added Dll4 to human airway epithelial cultures (EpiAirway, MatTek). This resulted in increased Muc5AC-producing cells, paralleling the results seen in mouse tracheal cultures (Fig. 5A,B). To test the effects of blocking Notch signaling on human airway epithelium, we co-cultured airway samples in Dll4 and increasing concentrations of DBZ. This resulted in a dose-dependent decline in the number of Muc5AC-positive cells (Fig. 5B). When cultures were pre-treated with varying concentrations of DBZ before Dll4 addition, mucous cell production was again inhibited by increasing concentrations of DBZ in a dose-dependent fashion (Fig. 5A,B).

We next cultured human airway epithelium with recombinant IL13, an agent known to act directly on airway cells via a Stat6-dependent pathway to increase mucous cell numbers in a variety of human diseases (Kuperman et al., 2002). As expected, increased numbers of mucous cells were observed (Fig. 5A,B). Interestingly, pre-incubation with DBZ blocked IL13-induced mucous production (Fig. 5A,B). Cultures grown in IL13 alone contained 39±18% mucous cells whereas cultures pre-incubated with 1 mM DBZ and IL13 resulted in only 2.8±1.9% of cells expressing Muc5AC (P=0.039, n=3). Therefore, we show that antagonizing Notch signaling blocks mucous cell differentiation induced by a factor that is known to contribute to human airway inflammation.

To determine if Notch signaling induces mucous cell differentiation through the Stat6-dependent pathway utilized by IL13, we harvested trachea from E14.5 *Stat6*$^{-/-}$ embryos and cultured them in the explant assay system. As previously shown, incubation with Dll4 or IL13 increased the percentage of Muc5AC-producing cells in wild-type explants compared with control cultures (control=4%, IL13=20%, P<0.0001, Dll4=12%, P=0.015, n=15) (Fig. 6A-C). The epithelium of *Stat6*$^{-/-}$ trachea incubated with control media contained 6% mucous cells, indicating that mucous cell differentiation spontaneously occurs in the absence of Stat6 (Fig. 6A′). Incubation of *Stat6*$^{-/-}$ trachea with IL13 did not result in increased mucous cell numbers as expected (8%, P=0.477, n=15) (Fig. 6B′). Surprisingly, Dll4 increased the percentage of Muc5AC-producing cells in *Stat6*-null trachea (37%, P<0.0001, n=15) (Fig. 6C′) Notch-induced mucous cell differentiation therefore acts through a Stat6-independent mechanism. This is consistent with the persistence of HNF-3β in mucous epithelial cells, a Stat6 target the downregulation of which results in mucous metaplasia (Wan et al., 2004). Furthermore, Notch inhibition of mucous metaplasia using γ-secretase inhibitors blocks IL13 Stat6-dependent mucous metaplasia (Fig. 5). Interestingly, DLL4 addition resulted in significantly more mucous metaplasia in *Stat6*$^{-/-}$ trachea compared with its addition in control trachea.

