The loss of Hoxa5 function promotes Notch-dependent goblet cell metaplasia in lung airways

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Summary

Hox genes encode transcription factors controlling complex developmental processes in various organs. Little is known, however, about how HOX proteins control cell fate. Herein, we demonstrate that the goblet cell metaplasia observed in lung airways from Hoxa5−/− mice originates from the transdifferentiation of Clara cells. Reduced CC10 expression in Hoxa5−/− embryos indicates that altered cell specification occurs prior to birth. The loss of Hoxa5 function does not preclude airway repair after naphthalene exposure, but the regenerated epithelium presents goblet cell metaplasia and less CC10-positive cells, demonstrating the essential role of Hoxa5 for correct differentiation. Goblet cell metaplasia in Hoxa5−/− mice is a FOXA2-independent process. However, it is associated with increased Notch signaling activity. Consistent with these findings, expression levels of activated NOTCH1 and the effector gene HEY2 are enhanced in patients with chronic obstructive pulmonary disease. In vivo administration of a γ-secretase inhibitor attenuates goblet cell metaplasia in Hoxa5−/− mice, highlighting the contribution of Notch signaling to the phenotype and suggesting a potential therapeutic strategy to inhibit goblet cell differentiation and mucus overproduction in airway diseases. In summary, the loss of Hoxa5 function in lung mesenchyme impacts on epithelial cell fate by modulating Notch signaling.

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Key words: Hox genes, Goblet cells, Notch pathway

Introduction

During development, the undifferentiated endodermal epithelium of the lung primordium will give rise to specialized cell types along the anterior-posterior axis of the respiratory tree. Trachea and bronchi are lined by a pseudostratified epithelium consisting of basal, ciliated and secretory cells, the latter including Clara cells and few goblet cells. Non-cartilaginous airways of the bronchial tree are covered by a simple columnar epithelium mainly composed of ciliated and Clara cells. In the distal lung, type 1 and type 2 cells constitute the alveolar epithelium. All these cells fulfill critical functions, including mucociliary clearance, gas exchange and maintenance of surface tension (Morrisey and Hogan, 2010). In response to chronic insults, differentiation of the bronchial tree epithelium is altered with an increased number of goblet cells. Mucus normally produced by goblet cells is critical for host defense, but in excess it represents a major cause of airway obstruction. Goblet cell metaplasia and mucus hypersecretion contribute to the morbidity and mortality of chronic pulmonary disorders like chronic obstructive pulmonary disease (COPD) (Fahy and Dickey, 2010).

Cell fate determination of the respiratory epithelium is under the concerted action of multiple molecules (Morrisey and Hogan, 2010; Whitsett et al., 2011). Depending on the pathology, the goblet cell population may exhibit a distinct mucin content and express a specific array of transcription factors (Plantier et al., 2011). Several intertwining molecular networks are involved in goblet cell differentiation. Classical knock-out experiments and tissue-specific conditional mutations in mice have contributed to our understanding of the mechanisms involved in the acquisition of respiratory cell fate. For instance, the mutation of Foxa2, a gene encoding a winged helix/forkhead box transcription factor, in the respiratory epithelial cells induces pulmonary eosinophilic inflammation and goblet cell metaplasia (Wan et al., 2004; Chen et al., 2009). Expression of an activated form of β-catenin, a central member of the Wnt canonical pathway, in respiratory epithelial cells causes goblet cell hyperplasia and reduced Foxa2 expression (Mucenski et al., 2005). The Notch pathway has also emerged as a key player in airway epithelial cell fate. The mosaic mutations in airway epithelium of Pofut-1, an O-fucosyltransferase essential for Notch-ligand binding, and Rhypk, a transcriptional effector, lead to goblet cell metaplasia with reduced FOXA2 expression (Tsao et al., 2011). In contrast, overexpression of the active intracellular domain of the mouse NOTCH1 receptor (N1ICD) in lung epithelium promotes goblet cell metaplasia without change in FOXA2 expression.
Altogether, these data demonstrate the prevalent role of Foxa2 and the importance of thresholds in Notch pathway activation in the determination of airway epithelial cell fate (Guseh et al., 2009; Rock et al., 2011).

