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RESEARCH ARTICLE

Jagged1 is the Major Regulator of Notch-Dependent Cell Fate in Proximal Airways

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Background: The Notch signaling pathway plays complex roles in developing lungs, including regulation of proximodistal fates, airway cell specification and differentiation. However, the specific Notch-mediated signals involved in lung development remain unclear. Results: Here we report that *Jagged1* is expressed in a subset of bronchial and bronchiolar epithelial cells, where it controls proximal airway cell fate and differentiation. In agreement with previous studies involving disruption of all Notch signaling, we found that deletion of *Jagged1* in airway epithelium increased the number of ciliated cells at the expense of Clara cells, a phenotype associated with downregulation of *Hes1*. Deletion of *Jagged1* also led to an increased number of pulmonary neuroendocrine cells (PNEC), suggesting that Jagged1/Notch signaling inhibits PNEC cell fate. As expected, *Jagged1* deletion did not affect alveolar cell differentiation, although alveolar septation was impaired, likely an indirect effect of proximal airway defects. Finally, in the postnatal lung, *Jagged1* deletion induced mucous metaplasia, accompanied by downregulation of *Hes1* and *Hes5*. Conclusions: Our results demonstrate that Jagged1-mediated Notch signaling regulates multiple cell fate decisions as well as differentiation in the respiratory system to coordinate lung development and to maintain a balance of airway cell types in adult life. *Developmental Dynamics 242:678–686, 2013*.

Key words: Jagged1; Notch; airway cell fate; mucous metaplasia; lung

Key Findings:

- Jagged1 regulates airway cell fate decision in the developing lung.
- Jagged1 prevents mucous metaplasia in the postnatal lung.
- Jagged1 influences alveologenesis.

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INTRODUCTION

Development of the mammalian respiratory system requires coordinated differentiation of multiple cell types that form conducting airways and alveoli, vasculature, as well as interstitial tissue. The Notch signaling pathway controls cell fate specification, differentiation, proliferation, as well as apoptosis during development. Notch genes encode transmembrane receptors that interact with membrane-bound DSL ligands of the Delta and Serrate/Jagged families. There are four Notch receptors (Notch1, 2, 3, 4) and five DSL ligands (Dll1, 3, 4, and Jagged1, 2) in mammals. Notch receptors and ligands, as well as Notch signaling modulators, are broadly expressed in developing lungs (Post et al., 2000; Kong et al., 2004; K. Xu et al., 2010), suggesting that Notch signaling may play a critical role(s) in lung development. Indeed, Notch signaling has been shown to promote proximal cell fates during early lung morphogenesis. In this regard, Notch

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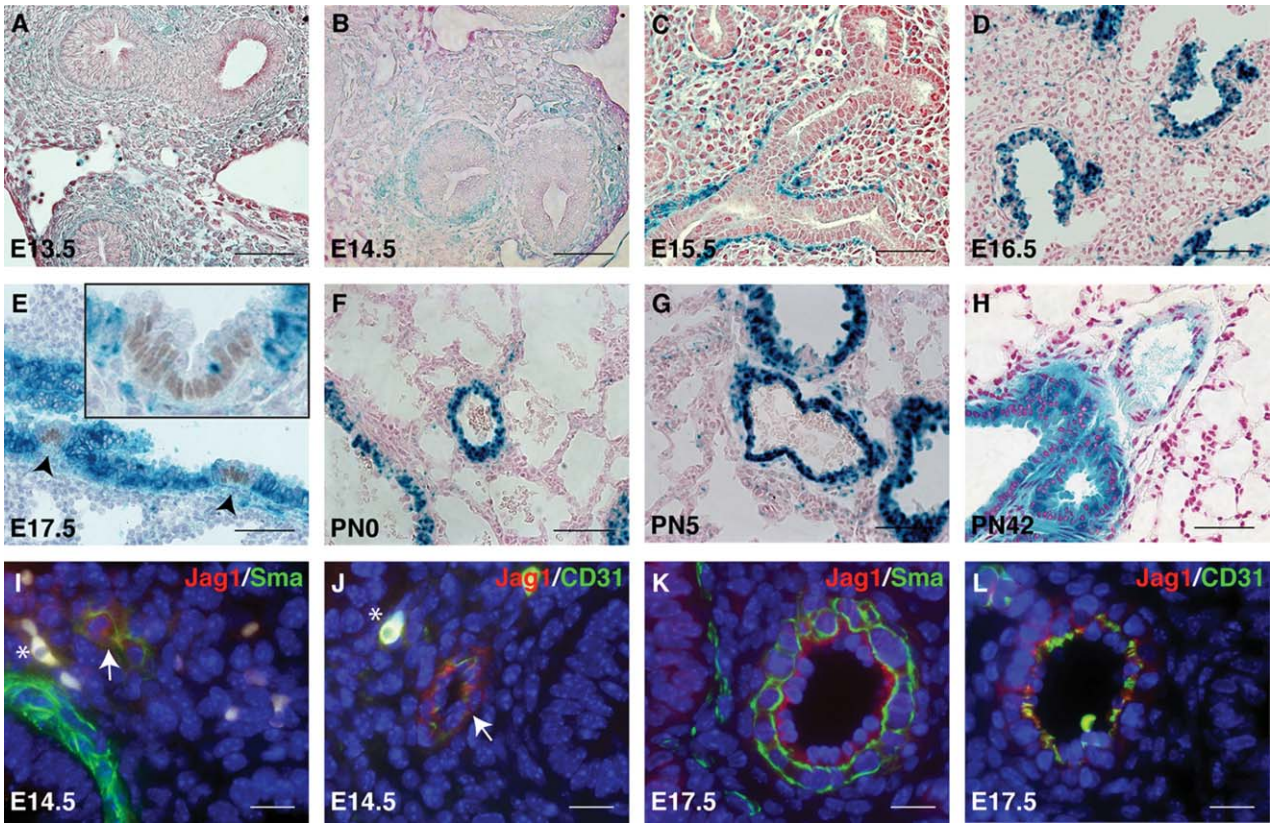