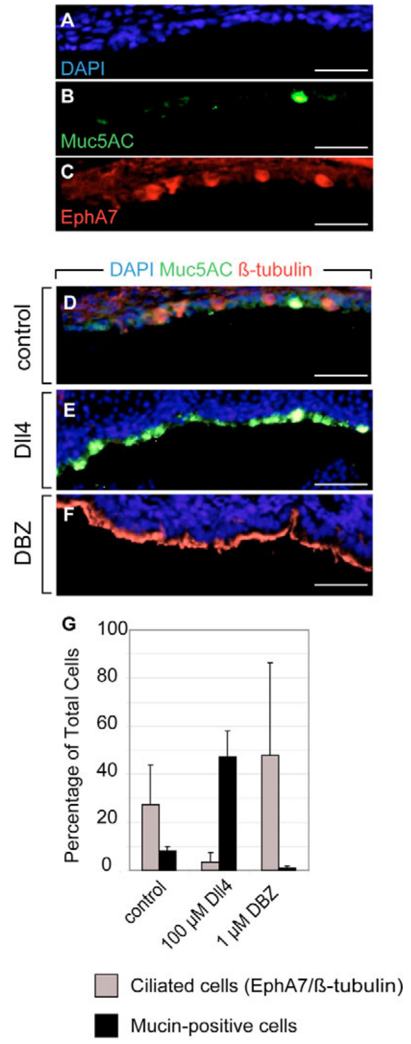

Fig. 4. Notch agonists and antagonists alter ciliated and mucous cell numbers in mouse tracheal explants. (**A-C**) E14.5 mouse tracheas (A) cultured in vitro for 10 days display normal mucous (B) and ciliated cell (C) differentiation. (**D-F**) Immunohistochemistry of tracheal explants: mucous cells (green) and ciliated cells (red) were present in control explants (D). Culture with the Notch ligand Dll4 results in increased mucous cell differentiation (green, E). Addition of a Notch signaling antagonist, DBZ, results in increased ciliated cell differentiation (red, F). (**G**) Incubation of tracheal explants in Dll4 increases mucous cells while decreasing ciliated cells. Addition of DBZ, the Notch antagonist, results in the near absence of Muc5AC-positive cells. Scale bars: 25 µm.

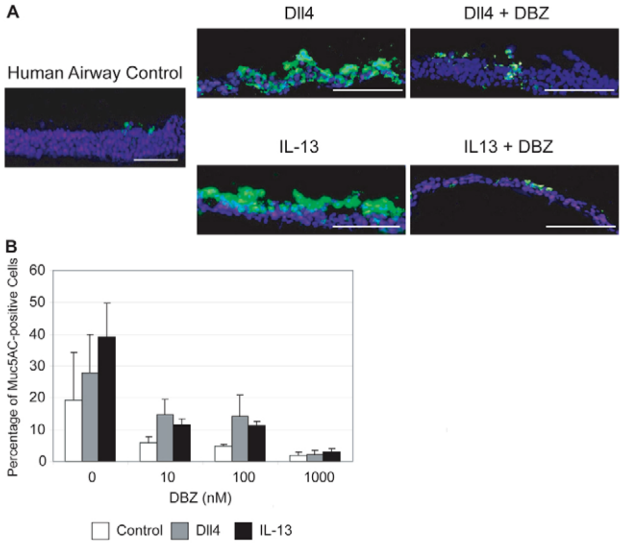

Fig. 5. Notch antagonists decrease mucous cell differentiation in human airway epithelial cultures. (**A**) Human airway cultures incubated with Dll4 or IL13 (middle column) show substantial Muc5AC (green) immunostaining compared with control (left panel). DBZ addition to Dll4 or IL13 (right-hand column) decreased Muc5AC staining. (**B**) Increasing concentrations of DBZ decreased Muc5AC staining in control cultures (white) and in those cultures incubated with either Dll4 (gray) or IL13 (black). Scale bars: 100 µm.

Notch misexpression and Stat6 activation in the airway epithelium both result in mucous metaplasia. Three hypothetical relationships could explain the interaction of these two pathways (Fig. 6D); Notch could potentially function upstream of Stat6, downstream of Stat6, or in an independent and parallel pathway. The above results indicate that Notch activation is sufficient to induce mucous metaplasia in the absence of Stat6, ruling out the first model. The second model has not been directly tested, but microarray analysis of gene expression changes in Stat6-dependent mouse asthma models reveals no significant change in Notch target genes, including Hes1, Hey1 and Hey2 (Kuperman et al., 2005). Additionally, we do not observe changes in the Stat6-target HNF-3β in NotchIC transgenic lungs. However, we do observe mucous cell differentiation in the absence of Stat6. In aggregate, these data support the model in which Notch and Stat6 signaling operate in parallel and independent pathways to regulate mucous metaplasia.