Lung branching morphogenesis and epithelial cell fate determination require reciprocal interactions between the contiguous epithelium and the lung mesenchyme (Alescio and Cassini, 1962; Shannon et al., 1998). Despite accumulated evidence showing that mesenchyme can instruct epithelial differentiation, the nature of the mesenchymal factors involved still remains elusive. Hox genes encode transcription factors specifying regional identity along the body axes and regulating morphogenesis during animal development (Pourquié, 2009). In human and mouse, 39 Hox genes are characteristically organized in four clusters and classified in 13 paralog groups. Several Hox genes, predominantly from paralog groups 2 to 6, are expressed in a distinct spatio-temporal fashion during lung ontogeny (Bogue et al., 1994; Mollard and Dziadek, 1997). Their expression is mainly restricted to lung mesenchyme. Except for Hoxa5, the lack of overt lung phenotype in single mutants suggests that, taken individually, these Hox genes do not play a predominant role in lung ontogeny. Functional redundancy by other Hox genes may mask anomalies (Rossel and Capecci, 1999).

The Hoxa5 mutation results in panoply of phenotypes indicative of the broad range of Hoxa5 actions throughout life (Jeanotte et al., 1993; Aubin et al., 2002; Garin et al., 2006; Gendronneau et al., 2012). Most Hoxa5–/– mice die at birth from respiratory distress due to tracheal and lung dysmorphogenesis (Aubin et al., 1997). Surviving mutants display lung airspace enlargement and goblet cell metaplasia (Mandeville et al., 2006). Hoxa5 expression is confined to the mesenchyme of the entire respiratory tract suggesting that it provides regional cues to the contiguous epithelia and participates to cell fate determination (Aubin et al., 1997; Couble et al., 2010). Herein, we have focused on the cellular and molecular mechanisms underlying goblet cell metaplasia in Hoxa5–/– mice. The loss of Hoxa5 function induces Clara to goblet cell transdifferentiation, a FOXA2-independent process accompanied by an increased activity of Notch signaling. Overall, our data indicate that mesenchyme-expressed Hoxa5 participates to epithelial secretory cell fate by controlling the Notch pathway.

Materials and Methods

Mice, genotyping and tissue collection

The Hoxa5 mutant mouse line was maintained in the MFl-12/9/Sv-C57BL/6 background or in the 129/Sv inbred background (Jeanotte et al., 1993). Transgenic mice carrying the TCF3-Lef1lacZ reporter gene, provided by Dr. Dufoit, were used to study the impact of the Hoxa5 mutation on Wnt canonical signaling (Mohamed et al., 2004). Age of the embryos was estimated by considering the morning of the day of the vaginal plug as embryonic (E) day 0.5. Experimental specimens were genotyped by Southern blot analysis.

Lungs from control and mutant embryos were collected at E18.5. For adult mice, lungs were snap-frozen in liquid nitrogen (LN2) for RNA extraction, and paraffin-embedded (Jaffe et al., 1990). Experimental specimens were genotyped by Southern blot analysis.

Human specimens

Human lung samples from anonymous patients were provided by the “Banque de Tissus du Réseau en Santé Respiratoire du FRSQ” from l’Institut universitaire de cardiologie et de pneumologie de Québec (IUCPQ, Canada). Specimens were characterized on the basis of clinical history, physical examination, pulmonary function tests and chest X-ray. The study was approved by IUCPQ ethics committee and written informed consent was obtained from patients before entry into the study.

Naphthalene injury model

Wild-type (wt) and Hoxa5–/– mice of 8 weeks received one intraperitoneal injection of 275 mg/kg of naphthalene (Sigma-Aldrich) or the equivalent volume of corn oil as vehicle (Zemke et al., 2009). At day 1 (wt, n=6; Hoxa5–/–, n=6), day 3 (wt, n=7; Hoxa5–/–, n=8), day 14 (wt, n=10; Hoxa5–/–, n=9), day 42 (wt, n=8; Hoxa5–/–, n=10), and day 84 (wt, n=5; Hoxa5–/–, n=6) after naphthalene injection, mice were euthanized and their lungs were collected. Vehicle-injected mice were sacrificed at day 3 after injection (wt, n=8; Hoxa5–/–, n=9).

Treatment with the γ-secretase inhibitor GSI L685,458

Control and Hoxa5–/– mice were anesthetized before receiving intranasally at postnatal day (D) 30 and D37, 50 μl of 0.3 mg/kg of GSI L685,458 (Sigma-Aldrich; wt, n=10; Hoxa5–/–, n=11) or 50 μl of 1.5% DMSO as vehicle (wt, n=11; Hoxa5–/–, n=11). Mice were sacrificed 3 days (D40) after the second instillation of GSI L685,458 (Kang et al., 2009).

Quantitative RT-PCR experiments

Total RNA was isolated from lungs of E18.5 embryos, D30 and D60 mice with TRIzol reagent (Invitrogen). cDNA was synthesized with the Superscript II Reverse Transcriptase (Invitrogen) using 2 μg of total RNA and random primers. Quantitative PCR (qPCR) was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) and a thermal cycler ABI PRISM 7000. The Rpl19 gene was used as control. Primer sequences are listed in supplementary material Table S1.