Fig. 1. Expression of *Jagged1* in the developing lung. **A–H:** Representative photomicrographs of X-galactosidase (X-gal) staining in lung sections from *Jag1*$^{\beta\text{-}Geo/+}$ mice at the indicated developmental stages. Double staining of X-gal (stained in blue) and anti-Mash1 (stained in brown) are shown in E. Neuroepithelial bodies (NEB) are indicated by arrowheads in E and shown in high-magnification image (inset in E). **I–L:** Immunofluorescence double staining of Jagged1 and smooth muscle actin-α (Sma) (I,K), Jagged1 and CD31 (J,L). Arrows point to cells showing co-staining of Jag1 and Sma (I), Jage1 and CD31 (J). *Indicates autofluorescence in red blood cells. Scale bars = 50 μm in A–H and 10 μm in I–L.

controls the balance of neuroendocrine versus non-neuroendocrine and ciliated versus secretory cell types in the developing airways, as well as to prevent mucous metaplasia in the postnatal lung (Ito et al., 2000; Shan et al., 2007; Tsao et al., 2008, 2009, 2011; Guseh et al., 2009; Morimoto et al., 2010, 2012). Recently, Notch was found to control differentiation of basally located adult airway stem cells (Rock et al., 2011). In the distal lung, Notch signaling is dispensable for alveolar epithelial differentiation and ectopic expression of constitutively active Notch1 or Notch3 inhibits differentiation and maturation of alveolar epithelium (Dang et al., 2003; Guseh et al., 2009). Despite this, *Lfng*-dependent Notch signaling is required for alveologenesis, likely through its effect on Notch-dependent myofibroblast differentiation (K. Xu et al., 2010). Thus, Notch plays multiple roles and functions at different stages of pulmonary development. While Notch2 appears to be the primary receptor for regulating the Clara/ciliated cell decision and Notch1, 2, and 3 function redundantly to repress NE specification (Morimoto et al., 2012), it is not known which ligand(s) is involved. Here we report that Jagged1 is highly expressed in proximal airways. Spatial-temporal deletion of *Jagged1* in the airway epithelium during mouse embryonic development causes altered cell fate specification with an excess of PNEC, as well as ciliated and mucosal cells, at the expense of Clara cells. Inactivation of Jagged1 also resulted in defective alveologenesis in the distal lung. Thus, Jagged1 functions during lung development to control Notch-dependent differentiation of most airway cell types.

RESULTS

Dynamic Expression of Jagged1 in the Developing Lung

We previously showed that Jagged1 is broadly expressed in proximal airway epithelial cells during the canalicular stage (E16.5–E17.5) of mouse lung development, whereas expression of Dll1 is restricted to the pulmonary neuroendocrine cells (K. Xu et al., 2010). Here we further investigated expression of Jagged1 throughout mouse lung development by monitoring β-Gal in *Jagged1*$^{\beta\text{-}Geo/+}$ knock-in mice (K. Xu et al., 2010). At embryonic day 13.5 (E13.5), β-Gal activity was mostly restricted to mesenchyme and a few scattered airway epithelial cells. This expression persists through E14.5, with positive cells mostly found surrounding airway epithelium (Fig. 1A,B). Immunofluorescence double staining showed co-localization of Jagged1 with the endothelial cell marker CD31, and in some mesenchymal cells, with smooth muscle actin α (Sma) (Fig. 1I,J). At E15.5, β-Gal expression remained high in mesenchymal cells immediately adjacent to conducting airways, but was not seen in distal buds. Indeed, β-Gal-positive cells were present in blood

vessels but not in airway epithelium (Fig. 1C). By E16.5, reporter expression turned on in bronchial and bronchiolar epithelium (Fig. 1D). At this stage, Jagged1 expressers are thought to be ciliated cells, juxtaposed with Notch1-expressing Clara cells (Tsao et al., 2009). Interestingly, X-gal staining is not seen in cells immediately adjacent to neuroendocrine cells of the neuroepithelial body (NEB) (Fig. 1E, inset). Clara cells associated with fetal NEBs (also called SPNC/Clara-like cells) represent a distinct set of progenitor cells that can self-renew or give rise to Clara and ciliated cells in the adult lung (Guha et al., 2012; Morimoto et al., 2012). Notably, SPNC/Clara-like cells, which express Notch1, Notch3, and probably Notch2, and directly contact Dll1-expressing PNECs (Ito et al., 2000; K. Xu et al., 2010; Guha et al., 2012; Morimoto et al., 2012), may also contact Jagged1-expressing cells at the border of the NEB. Double staining was also used to test for expression of Jagged1 in vascular cells. High-level Jagged1 expression co-localized with CD31, while low-level Jagged1 immunoreactivity was detected in smooth muscle cells (Fig. 1K,L). During the saccular stage of lung development (E18.5 to postnatal day 5), β-Gal activity was very weak or undetectable in the distal lung, while vascular smooth muscle cells and endothelial cells showed robust staining. This pattern of Jagged1 expression continued beyond birth and into adulthood (Fig. 1F–H). We also detected Jagged1 expression by immunohistochemistry, confirming our β-Gal gene-reporter-based expression data (Fig. 2A). Thus, Jagged1 expression in the developing lung is dynamic, switching from mesenchymal cells to airway epithelium. The most prominent expression domain seen in airway epithelium started at approximately E16.5 suggesting that Jagged1 may play an important role(s) in proximal airway development. Since multiple Notch receptors are expressed in developing airway cells, and in many cases these cells are in direct contact with Jagged1-expressing neighbors, Jagged1-dependent Notch signaling could regulate cell fate specification and differentiation of multiple airway cell types.