DISCUSSION

Notch and lung cell fate

The mechanisms involved in generating and maintaining cell-type diversity in the mammalian lung are poorly understood. We have demonstrated that the Notch pathway is involved in the modulation of lung cell types in both the developing and mature lung epithelium. Moreover, we found that Notch functions contextually, operating in different ways at different sites in the developing lung. Proximally, Notch acted in the airway to alter the proportions of ciliated and mucous cell fate in both the embryonic and adult epithelium (Fig. 7A). Distally, Notch activation appeared to prevent differentiation of lung-bud-tip progenitors into alveolar cells (Fig. 7B). In addition, ectopic Notch signaling robustly inhibited distal progenitor cell replication and only marginally increased rates of distal cell apoptosis.

Notch signaling prevents alveolar development

During early lung development, lung progenitors located at the distal lung-bud tip produce branching airways. After E16.5, branching is largely complete and distally located progenitors of the lung-bud tip produce the alveolar saccules. It is unknown whether proximal and distal progenitors of the lung-bud tip comprise a single population of cells or whether there are two distinct populations of progenitors. However, it is known that Hes1 is only weakly expressed distal to the bronchioalveolar duct junction during embryogenesis and in the adult lung. By contrast, we have shown that transgenic Notch misexpression results in Hes1 protein expression distal to the BADJ and also results in abnormal cysts comprised of aberrant Nkx2.1$^+$

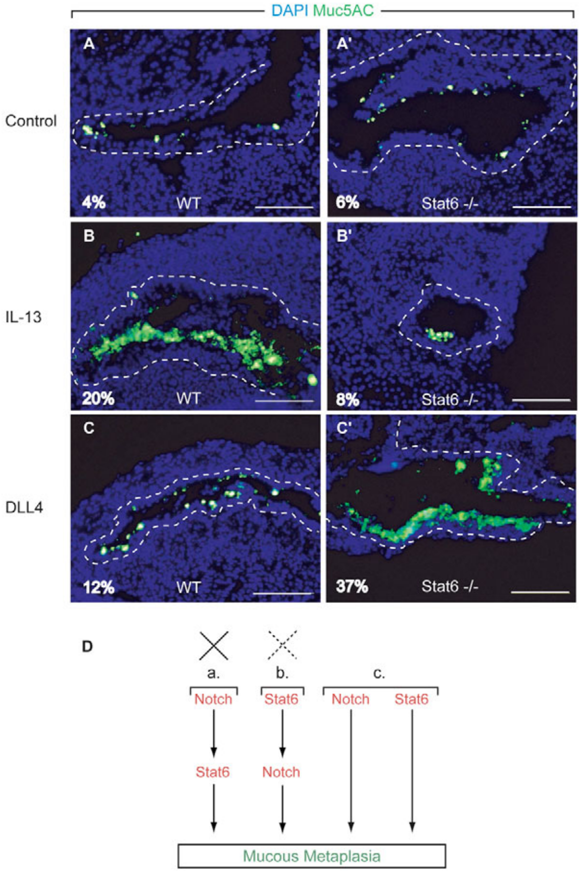

Fig. 6. Stat6 is not necessary for DLL4-induced mucous metaplasia. (**A-C**) Immunohistochemistry of control tracheas that were harvested at E14.5 and cultured for 7 days. Tracheas displayed normal, low levels of mucous differentiation, identified by Muc5AC staining (green) (A). Addition of IL13 (B) or DLL4 (C) induced mucous metaplasia. (**A′-C′**) Immunohistochemistry of *Stat6*$^{-/-}$ tracheas that were harvested at E14.5 and cultured for 7 days. Tracheas displayed normal, low levels of mucous differentiation (A′). Addition of IL13 (B′) failed to induce mucous metaplasia as predicted. Addition of DLL4 (C′) induced mucous metaplasia. (**D**) Possible models for the interaction of Notch and the IL13/Stat6 signaling pathway, which both result in mucous metaplasia. Notch signaling cannot be upstream of Stat6 activation as Notch-induced metaplasia occurs in *Stat6*-null trachea. Notch signaling might be downstream of Stat6 signaling, although this is unlikely because Stat6 activation is not associated with Notch target induction. Therefore, Notch and Stat6 signaling may represent two parallel pathways for inducing mucous metaplasia. Scale bars: 100 μm.