Immunohistochemistry (IHC) and immunofluorescence (IF) analyses

Paraffin-embedded lungs were serially sectioned at 4 μm for e18.5 and 6 μm for postnatal specimens. Immunostaining experiments were performed as described (Gendronneau et al., 2012). Slides were counterstained with either Alcian blue (for detection of acid mucus-producing cells) and nuclear Fast Red or Periodic Acid–Scheiff stain and Hematoxylin.

Primary and secondary antibodies used are listed in supplementary material Table S2. For IF studies, nuclei were visualized by DAPI staining. To identify goblet cells by fluorescence, the periodic acid fluorescent Schiff (PAS) procedure was applied (Evans et al., 2004). Images were captured with an FV1000 confocal system or with a Leica DMR microscope coupled to a Qimaging camera.

Proliferation and apoptosis analyses

To assess the proliferation rate, mice were injected intraperitoneally with 250 μg/g BrDU (Sigma-Aldrich) in phosphate buffer (PBS). Lungs were collected 4 hours later, fixed in 4% PFA overnight and paraffin-embedded for IHC. Proliferation index was calculated as the number of BrDU-positive bronchial epithelial cells divided by the number of bronchial epithelial cells. A mean of 400 airway epithelial cells per specimen were counted.

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase (TdT) DNA end labeling method (Giroux and Charron, 1998).

β-galactosidase staining

Lungs were fixed in ice-cold 4% PFA, 0.2% glutaraldehyde in PBS for 1 hour. Samples were transferred into ice-cold 30% sucrose in PBS overnight and then in Tisue-Tek O.C.T. (Sakura Finetek). 10 μm cryostat sections were processed for X-gal staining and counterstained with nuclear Fast Red as described (Trainor et al., 1999).

Statistical analysis

To study the effect of the genotype on the number of MAC3-positive cells, a two-way ANOVA with repeated measures was applied. Student’s t test was performed to compare the number of BrDU-positive cells and the different cell types after naphthalene and GSI L685,458 treatments. When appropriate, Student’s t test or the nonparametric Wilcoxon test was performed for gene expression analyses. Statistical analyses were done using the SAS 9.2 statistical software (SAS Institute, Cary, NC). A significance level inferior to 5% (p<0.05) was considered statistically significant.

Results

Goblet cell metaplasia in Hoxa5–/– mice is not linked to an inflammatory response

We previously reported goblet cell hyperplasia and mucus hypersecretion in lungs from Hoxa5–/– mice, a phenotype associated with an inflammatory response (Mandeville et al.,
We further characterized the goblet cell phenotype using alcin blue (AB) staining of lungs from wt and \textit{Hoxa5}\textsuperscript{+/−} specimens at E18.5, D15 and D30 (Fig. 1A–F). At E18.5, goblet cells were barely found in trachea and primary bronchi of wt and \textit{Hoxa5}\textsuperscript{−/−} embryos. In D15 control specimens, goblet cells remained scattered along the bronchial epithelium, whereas numerous AB-positive cells were observed in the bronchi of \textit{Hoxa5}\textsuperscript{−/−} mice. Goblet cell hyperplasia worsened with age as shown by the intense AB staining in D30 \textit{Hoxa5}\textsuperscript{−/−} samples. Goblet cells were mainly located along intrapulmonary
conducting airways, extending from the primary bronchi to the bronchioi where basal cells were sparsely found (data not shown). No MUC5AC immunostaining was detected along the respiratory tract of wt animals whereas MUC5AC-positive cells were abundant in Hoxa5−/− mice (Fig. 1G,H). In lungs from D30 Hoxa5−/− animals, qPCR analysis revealed a significant increase in expression levels for the anterior gradient 2 (Agr2) and the calcium-activated chloride channel family member 3 (Clica3) genes, both involved in the regulation of goblet cell hyperplasia (Fig. 1M,N) (Nakanishi et al., 2001; Chen et al., 2009).

Goblet cell hyperplasia often reflects pulmonary inflammation. We evaluated the inflammatory status of our Hoxa5 mutant mouse colony by assessing macrophage recruitment to the lung by IHC with the MAC3 macrophage marker. In contrast to our previous observations, there was no significant difference in the number of MAC3-positive cells between wt and Hoxa5−/− specimens at E18.5 and D30 (Fig. 1I–L,O) (Mandeville et al., 2006). Moreover, qPCR analysis did not reveal significant changes in expression for the T helper (Th) type 2 cytokines, Il-6 and Il-13, and the chemokine Ccl11 genes between wt and mutants, indicating that goblet cell hyperplasia occurred without induction of proinflammatory mediators (Fig. 1P–R) (Zhu et al., 1999; Dent et al., 2004). Thus, inflammation did not contribute to goblet cell hyperplasia in Hoxa5−/− mice. The airway phenotype observed likely resulted from the impact of the Hoxa5 mutation on the respiratory epithelium.