Jagged1 Mutation Causes Altered Cell Fate Specification in Proximal Airways

To define the function of epithelial-expressed Jagged1 in developing lungs, we used the *Spc-rtTA; Tet-O-Cre* airway-specific and inducible bitransgenic system (Mancini et al., 2005; Perl et al., 2002). The Spc promoter drives transgene expression specifically in lung endoderm, first in progenitor cells of primary lung buds, and later at higher levels in type II alveolar cells of the distal lung (Okubo and Hogan, 2004). Upon systemic administration of a tetracycline analog, doxycycline (Dox), the rtTA/Dox complex activates expression of Cre recombinase, which can trigger deletion of *Jagged1* exclusively in airway epithelial cells of $Jag1^{flox/flox}$*; Spc-rtTA; Tet-O-Cre* triple transgenic mice (hereafter referred to as $Jag1^{cKO}$). We treated pregnant females with Dox from E7.5 to E17.5 so that *Jagged1* would be deleted in airway cells of $Jag1^{cKO}$ embryos. Indeed, anti-Jagged1 immunostaining showed decreased expression in proximal airways, but not in arteries of E17.5 $Jag1^{cKO}$ mutant lungs (Fig. 2A,B). The decreased level of Jagged1 was confirmed by real-time RT-PCR (Fig. 3A,B). We next examined cell fate and differentiation in proximal airways of E17.5 $Jag1^{cKO}$ lungs by staining for molecular markers of each cell type. Compared to $Jag1^{flox/flox}$*; Spc-rtTA* controls, the $Jag1^{cKO}$ lung contained far fewer secretory Clara cells (stained positive for CC10) in bronchi and bronchioles (Fig. 2C,D,M). In contrast, deletion of *Jagged1* caused an increase in the number of ciliated cells (marked by β-tubulin) in proximal airways (Fig. 2E,F,M). Thus, Jagged1 controls the balance between secretory and ciliated cells in the developing airway. Interestingly, this phenotype is similar to that seen in knockouts for *Rbpjκ or Pofut1*, which completely disrupt all canonical Notch signaling, as well as in the *Notch2* mutant (Tsao et al., 2009; Morimoto et al., 2012). In developing airways, neuroendocrine (NE) differentiation depends on basic helix-loop-helix (bHLH) transcription factors. In this context, Dll1-Notch signaling is thought to regulate the NE versus non-NE cell fate decision (Ito et al., 2000). We, therefore, analyzed pulmonary neuroendocrine cell (PNEC) differentiation in $Jag1^{cKO}$ mutants using anti-Mash1/Ascl1 and anti-CGRP immunostaining (Fig. 2G–L). $Jag1^{cKO}$ lungs showed a modest increase in the number of NEBs located within bronchi/bronchioles while the number of PNECs per NEB was not affected. In contrast, there was a significant increase in the number of $CGRP^{+}$ cells in distal lungs of $Jag1^{cKO}$ mice (Fig. 2M). This increased number of PNECs reveal that Jagged1 is involved in regulating NE versus non-NE cell fate in developing airways, likely through induction of Hes1, a bHLH transcription factor and downstream target of canonical Notch signaling (Ito et al., 2000; Morimoto et al., 2012). Indeed, a survey of gene expression at E17.5 by quantitative RT-PCR showed that *Hes1* is the most downregulated gene among selected Notch signaling pathway targets in $Jag1^{cKO}$mutant lungs (Fig. 3A). In addition, the nuclear Hes1 seen in control airways is barely detectable in $Jag1^{cKO}$ mutants (Fig. 3C,D).

Despite an imbalance in proximal airway cell types, the vast majority of $Jag1^{cKO}$ mice survived into adulthood. We next examined the balance of cell types in conducting airways of adult $Jag1^{cKO}$ lungs. A decreased percentage of Clara cells persisted in adult mutant lungs (Fig. 4A,B,G). Interestingly, anti-Muc5AC immunostaining and Alcian Blue staining showed that $Jag1^{cKO}$ mutant mice developed mucous metaplasia in the conducting airway, starting from 6 weeks of age (Fig. 4C–G). Thus, deletion of *Jagged1* caused an increased production of goblet cells at the expense of Clara cells. This result is in agreement with the finding that Notch signaling through Hes5 restricts mucin gene expression (Tsao et al., 2011). Indeed, quantitative RT-PCR revealed decreased expression of Hes1 and Hes5 in the $Jag1^{cKO}$ mutant lung at 4 weeks after birth (Fig. 3B). When compared to *Pofut1* conditional knockouts, using a *Tgfb3-Cre* deleter (Tsao et al., 2011), the mucous metaplasia observed in $Jag1^{cKO}$mutant