Ecadherin$^+$ HNF-3β$^+$-expressing epithelial cells in lieu of normal distal lung alveolar progenitor cells or normal differentiated alveolar type 1 and type 2 cells. We suggest that the normal downregulation of Notch in cells distal to the BADJ is necessary for the differentiation of type 1 and type 2 alveolar cells. Interestingly, the abnormal epithelial cells of transgenic cysts retained lung identity, as evidenced by Nkx2.1 expression, and a small subset of these cells co-expressed CC10 and Sox2, identifying them as differentiated airway Clara cells (data not shown).

In contrast to normal lung-bud-tip progenitors, the aberrant cystic epithelial cells did not proliferate and had small but significant increases in their rate of apoptosis. This finding is consistent with recent studies that show that Notch inhibition increases the rate of replication of distal lung progenitors (Tsao et al., 2008). We also observed that ectopic smooth muscle surrounds distal cystic epithelium. This might indicate that constitutive epithelial Notch signaling activates an epithelial program that instructs adjacent mesenchymal cells to differentiate into smooth muscle. Alternatively, Notch activation in distal epithelial progenitors might block an epithelial-to-mesenchymal signal that normally prevents distal smooth muscle differentiation.

The progenitor-progeny hierarchy in the mammalian lung remains poorly understood. Furthermore, it is unclear whether proximal and distal cells are derived from common or distinct progenitor populations, as no clonal analysis of lung-bud-tip cells has been definitively established. In a single lineage model, a proximal progenitor cell gives rise to the airway epithelium as the embryonic lung branches and develops. Later in development, the same progenitor would give rise to a distal progenitor, which would be responsible for generating distal alveolar cell types (Fig. 7C). In a dual-lineage model, proximal and distal progenitors would both be present early in lung development. Only the proximal progenitor would be active during branching morphogenesis, and later the distal progenitors would be activated and give rise to distal alveolar cells (Fig. 7C).

In either model, Notch misexpression prevents the execution of a distal differentiation program and modulates cell fate in the proximal differentiation program. However, in distal cystic cells, it is unclear what cell fate distal progenitor cells have adopted in response to ectopic Notch signaling. One possibility is that Notch signaling results in a proximalization of the distal lung such that distal cells have acquired a proximal fate. Such a model would predict the ectopic expression of proximally restricted markers in distal cysts. Indeed, cystic epithelial expression of E-Cadherin, Hes-1 and HNF-3β reflect proximal identity. However, the majority of cystic epithelial cells did not express markers of a completely differentiated airway epithelium such as CC10, β-tubulin or Muc5AC. A small population of cyst cells did, however, co-express SOX2 and CC10, indicating a fully executed Clara-cell differentiation program. Conversely, in airway epithelium, Notch misexpression induced mucous cell metaplasia, changing the relative proportions of differentiated cell fates that are normally present.

Whether the cysts represent proximalization of the distal lung progenitors remains unclear. However, cyst cells clearly lack markers of distal progenitor cells and distal alveolar cells. In addition, the cyst cells seem to have some characteristic markers of proximal epithelial cells but not others. They may represent arrested or trapped airway progenitor cells, partially differentiated proximal progenitor cells that have arrested, or cells which ordinarily would have acquired an alveolar differentiation program that have been so abnormally disrupted that their cell fate does not correlate to a recognizable cell type in the normal embryo. A genetic system that permits regulated misexpression of Notch using tetracycline activation would permit us to assess whether these Notch-affected cells are capable of re-expressing their progenitor markers and differentiating into alveoli. This approach has been successfully used in the pancreas to show that this is indeed the case (Stanger et al., 2005). Given the similarities in the branching morphogenesis of the pancreas and the lung (Zhou et al., 2007), we speculate that the Notch-induced cystic cells would represent arrested progenitors cells that could complete their normal alveolar differentiation program upon suppression of the ectopic Notch stimulus.