The Hoxa5 mutation induces a developmental defect causing Clara to goblet cell transdifferentiation

To define the origin of goblet cells in the airway epithelium of Hoxa5−/− mice, we examined cellular proliferation and apoptosis in proximal airways from D30 wt and Hoxa5−/− specimens. Goblet cell hyperplasia in mutants occurred without evidence of increased proliferation of goblet cells or augmented apoptosis of other cell types, as assessed by BrdU (Fig. 2A–C) and TUNEL labeling (data not shown), respectively.

Models developed to mimic pathological conditions have shown that Clara, ciliated and basal cells can display plasticity and contribute to mucous cell hyperplasia (Evans et al., 2004; Tyner et al., 2006; Chen et al., 2009; Turner et al., 2011; Rock et al., 2011). To assess the integrity of the airway epithelium of Hoxa5−/− mice, the relative abundance of these cell types was examined in lungs from D30 and D60 wt and mutant mice (Fig. 2; data not shown). In areas of mucous cell hyperplasia, no major change in the number and distribution of basal cells was observed when using the transcription factor p63 as marker (Fig. 2D–F). A comparable observation was made for ciliated cells detected by FOXJ1, a forkhead transcription factor required for the assembly of the ciliary apparatus (Chen et al., 1998). In Hoxa5−/− specimens, ciliated cells appeared intermingled with goblet cells raising questions about the localization of Clara cells (Fig. 2G–I). Immunostaining with the Clara cell marker CC10 (encoded by the Scgb1a1 gene) showed the widespread distribution of Clara cells along the proximal airways of wt mice with a strong cytoplasm labeling. A weaker CC10 immunostaining was detected in Hoxa5−/− specimens. Moreover, AB-positive cells displayed CC10 co-staining, which was restricted to the apex of the cells (Fig. 2J–M). qPCR analysis confirmed the decreased Scgb1a1 expression in lungs from D60 Hoxa5−/− mice (Fig. 2N). The co-localization of AB staining with CC10 labeling suggested that mucus-producing cells might derive from Clara cells.

To corroborate the dual identity of goblet cells in Hoxa5−/− specimens, IF co-staining experiments were performed on D60 wt and Hoxa5−/− mice using CC10 antibody with periodic acid fluorescent Schiff (PAF), the latter revealing intracellular mucins, or with an AGR2 antibody (Fig. 3). Airway epithelium of wt mice exhibited CC10 labeling of Clara cells’ cytoplasm and weak or no PAF and AGR2 staining (Fig. 3A–C,G–I). In Hoxa5−/− mice, PAF- and AGR2-positive cells were abundant and most of them co-localized with CC10-positive cells (Fig. 3D–F,J–L). Again, CC10 staining was confined to the apex of the mucus cells even in the few goblet cells detected in wt specimens (Fig. 3C,F,L). Thus in absence of Hoxa5 function, goblet cell metaplasia occurs via Clara to goblet cell transdifferentiation.

To determine if the transdifferentiation of Clara cells results from impaired cell specification during development, we examined the respiratory epithelial cell types in E18.5 wt and Hoxa5−/− embryos. No major difference in the proportion and the proximo-distal distribution of p63-positive basal cells and βIV-tubulin-positive ciliated cells was observed (Fig. 4A–D). Consistent with the AB staining, no PAF-positive cells were detected in E18.5 wt and Hoxa5−/− specimens (data not shown). However, a reduced CC10 signal was seen in Hoxa5−/− lungs (Fig. 4E,F). qPCR analysis of Scgb1a1 expression also showed decreased levels in E18.5 Hoxa5−/− lungs (p=0.06) (Fig. 4I). To define if airway epithelial cells were stalled in a progenitor state in Hoxa5−/− embryos, the Stage-Specific Embryonic Antigen-1 (SSEA1) antibody, a marker of mouse embryonic stem cells, was used (Xing et al., 2010). No difference in SSEA1 staining intensity and distribution was observed between wt and Hoxa5−/− specimens (Fig. 4G,H). Thus, the goblet cell metaplasia observed in lungs from Hoxa5−/− mice results from Clara cell transdifferentiation, an event that initiates prior to birth.