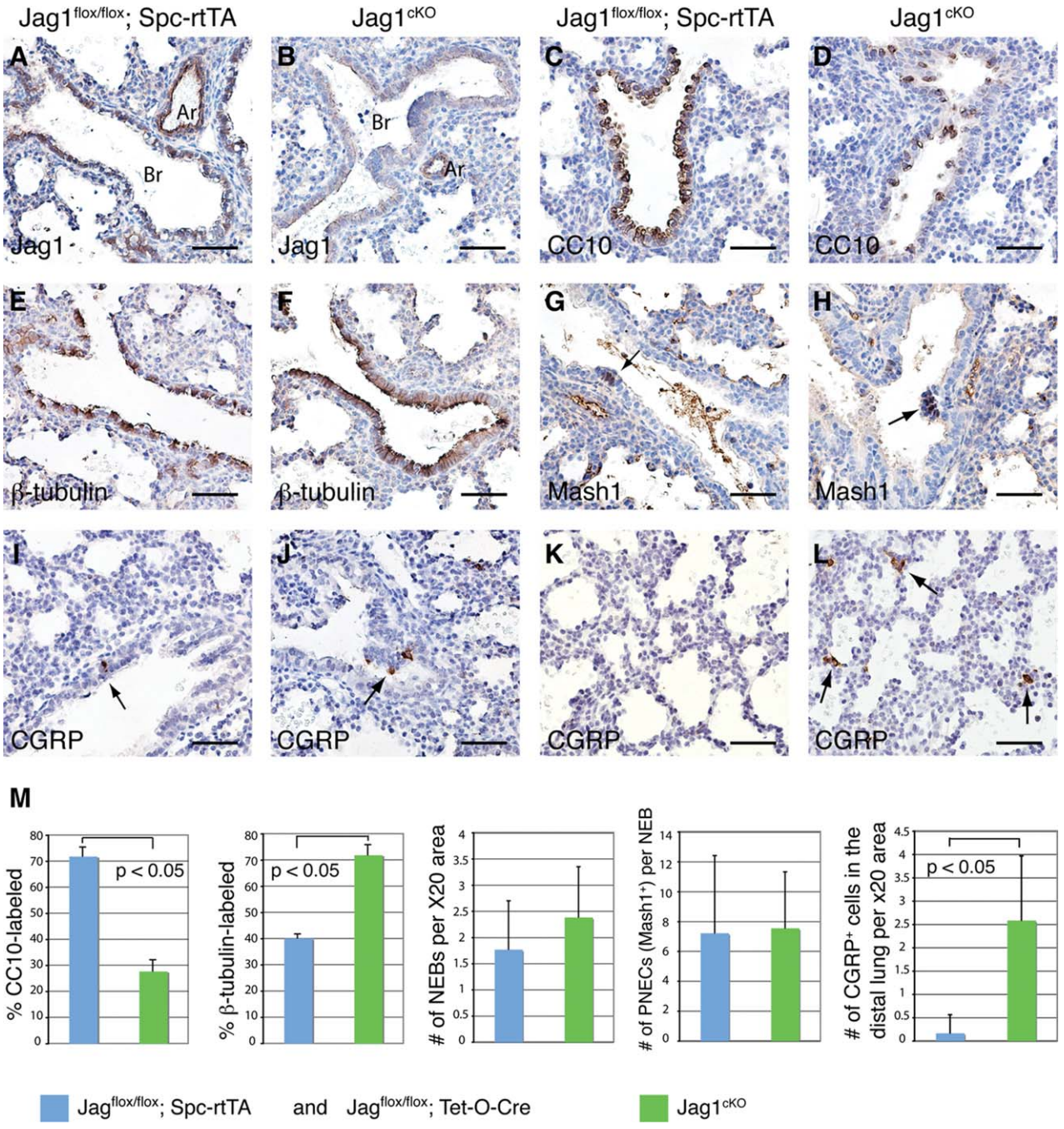

Fig. 2. Deletion of *Jagged1* in lung epithelium disrupts the balance of proximal airway cell types. **A–L:** Representative photomicrographs of immunohistochemistry for Jagged1, CC10, β-tubulin, Mash1, and CGRP in E17.5 lung sections of *Jag1$^{flox/flox}$; Spc-rtTA* (**A,C,E,G,I,K**) and *Jag1^{cKO}* mice (**B,D,F,H,J,L**) treated with Doxycycline from E7.5 to E17.5. CGRP staining in the distal lung are shown in K and L. Br: bronchiole; Ar: artery. Arrows point to neuroepithelial bodies (NEB) in G–J. Scale bars = 50 μm. **M:** Quantification of labeled cells. CC10$^+$ and β-tubulin$^+$ cells are quantified and normalized as percentage of total cells in bronchi/bronchioles. NEBs are quantified in ×20 magnification photographs (n = 13) of proximal airways. Mash1$^+$ PNECs are quantified per NEB. Numbers of CGRP$^+$ cells in the distal lung are quantified in ×20 magnification photographs (n = 12). Control mice included *Jag1$^{flox/flox}$; Spc-rtTA* and *Jag1$^{flox/flox}$; Tet-O-Cre*. Data are presented as mean ± standard deviation derived from at least three animals of each group. *P* values are calculated using the Student's *t*-test.

mice appeared less severe. This may well be due to relatively low efficiency of Cre induction in the bi-transgenic system, activated in embryos via placental transfer of doxycycline. Some pulmonary progenitor cells may escape Dox-induced deletion of *Jagged1* during embryonic development, and give rise to progeny cells carrying wild-type *Jagged1* in conducting airways of adult lung.

Defective Alveolar Septation in *Jag1^{cKO}* Mutant Lungs

By the canalicular/early saccular stage of lung development, Jagged1 expression is confined to bronchial