We further speculate that the ectopic SOX2 and CC10 cells may represent proximal airway epithelial cells that properly differentiated in the airway, but which were subsequently passively drawn into

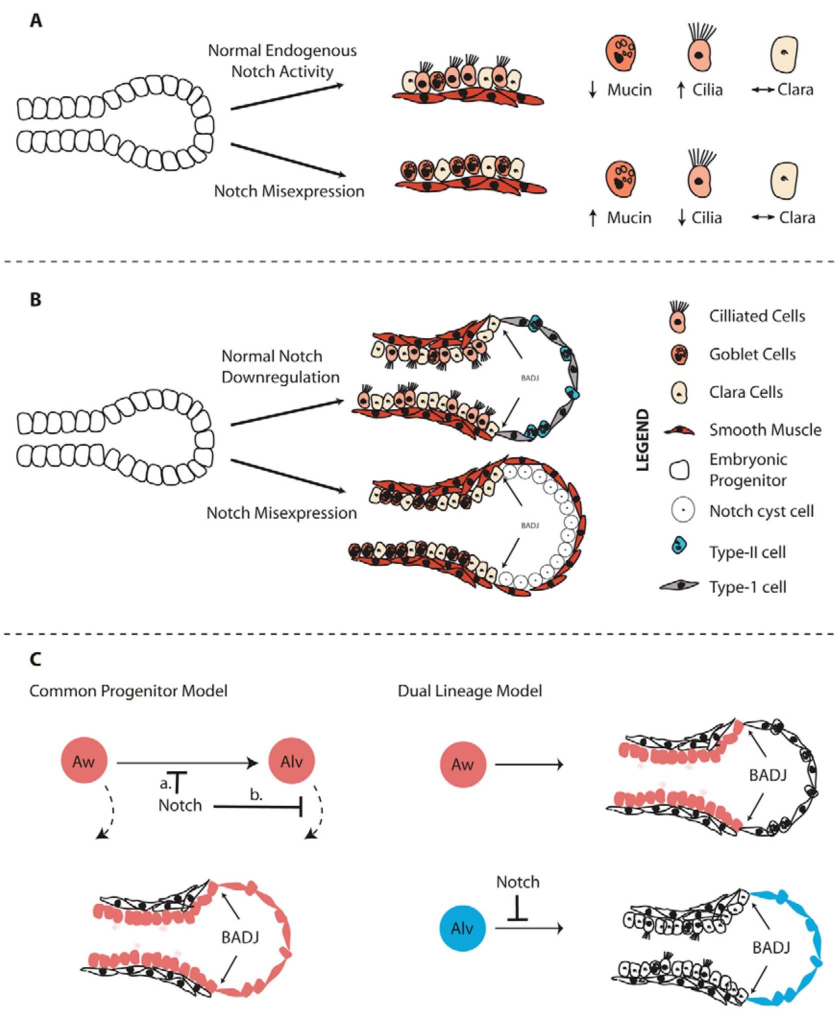

Fig. 7. Models of Notch action in mouse lung development. (**A**) Notch misexpression increases mucous cell differentiation and inhibits ciliated cell differentiation in proximal airway epithelium. (**B**) Notch downregulation is required for alveolar development. Constitutive Notch misexpression inhibits alveolar development and results in a dilated cystic epithelium. (**C**) Schematic representations of possible common and dual lineage progenitor models for lung development. In the common lineage model, a single progenitor lineage produces proximal and distal cell types. Notch signaling could block the transition from a proximal progenitor to a distal progenitor (a) or could block the differentiation of an already established distal progenitor (b) in this model. In the dual lineage model, in which there are distinct proximal and distal progenitors, Notch signaling may specifically block the differentiation of distal progenitors, whereas it would only modulate the specific cell-fate distribution of proximal progenitor progeny.

distal cysts. Alternatively, they may represent airway epithelial cells that differentiated from arrested progenitor cells. Assuming there is a single progenitor cell population for producing differentiated proximal and distal cells, this hypothesis would lead us to surmise that the distal cystic epithelial cells are trapped in a pre-airway state and have not yet initiated an alveolar program (Fig. 7C). The other possibility is that the progenitor cells have started their alveolar program, but are blocked from executing it due to Notch misexpression. This seems less likely due to the presence of some proximal markers in distal cystic cells. Notch misexpression and its effects on lung-bud-tip progenitors will be easier to interpret after single cell lineage analysis of the lung-bud-tip cells is available.