A role for Hoxa5 in lung repair

Accumulation of evidences supports the notion that lung developmental processes also govern regeneration of lung tissue in pathological conditions. To assess the capacity of lung epithelium from Hoxa5−/− mice in restoring its integrity, a naphthalene-induced lung injury approach was tested in wt and Hoxa5−/− mice (Van Winkle et al., 1995). Clara cells expressing the cytochrome P-450 monooxygenase CYP2F2 are the primary targets for the cytotoxicant naphthalene, CYP2F2 being essential for the bioactivation and toxicity of naphthalene in lung. Naphthalene-resistant variant Clara cells show multipotent differentiation and the ability to regenerate the destroyed airway epithelium (Hong et al., 2001). Lungs from wt and Hoxa5−/− treated animals were analyzed after 1, 3, 14, 42 and 84 days. Epithelial regeneration was monitored by CC10 IHC combined to AB staining. As expected, injection of vehicle did not produce apparent damage in wt and Hoxa5−/− mice three days after injection. In Hoxa5−/− mice, AB staining was found significantly higher while CC10 signal was weaker (Fig. 5A,B). On days one and three following naphthalene injection, airway epithelial injuries appeared similar in wt and Hoxa5−/− mice (Fig. 5C,F). A majority of Clara cells were sloughed from the basement membrane and the bronchi were lined by a simple squamous epithelium composed of FOXJ1-positive cells.
Fig. 2. Characterization of the respiratory epithelium of $Hoxa5^{-/-}$ mice. (A–C) BrdU-labeled airway cells (arrowheads) from wt and $Hoxa5^{-/-}$ D30 mice. BrdU labeling index was similar for both genotypes. Values are expressed as mean ± SD (n=4–5 animals/group). (D–I) Correct specification of basal and ciliated cells as shown respectively by immunostaining with p63 (arrowheads; D–F) and FOXJ1 (arrowheads; G–I) in lungs from wt and $Hoxa5^{-/-}$ D30 mice. (J–M) Decreased CC10-staining in $Hoxa5^{-/-}$ D30 mice. AB-stained goblet cells displayed weak but positive CC10 immunolabeling in mutants (arrowheads, the arrow points to a Clara cell; M). (N) qPCR analysis of $Scgb1a1$ expression in lungs from wt and $Hoxa5^{-/-}$ D60 mice revealed decreased expression in mutants. Values are expressed as mean ± SEM (n=6–8 animals/group). *p<0.05. Scale bars, 50 µm (A,B,D,E,G,H,J,K), 25 µm (F,I,L,M).
Goblet cells in the airways of Hoxa5<sup>2/2</sup> mice were totally exfoliated indicating that in addition to CC10, they might also express CYP2F2 (Li et al., 2011). Starting on day 14 onwards, CC10-positive cells were detected with an irregular distribution along the airways (Fig. 5G–L). Similar to our observations with untreated mice, Hoxa5<sup>2/2</sup> mice exhibited a weak CC10 immunoreactivity when compared to controls (Fig. 3, Fig. 5G–L). On day 84 after naphthalene injection, goblet cells reappeared along the airway epithelium of Hoxa5<sup>2/2</sup> mice (Fig. 5K,L). CD3 immunostaining revealed areas invaded by lymphocytes in lungs from wt and Hoxa5<sup>2/2</sup> mice, suggesting that inflammation is not a predominant causal factor for goblet cell metaplasia in Hoxa5<sup>2/2</sup> specimens (supplementary material Fig. S1A–C).

To define how the different cell types recovered from the naphthalene-induced injury, cell quantification was performed (Table 1). At days 14, 42, and 84 after injury, the airway epithelium of naphthalene-treated Hoxa5<sup>2/2</sup> mice contained a significant lower number of Clara cells than wt specimens, which corroborated the histological data. No difference in the number of FOXJ1-positive ciliated cells was observed between wt and Hoxa5<sup>2/2</sup> mice, whereas the number of AB-positive goblet cells was significantly higher in Hoxa5<sup>2/2</sup> mice at day 84 after naphthalene injection. qPCR analysis of Scgb1a1, Cyp2f2, Agr2 and Foxj1 expression levels was also performed to appreciate lung airway re-epithelialisation (Fig. 5M–O; data not shown). As expected, Hoxa5<sup>2/2</sup> mice injected with vehicle displayed reduced Scgb1a1 and Cyp2f2 expression and increased Agr2 transcript levels when compared to wt. At day 3 after injury, Scgb1a1, Cyp2f2 and Agr2 expression was barely detectable in both wt and mutant lungs. At days 14 and 42 after injury, airway epithelium recovery was characterized by a tendency to

![Image](https://example.com/image1.png)

Fig. 3. Clara to goblet cell transdifferentiation in Hoxa5<sup>2/2</sup> airway epithelium. (A–L) Proximal airway sections from wt and Hoxa5<sup>2/2</sup> D60 mice. Clara cells were detected by CC10 immunolabeling (green) and goblet cells by PAF staining or AGR2 immunolabeling (red). Nuclei were counterstained with DAPI. Co-localization of CC10 and PAF or AGR2 within Hoxa5<sup>2/2</sup> airway epithelial cells is shown by the yellow color (arrowheads; C,F,I,L). Scale bar, 25 μm.