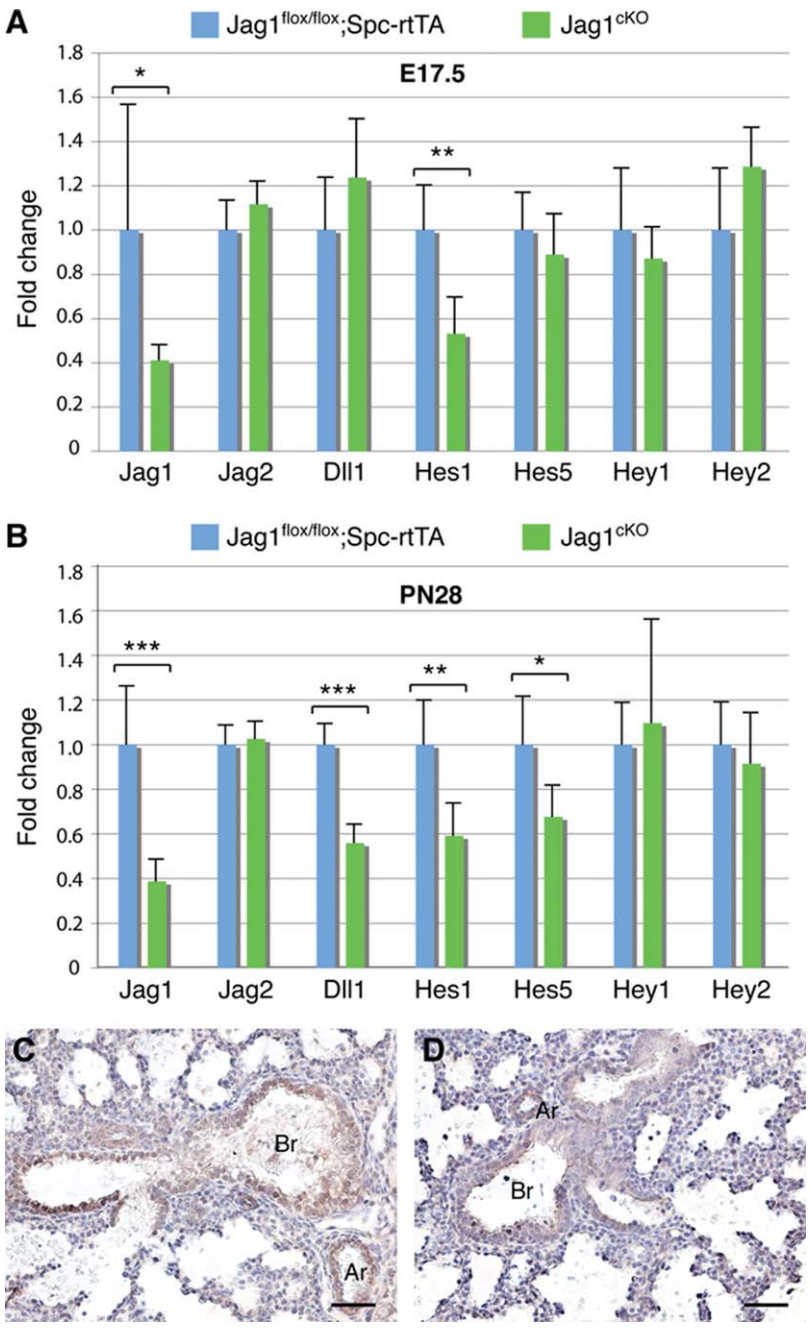

Fig. 3. Altered expression of Notch signaling pathway genes in *Jag1*^{cKO} mutant lungs. **A,B:** Real-time RT-PCR of *Jag1, Jag2, Dll1*, and selected Notch target genes in *Jag1*$^{flox/flox}$*; Spc-rtTA* and *Jag1*^{cKO} mutant lungs at E17.5 (A) and postnatal day 28 (B). Animals were treated with Dox the same as in Figure 2. Data are presented as mean ± standard deviation (n = 3 per group). *P* values are calculated using the Student's *t*-test. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005. **C,D:** Representative photomicrographs of anti-Hes1 immunostaining in E17.5 lung sections of *Jag1*$^{flox/flox}$*; Spc-rtTA* (C) and *Jag1*^{cKO} mice (D). Br: bronchiole; Ar: artery. Scale bars = 50 μm.

and bronchiolar epithelia, and deletion of *Jagged1* caused an imbalance of cell types within proximal airways. Next, we examined distal lung development in the *Jag1*^{cKO} mutant mice. Immunostaining of E17.5 lung sections from *Jag1*^{cKO} mutants showed grossly normal expression of type II alveolar cell (SPC) and type I alveolar cell (T1α/podoplanin) markers. While *Jag1*^{cKO} mutants appear to have slightly thicker alveolar walls, the percentage of SPC^{+} type II cells (out of the total number of distal lung cells) is similar in mutant (15%) and control (14%) lungs (Fig. 5A,B). The continuous lining of T1α^{+} cells on the apical side of each alveolus suggests that proper differentiation and maturation of type I cells occurs in mutant lungs (Fig. 5C,D). Capillaries are an integral component of alveoli. Given that Notch signaling controls vascular development (Herbert and Stainier, 2011), we examined the capillary network in *Jag1*^{cKO} mutant lungs. As shown in Figure 5E,F, anti-CD31 immunostaining revealed no obvious abnormalities in microvasculature. Thus, Jagged1-mediated Notch activation is dispensable for alveolar epithelial cell differentiation, and distal lung development seems to proceed normally in prenatal *Jag1*^{cKO} mutant lungs. However, in 6 out of 9 *Jag1*^{cKO} mutants examined, alveolar septation was disrupted to some degree. For instance, one week after birth, some mutant lungs showed impaired alveolarization, with relatively large alveolar saccules and a decreased number of growing septae. By 3 weeks of age, the majority of mutant lungs exhibited an emphysema-like phenotype due to largely failed secondary septation of alveoli (Fig. 5K–P, U). PDGFRα signaling controls myofibroblast differentiation, which is required for alveolarization in the distal lung (Bostrom et al., 1996; Lindahl et al., 1997; Kimani et al., 2009). We found similar staining intensity and distribution of PDGFRα and smooth muscle actin-α, markers for myofibroblast progenitors and differentiated myofibroblasts, in *Jag1*^{cKO} and control lungs (Fig. 5G–J), indicating that deletion of *Jagged1* had no obvious effect on myofibroblast differentiation. To rule out the possibility that Dox-rtTA toxicity caused alveolar defects in *Jag1*^{cKO} mice (Morimoto and Kopan, 2009), we conducted additional loss-of-Jag1 experiments. When Dox were fed from E7.5 to E15.5, *Jag1*^{cKO} mutants still showed defective alveolar septation, while *Jag1*$^{flox/flox}$*; Spc-rtTA* controls appeared normal (Fig. 5Q,R). We also examined whether Dox-rtTA toxicity interferes with alveologenesis by feeding Dox from E16.5 to postnatal day 10. Under these conditions, all three *Jag1*$^{flox/flox}$*; Spc-rtTA* lungs examined by H&E staining showed normal alveolar structure (Fig. 5S,T,U). We conclude that Dox-rtTA toxicity does not