Notch and airway cell-fate choice

Notch promotes mucous cell production and decreases the number of ciliated cells in the mammalian airway. Constitutive Notch expression increased mucous cells and eliminated ciliated cells in our in vivo assay. Moreover, using agonists and antagonists of Notch signaling, we demonstrated that this effect can be reproduced in embryonic mouse tracheal cultures and adult human epithelial cell cultures.

The effect of Notch agonists and antagonists on adult human airway epithelial cells demonstrates that the very same developmental pathway can similarly regulate adult-cell- and embryonic-cell-fate choice in the airway. Notch, in the adult, may work on local, as of yet poorly characterized, progenitor cells in the adult airway epithelium to produce the correct proportions of ciliated and mucous cells. This raises the possibility that Notch may fine-tune and remodel cell-fate distribution in the airway after injury and during maintenance and repair. Further study is necessary to better define how this occurs. Genetic lineage tracing is necessary to identify the exact progenitor cells involved and their lineage relationships to differentiated airway cells. This will help define exactly which cells receive and produce Notch signals.

Notch, via Hes1, has been shown to act in a dichotomous fashion to specify neuroendocrine and non-neuroendocrine cell fates in the lung airway. Hes1 and Mash1 are known to work antagonistically to influence cell fate in several organ systems. In the lung, Hes1 expression is known to direct progenitors to a non-neuroendocrine fate. Neuroendocrine cells do not express Hes1, but instead express high levels of Mash1 that antagonize Hes1 expression (Borges et al., 1997; Ito et al., 2000). Molecularly, Notch signaling activates Hes1 expression, which binds and inhibits promoter regions of Mash1 (Ito et al., 2000). Notch, in this way, serves to inhibit neuroendocrine differentiation. We now demonstrate that Notch, among the non-neuroendocrine lineages, promotes mucous cell differentiation and inhibits the differentiation of ciliated cells in the airway epithelium (Fig. 7B).

How and why Notch has different effects on early (proximal) and late (distal) lung progenitors at different times during development remain entirely open questions. This difference may be a result of different cellular competences of early and late lung-bud progenitors to Notch signaling. Furthermore, as previously mentioned, airway and alveolar progenitors may consist of a single population or two distinct populations of progenitor cells. In addition, Wnts, Hhs and BMPs have all been previously demonstrated to cooperate with Notch. Each is expressed differently in the airway and alveolus. How these pathways operate in concert with Notch signals to regulate specific populations of progenitor cells is an open question and merits further study.

Implications for obstructive lung disease

Many obstructive lung diseases, including chronic bronchitis, cystic fibrosis and asthma, are characterized by mucous metaplasia and mucous hypersecretion, leading to airflow obstruction and increased susceptibility to infection. Irritant-induced cytokine release by T_H2 lymphocytes and IL13/Stat6-mediated secretory processes result in the excess creation of mucus and mucus-producing cells (Kuperman et al., 2002). In addition, there is a known inverse correlation between HNF3β expression and goblet cell hyperplasia in both mouse models of mucous metaplasia and human disease (Wan et al., 2004). These results suggest that Notch either acts independently of Stat6, the only previously described pathway that regulates mucous metaplasia, or downstream of the Stat6 pathway. Our work suggests that inhibitors of Notch signaling might act by a novel and dominant mechanism to decrease mucosecretion by decreasing mucous cell numbers and simultaneously improve mucociliary clearance by increasing ciliated cell numbers. Agents that work independently or downstream of the Stat6 pathway may provide new therapeutic targets in airway diseases associated with mucous metaplasia. Further study is necessary to better define the role of excessive Notch signaling in human airway diseases. The creation of lineage-specific CreER driver lines for Clara and basal cells will enable studies that determine which of these specific progenitor-cell populations are responsive to Notch signaling.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/10/1751/DC1

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