![Image](https://example.com/image2.png)

Fig. 4. Characterization of airway epithelium in Hoxa5<sup>2/2</sup> embryos. Correct specification of basal (A,B) and ciliated cells (C,D), shown respectively by immunostaining with p63 (red) and βIV-tubulin (red) in lungs from wt and Hoxa5<sup>2/2</sup> E18.5 embryos. (E,F) CC10 immunolabeling (green) showed a reduced number of Clara cells in Hoxa5<sup>2/2</sup> specimens. (G,H) SSEA1 immunostaining (red) was similar for both genotypes. (I) qPCR analysis for Scgb1a1 expression in lungs from E18.5 wt and Hoxa5<sup>2/2</sup> embryos indicated decreased expression in mutants. Values are expressed as mean ± SEM (n=7 animals/group). Scale bars, 50 μm (A–D), 100 μm (E–H).
Fig. 5. See next page for legend.
decreased expression of Scgb1a1 and Cyp2f2 in Hoxa5-/- lungs. No significant difference was observed for Foxj1 expression from day 3 to day 42 after injury, supporting the IHC data (Table 1; data not shown). Similar Agr2 expression levels were observed in wt and Hoxa5-/- lungs up to day 42 after naphthalene injection, consistent with the lack of detectable goblet cells at this time point and suggesting that abnormal Clara cell differentiation precedes goblet cell metaplasia (Fig. 5O).

Thus, the lack of Hoxa5 function did not prevent lung airway re-epithelialisation after naphthalene injection. However, the cellularity of the regenerated epithelium in naphthalene-treated Hoxa5-/- mice remained similar to that from untreated mutant animals indicating that mesenchymal Hoxa5 expression is necessary for correct lung airway re-epithelialisation and differentiation.

Impact of the loss of Hoxa5 function on regulators involved in lung epithelial cell differentiation and specification

To determine the mechanisms by which Hoxa5 controls goblet cell differentiation, we analyzed the expression of transcription factors known to govern lung epithelial cell differentiation in airways from D30 wt and Hoxa5-/- mice (Fig. 6). SPDEF expression was markedly increased at sites of goblet cell metaplasia in Hoxa5-/- mice (Fig. 6A–C). A similar observation was made for the forkhead box transcription factor FOXA3, a nuclear marker of goblet cells (Fig. 6D–F) (Park et al., 2011). In contrast, no difference was detected for NKX2.1 expression in basal and secretory cells, but not in goblet cells (Fig. 6G–I) (Whitsett et al., 2011; Plantier et al., 2011). We also looked at GATA6 expression as GATA6 and NKX2.1 act synergistically in regulating pulmonary epithelial differentiation (Zhang et al., 2007). GATA6 expression was detected in most cells, including goblet cells, along the airway epithelium with no major difference between wt and Hoxa5-/- mice (Fig. 6J–L).

The loss of FOXA2 expression is associated with goblet cell metaplasia (Wan et al., 2004; Chen et al., 2009). Surprisingly, we did not observe any impact of the Hoxa5 mutation on FOXA2 staining even if we repeated the experiments with two distinct

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**Table 1. Percentage of Clara, ciliated, and goblet cells in bronchial airways after naphthalene-induced injury.**

|                      | 3 days post vehicle | 3 days post NAPH | 14 days post NAPH | 42 days post NAPH | 84 days post NAPH |
|----------------------|---------------------|------------------|-------------------|-------------------|-------------------|
|                      | wt n=7              | Hoxa5-/- n=5     | wt n=7            | Hoxa5-/- n=6      | wt n=5            |
| CC10+ cells          | 43.4±5.5            | 24.5±2.6**       | 32.3±6.2          | 20.8±4**          | 31.2±6.5          |
| FOXJ1+ cells         | 3.3±4.8             | 14.9±9.3*        | 6.8±6.4           | 6.7±4.5           | 5.4±2.7           |
| Alcian blue+ cells   |                     | n.d.             | n.d.              | 7.4±5.6           | 6±4.6             |

Values are expressed as % of the total number of cells. A minimum of 1000 cells were counted per specimen.