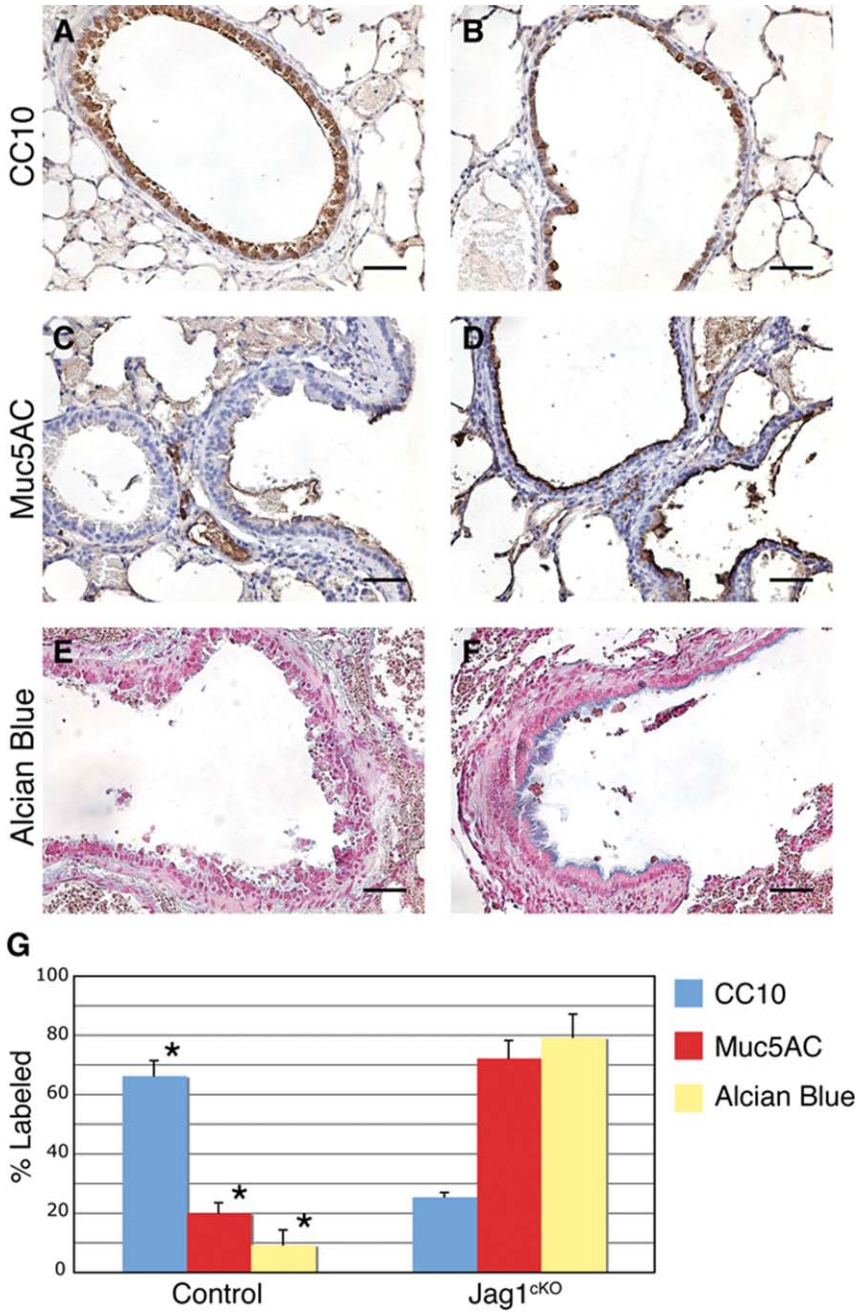

Fig. 4. Decreased number of Clara cells and mucous metaplasia in adult *Jag1*^{cKO} mutant lungs. **A–F:** Representative photomicrographs of anti-CC10, anti-Muc5AC immunostaining, and Alcian Blue staining in lung sections of *Jag1*$^{flox/flox}$; *Spc-rtTA* (A,C,E) and *Jag1*^{cKO} (B,D,F) mice at 6 months of age. Animals were treated with Dox the same as in Figure 2. Scale bars = 50 μm. **G:** Quantification of the labeled cells. Positively stained cells are quantified and normalized as percentage of total cells in the bronchi. Data are presented as mean ± standard deviation derived from at least two animals of each genotype. *P* values are calculated using the Student's *t*-test. *$P < 0.05$.

contribute significantly to alveolar defects observed in the *Jag1*^{cKO} mutants. Perhaps our mice were less susceptible to Dox-rtTA toxicity because they were on a mixed genetic background that differed from the background tested by Morimoto and Kopan. Interestingly, deletion of *Jagged1* in the alveolar epithelial cells only (by administration of Dox starting at E16.5 or E17.5) had no effect on alveolar development (Fig. 5S,T,U and data not shown), indicating that the Jagged1-mediated signal that directly or indirectly influences alveolarization is likely coming from proximal airway cells.

DISCUSSION

Multiple Notch receptors and ligands are expressed in the developing lung, and canonical Notch signaling has been shown to regulate airway lineage specification and differentiation. Here we show that Jagged1 is required for correctly balanced cell fate specification in conducting airways. Interestingly, Jagged1 appears to regulate multiple cell fate decisions in the lung epithelial hierarchy (summarized in Fig. 6). PNEC is one of the first specified cell types during airway morphogenesis. Dll1-mediated "lateral inhibition" is thought to control the NE versus non-NE cell fate balance (Ito et al., 2000). However, we report here that deletion of *Jagged1* in non-NE cells caused an increased number of NE cells to form. Since Jagged1 is expressed in cells juxtaposed to SPNC cells, it is likely that Jagged1 can activate Notch in SPNC cells and prevent them from adopting an NE fate. Currently, we do not have a clear explanation for why the *Jag1*^{cKO} shows ectopic CGRP^{+} cells in the distal lung. Also, during subsequent airway epithelial development, Jagged1 is expressed in ciliated cells, while Notch1 and Hes1 are highly expressed in non-ciliated cells, often in an alternating pattern (Tsao et al., 2009). Here we report that deletion of Jagged1 caused excessive ciliated cells to form at the expense of Clara cells, supporting a lateral inhibition model whereby Jagged1 in ciliated cells activates Notch receptors in neighboring cells and thereby blocks a default ciliated fate (Morimoto et al., 2010, 2012). In the distal lung, deletion of *Jag1* had no effect on differentiation and maturation of alveolar epithelial cells. However, *Jag1*^{cKO} lungs showed defective alveolar septation. Unlike *Lfng* null mice, *Jag1*^{cKO} mice show timely differentiation of myofibroblasts, and a less severe alveolar phenotype. Given that Fringe facilitates Delta-mediated Notch activation and inhibits Jagged/Serrate-mediated Notch signaling (Haines and Irvine, 2003), loss of *Lfng* and loss of *Jag1* would represent very

