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 seption 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. © 2013 Wiley Periodicals, Inc.

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.

Accepted 17 March 2013

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
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, Lfung-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 Jagged1β-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–H). Immunofluorescence double staining showed localization of Jagged1 with the endothelial cell marker CD31 (I–L). Arrows point to cells showing co-staining of Jag1 and Sma (I), Jagged1 and CD31 (J). *Indicates autofluorescence in red blood cells. Scale bars = 50 μm in A–H and 10 μm in I–L.
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 bi-transgenic 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 Jag1flax/fox; Spc-rtTA; Tet-O-Cre triple transgenic mice (hereafter referred to as Jag1KO). We treated pregnant females with Dox from E7.5 to E17.5 so that Jagged1 would be deleted in airway cells of Jag1KO embryos. Indeed, anti-Jagged1 immunostaining showed decreased expression in proximal airways, but not in arteries of E17.5 Jag1KO 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 Jag1KO lungs by staining for molecular markers of each cell type. Compared to Jag1flax/fox; Spc-rtTA controls, the Jag1KO 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 Rbpj 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 Jag1KO mutants using anti-Mash1/Ascl1 and anti-CGRP immunostaining (Fig. 2G–I). Jag1KO 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 Jag1KO 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 Jag1KO mutant lungs (Fig. 3A). In addition, the nuclear Hes1 seen in control airways is barely detectable in Jag1KO mutants (Fig. 3C,D).

Despite an imbalance in proximal airway cell types, the vast majority of Jag1KO mice survived into adulthood. We next examined the balance of cell types in conducting airways of adult Jag1KO lungs. A decreased percentage of Clara cells persisted in adult mutant lungs (Fig. 4A,B,G). Interestingly, anti-Muc5AC immunostaining and Alcin Blue staining showed that Jag1KO 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 Jag1KO 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 Jag1KO mutant
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 JaglKO Mutant Lungs

By the canalicular/early saccular stage of lung development, Jagged1 expression is confined to bronchial...
and bronchiolar epithelia, and deletion of Jagged1 caused an imbalance of cell types within proximal airways. Next, we examined distal lung development in the Jag1cKO mutant mice. Immunostaining of E17.5 lung sections from Jag1cKO mutants showed grossly normal expression of type II alveolar cell (SPC) and type I alveolar cell (T1α/podoplanin) markers. While Jag1cKO 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 Jag1cKO 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 Jag1cKO mutant lungs. However, in 6 out of 9 Jag1cKO 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 (Boström 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 Jag1cKO 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 Jag1cKO mice (Morimoto and Kopan, 2009), we conducted additional loss-of-Jag1 experiments. When Dox were fed from E7.5 to E15.5, Jag1cKO mutants still showed defective alveolar septation, while Jag1flox/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 Jag1flox/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
contribute significantly to alveolar defects observed in the Jag1cKO 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 Jag1cKO 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, Jag1cKO lungs showed defective alveolar septation. Unlike Lfng null mice, Jag1cKO mice show timelier 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\textsuperscript{flox/flox}, Spc-rtTA and Jag1\textsuperscript{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\textsuperscript{flox/flox}, Spc-rtTA (K,M,O) and Jag1\textsuperscript{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\textsuperscript{flox/flox}, Spc-rtTA (Q) and Jag1\textsuperscript{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\textsuperscript{flox/flox}, Spc-rtTA (S) and Jag1\textsuperscript{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 andDll1 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.

**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. Jagged1flox 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 Jagged1Ko 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 RT² 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^(-ΔΔCT), where ΔCT=CTTested Gene - CTGAPDH. Data were derived from three animals per group (three 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-8857, 1:150), Muc5AC (Santa Cruz, sc-21701, 1:50), Tlo1 (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.

**ACKNOWLEDGMENTS**

K. Xu was funded by start-up funds from the Cancer Institute of the University of Mississippi Medical Center. S. E. Egan’s lab has been supported by grants from the Canadian Cancer Society and the Canadian Institute of Health Research. Finally, we thank Dr. Martin Post, Dr. Jeffrey Whitsett, and colleagues in Jackson for reagents and/or advice.

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