Data are presented as mean ± SD.

* p<0.05, ** p<0.01

n.d.: not determined.
antibodies (Fig. 6M–O; data not shown). FOXA2 staining was detected along the airway epithelium in Hoxa5+/− specimens, even in regions of goblet cell metaplasia. We also looked at FOXA2 expression in naphthalene-treated wt and Hoxa5+/− mice on day 84 after injection (supplementary material Fig. S1D–F). FOXA2 staining was similar for both genotypes and goblet cell metaplasia occurred in Hoxa5+/− specimens without reduced FOXA2 expression. Thus, the molecular mechanisms responsible for goblet cell metaplasia in Hoxa5+/− mice are FOXA2-independent.

β-catenin, a key regulated effector of the Wnt canonical pathway, plays a critical role in the differentiation of respiratory epithelial cells (Mucenski et al., 2005). We examined the expression of members from the Wnt signaling pathways (canonical and non-canonical) involved in lung organogenesis by qPCR in trachea/primary bronchi and lung from D30 wt and Hoxa5+/− mice (Li et al., 2002; Mandel et al., 2008; Goss et al., 2009; Forony et al., 2010; Xu et al., 2011). Wnt5a and Wnt2 expression was significantly augmented in Hoxa5+/− lungs when compared to controls, whereas a significant decrease in Wnt4 expression was observed in mutant lungs specimens (supplementary material Fig. S2A–C). No change in expression levels for the sflr1 gene encoding a Wnt signaling inhibitor was observed in mutant specimens but a tendency to diminished expression of the Wnt inhibitory factor 1, Wif1, gene was detected in Hoxa5+/− trachea and primary bronchi specimens (supplementary material Fig. S2D,E). To assess the impact of the Hoxa5 mutation on the Wnt canonical pathway in lung epithelium, we used the TCF/Lef-lacZ transgenic reporter mouse line as a functional read-out of the activity of the cascade (Mohamed et al., 2004). As reported, lacZ expression was mainly confined to the epithelium and the staining was faint along the airways and in the pulmonary epithelium of E18.5 and D30 Hoxa5+/+ TCF/Lef-lacZ+ specimens (Dean et al., 2005). In Hoxa5+/− TCF/Lef-lacZ+ specimens, lacZ expression was more pronounced in intensity and in the number of positive cells suggesting that Hoxa5 may influence the activity of the Wnt/β-catenin cascade in lung epithelium (supplementary material Fig. S2F–K). However, we could not decisively establish that the X-gal staining colocalized with regions of goblet cell metaplasia, inferring that increased Wnt canonical signalling did not directly contribute to goblet cell metaplasia in Hoxa5+/− specimens.

Hoxa5 action in lung epithelial differentiation and Notch signaling

As N1ICD overexpression in mouse lung epithelium results in goblet cell metaplasia without change in FOX2 expression, we hypothesized that Notch signaling may be involved in transducing mesenchymal Hoxa5 function, and triggering goblet cell metaplasia in airway epithelium from Hoxa5+/− animals (Guseh et al., 2009). Expression analysis of members of the Notch pathway was performed by qPCR in trachea/primary bronchi and lung from D30 wt and Hoxa5+/− mice (Fig. 7A–F). No change in expression was observed for the ligand Jag1 and for the transcriptional effectors Hes1 and Hey1. However, significant increased expression of the ligand Jag2 and the target gene Hey2 was detected in Hoxa5+/− lungs, while significant increased expression of the transcriptional regulator Rbpjk was observed in the trachea(primary bronchi from mutant specimens. We evaluated the functional status of the Notch signaling pathway in Hoxa5+/− airways by IHC experiments for N1ICD and HEY2. Expression of both proteins was markedly increased at sites of goblet cell metaplasia in Hoxa5+/− specimens (Fig. 7G–L). A strong N1ICD staining was also associated with regions of goblet cells in naphthalene-treated Hoxa5+/− mice on day 84 after injection (supplementary material Fig. S1G–I). Thus, specific activation of the Notch pathway in airway epithelium may underlie the goblet cell phenotype in Hoxa5+/− mice with HEY2 as a potential effector of goblet cell metaplasia.

Increased expression of activated-NOTCH1 and HEY2 in human pulmonary disease

To test whether increased Notch signaling is a hallmark of goblet cell hyperplasia associated with human pulmonary diseases, we performed N1ICD and HEY2 IHC experiments on bronchial tissues from COPD patients. They revealed a robust expression of N1ICD and HEY2 in areas of goblet cell metaplasia along the airway epithelium and in submucosal glands from COPD patients when compared to controls (Fig. 7M–R).