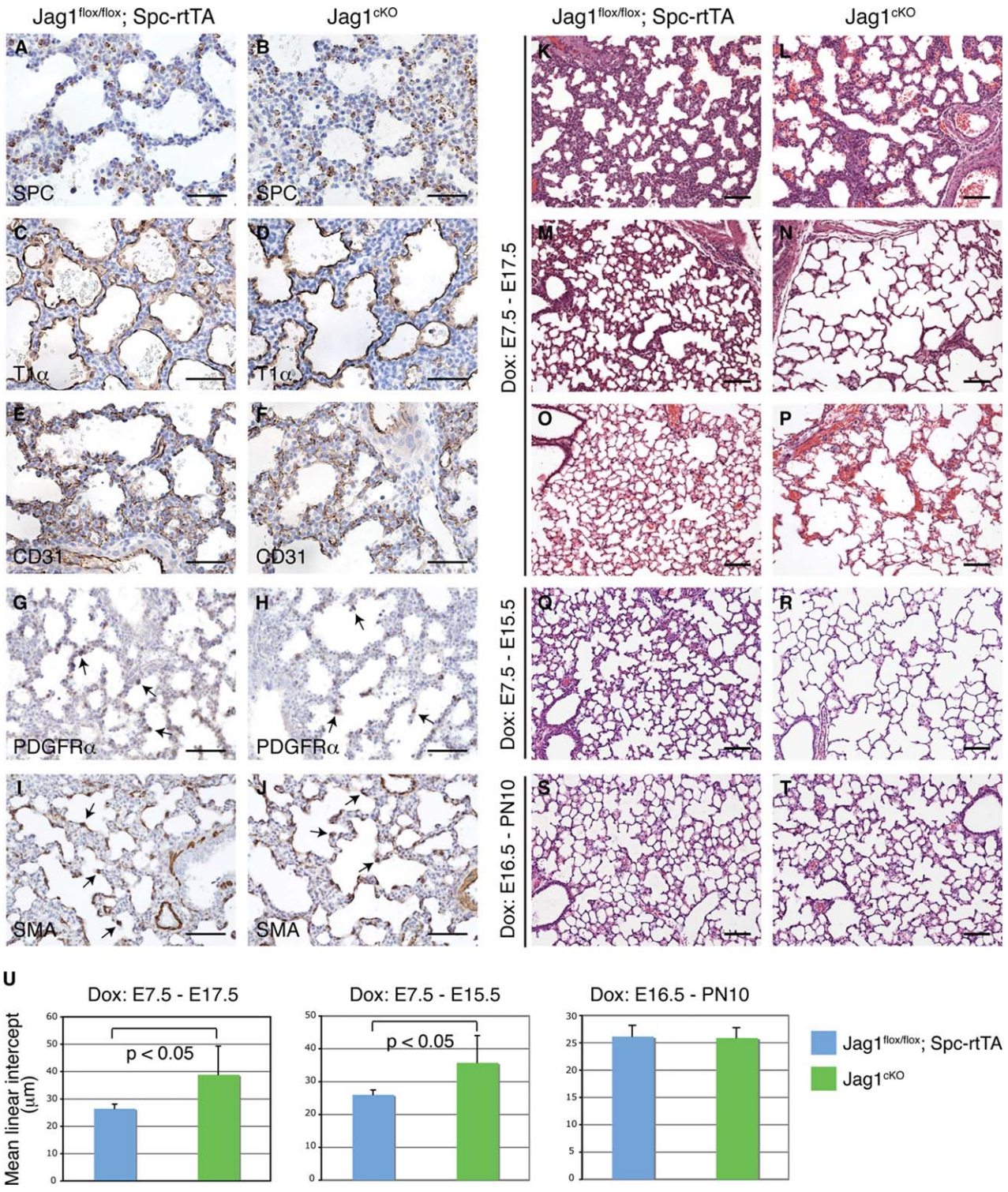

Fig. 5. Deletion of *Jagged1* in lung epithelium results in defective alveolarization despite grossly normal differentiation of distal lung cells. **A–J:** Representative photomicrographs of anti-SPC (A,B), anti-T1α (C,D), anti-CD31 (E,F), anti-PDGFRα (G,H) immunostaining in the *Jag1$^{flox/flox}$; Spc-rtTA* and *Jag1^{cKO}* lung sections at E17.5, and anti-smooth muscle actin-α (SMA) staining at postnatal day 7 (I,J). Dox was administered from embryonic day 7.5 to 17.5. **K–P:** Representative H&E-stained lung sections from *Jag1$^{flox/flox}$; Spc-rtTA* (K,M,O) and *Jag1^{cKO}* (L,N,P) mice at 1 week (K,L), 3 weeks (M,N), and 7 weeks (O,P) old. Animals were treated with Dox from E7.5 to E17.5. **Q,R:** Representative H&E staining of lung sections from *Jag1$^{flox/flox}$; Spc-rtTA* (Q) and *Jag1^{cKO}* (R) mice at 3 weeks of age with Dox treatment from embryonic day 7.5 to 15.5. **S,T:** Representative H&E staining of lung sections from *Jag1$^{flox/flox}$; Spc-rtTA* (S) and *Jag1^{cKO}* (T) mice at 3 weeks of age with Dox treatment from embryonic day 16.5 to postnatal day 10. **U:** Mean linear intercepts measured in lung sections from adult mice with Dox treatment of E7.5–E17.5, E7.5–E15.5, and E16.5–PN10. Values are means ± s.d. *P* values are calculated using the Student's *t*-test. Scale bars = 50 μm in A–J and 100 μm in K–T.

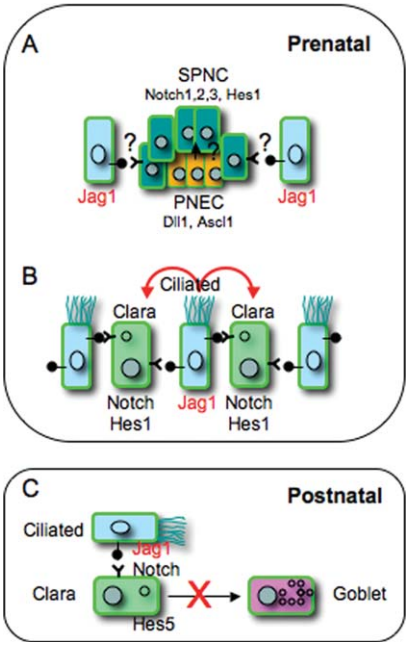

Fig. 6. Model for roles of Jagged1 in airway epithelial cell differentiation. **A:** During prenatal lung development, neuroendocrine (NE) versus Non-NE cell fate determination in the proximal airway is controlled by Notch signaling. In this context, both Jagged1 in cells flanking the NEB and Dll1 in PNECs may activate Notch in SPNC cells to prevent them from adopting NE fate. **B:** The cell fate decision between Clara and ciliated cells is mediated by Jagged1-dependent lateral inhibition, whereby Jagged1, expressed in ciliated cells, activates Notch in neighboring cells to suppress ciliated fate and promote Clara cell differentiation. **C:** In postnatal airways, Jagged1-mediated Notch signaling in Clara cells restricts goblet cell differentiation through Hes5. See the text for details.

different perturbations of the system, likely explaining the fairly different defect in alveolar development observed in each mutant. Intriguingly, Lfng is highly expressed in PNECs while Jagged1 expression is found surrounding the NEB. A previous report on NeuroD function during lung development suggested an intimate connection between the neuroendocrine compartment and distal lung development (Neptune et al., 2008). In this context, both Lfng and Jagged1 may well function within the NEB niche to send signals for proper distal lung morphogenesis.

In the postnatal lung, Notch signaling has been shown to prevent mucous metaplasia through bHLH transcription repressor Hes5 (Tsao et al., 2011). We observed an increased number of goblet cells in adult *Jag1*^{cKO} lungs, suggesting that Jagged1 contributes to suppression of goblet cell fate and mucin production. It would be interesting to determine whether Jagged1/Notch signaling functions to suppress transdifferentiation in adults, or if it controls lung fate specification only via lateral inhibition during development. Interestingly, deletion of *Jagged1* caused decreased expression of *Dll1*, as well as two Notch target genes, *Hes1* and *Hes5*, in adult lung tissue (Fig. 3D). Down-regulation of notch pathway genes, most significantly Dll1 and Hes5, was previously reported in human airway epithelium associated with smoking and chronic obstructive pulmonary disease (COPD) (Tilley et al., 2009). Our results further support the notion that Notch signaling may well actively maintain a balance of cell types lining conducting airways to prevent COPD in adult life.

EXPERIMENTAL PROCEDURES

Mouse Experiments

Mice were housed under standard condition and all animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. *Jagged1*^{flox} mice have been described previously (Mancini et al., 2005). To achieve deletion of *Jagged1* in the pulmonary epithelium, we used a tetracycline-inducible "Tet-On" transgenic system including two transgenic mouse lines, *Spc-rtTA* and *Tet-O-Cre* (Perl et al., 2002). For the deletion of *Jagged1* throughout the lung epithelium during development, pregnant females were administered with doxycycline-containing chow (0.625 g/Kg; Harlan-Teklad, Madison, WI) from day 7.5 to 17.5 of pregnancy. For the deletion of *Jagged1* in the distal lung epithelial cells only, doxycycline diet was given from pregnant day 17.5 to postnatal day 10.

Quantitative RT-PCR

Total RNA was prepared from lung tissues of control and *Jag1*^{cKO} mutant mice using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR was performed on a BioRad CFX96 Real-Time System using the RT^2 SYBR Green qPCR Master Mixes (Qiagen). We used the primer sequences for *Jag1, Jag2, Dll1, Hes1, Hes5, Hey1, Hey2, Ascl1*, and *Cgrp* as previously reported (J. Xu et al., 2010; Tsao et al., 2011; Voronova et al., 2011; Morimoto et al., 2012). The relative abundance of mRNA for each gene to GAPDH was determined by the equation $2^{-\Delta CT}$, where $\Delta CT = CT_{\text{Tested Gene}} - CT_{\text{GAPDH}}$. Data were derived from three animals per group (triple reactions for each sample).

Tissue Preparation, Histology, and Morphometry

Lung tissues at embryonic day 17.5 and postnatal day 7 were dissected and fixed in 10% buffered neutral formalin overnight and subsequently embedded in paraffin according to standard procedures. For adult lung tissues, inflation with 10% formalin at a constant fluid pressure of 25 cm H2O for 5 min was performed before fixation and embedding, as previously described (Braber et al., 2010). Five-micrometer-thick sections were stained with hematoxylin and eosin (H&E). Representative images of H&E staining were acquired with a Nikon Eclipse 80i microscope. Mean linear intercepts were measured as previously described (K. Xu et al., 2010).

X-Gal Staining

For X-gal staining, lung tissues were fixed in 2.7% formaldehyde, 0.02% Nonidet-P40 in PBS overnight, washed and infused with 30% sucrose, then embedded in OCT compound. Ten-micrometer-thick cryosections were stained with X-gal at 37°C for a few hours to overnight. Stained sections were further counterstained with eosin. In some experiments, lung tissues were whole-mount stained with X-gal, fixed in formalin, and embedded for paraffin sections, then counterstained with neutral red.

Immunohistochemistry

Immunostaining of lung sections was carried out as previously described (K. Xu et al., 2010). Staining was

performed on two sections per lung from at least three animals for each group. Presented are representative photomicrographs. Primary antibodies used for immunostaining were as follows: Jagged1 (Santa Cruz, Santa Cruz, CA: sc-6011, 1:100), Hes1 (Abcam, Cambridge, MA; ab71559, 1:200), smooth muscle actin-α (Abcam, ab5694, 1:400), CD31 (Abcam, ab28364, 1:50), PDGFRα (Santa Cruz, sc-338, 1:100), CC10 (Santa Cruz, sc-25555, 1:200), SPC (Santa Cruz, sc-13979, 1:200), CGRP (Santa Cruz, sc-8857, 1:150), Muc5AC (Santa Cruz, sc-21701, 1:50), T1α (University of Iowa, DSHB, Iowa City, IA; 1:1,000), Mash1 (BD Biosciences, San Jose, CA: 556604, 1:50), and β-tubulin (Sigma, St. Louis, MO; T7941, 1:200).

Statistics

All data are presented as mean ± standard deviation. Statistical analysis was performed using the two-tailed Student's *t*-test. *P* value of 0.05 or less was considered significant.

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