Attenuation of goblet cell metaplasia in Hoxa5+/− mice treated with a γ-secretase inhibitor

To directly assess the potential role of Notch signaling in the goblet cell phenotype of Hoxa5+/− mice, we used the cell permeable γ-secretase inhibitor (GSI) L685,458, which blocks Notch signaling by inhibiting the cleavage of the transmembrane domain of the receptor Notch by the γ-secretase and the liberation of active NICD (Kang et al., 2009). Controls and Hoxa5+/− mice were intranasally instilled with GSI L685,458 and sacrificed three days after treatment (Fig. 8A). Co-staining experiments with AB and CC10 revealed that the proportion of AB-positive cells was significantly diminished in airways of GSI-treated Hoxa5+/− mice when compared to vehicle-treated Hoxa5+/− mice (33% vs 13%) (Fig. 8B–H). No effect of the inhibitor was observed in wt mice. Agr2 and Cleta expression levels were both reduced in lungs from GSI-treated Hoxa5+/− mice, while Sbig1a1 expression was significantly restored in GSI-treated mutant mice (Fig. 8I–K). Thus, goblet cell metaplasia was attenuated in Hoxa5+/− mice by the Notch inhibitor GSI L685,458.

To validate the impact of the inhibitor on the Notch pathway, N1ICD immunostaining was performed on airway sections. The intensity of N1ICD labeling was diminished in GSI-treated compared to vehicle-treated Hoxa5+/− mice (Fig. 9A–F). Decreased Notch signaling activity was further confirmed by the down-regulation of the target gene Hey2 in GSI-treated Hoxa5+/− mice as revealed by IHC and qPCR (Fig. 9G–M). Altogether, the data support the notion that Notch activation mediates at least in part goblet cell metaplasia in Hoxa5+/− mice.

Discussion

The origin of goblet cells in Hoxa5+/− mice

We have previously shown that Hoxa5 is critical for lung formation (Aubin et al., 1997). The present study enlightens the role of Hoxa5 in airway epithelial cell fate. The loss of Hoxa5 function in airway mesenchyme hampers normal Clara cell differentiation leading to their transdifferentiation into goblet cells, an event occurring without change in epithelial proliferation. Despite evidences that ciliated cells could display plasticity contributing to mucous cell metaplasia (Tyner et al., 2006; Turner et al., 2011), our data are concuring with several studies showing that mucus-producing cells derive preferentially
from Clara cells (Evans et al., 2004; Park et al., 2007; Chen et al., 2009; Tsao et al., 2011). Considering that Clara cells were identified as epithelial progenitor cells in intrapulmonary airways and that Clara and goblet cells share similar machinery for protein secretion at their surface, the transdifferentiation of Clara cells into goblet cells in Hoxa5<sup>−/−</sup> mice is a logical outcome (Rawlins et al., 2009).

Studies of the mouse trachea established that goblet cell metaplasia might also originate from basal cells (Rock et al., 2011). In Hoxa5<sup>−/−</sup> mice, no goblet cell metaplasia was detected...
in the trachea where basal cells are the most abundant (data not shown). While all these data reveal how the airway epithelial cells are remarkably plastic and capable of extensive remodeling, our results did not provide evidence for a role of ciliated or basal cells in goblet cell metaplasia in \textit{Hoxa5}^{+/+} mice.

Goblet cell metaplasia is closely associated with pulmonary inflammation (Whitsett et al., 2011). Reduced Foxa2 expression is also coupled to goblet cell metaplasia and inflammation (Wan et al., 2004; Chen et al., 2010). In \textit{Hoxa5}^{+/+} mice, goblet cell metaplasia is neither accompanied by inflammation, nor by a change in FOXA2 expression. We previously reported that recruitment of inflammatory cells characterized the lung phenotype of \textit{Hoxa5}^{+/+} mice, a result not reproduced in the current analysis (Mandeville et al., 2006). A likely explanation for the disparity between the inflammation statuses of the mice is the improvement of the mouse husbandry conditions over the years, which positively impacted on the health of the animals. Interestingly, inflammatory cells were detected in wt and

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**Fig. 8. Attenuated goblet cell metaplasia in \textit{Hoxa5}^{+/+} mice after GSI treatment.** (A) Diagram of the experimental protocol. (B) \textit{Hoxa5}^{+/+} mice displayed less goblet cells after GSI treatment when compared to vehicle-treated mutants. Percentage of goblet cells was calculated as the number of AB-positive bronchial epithelial cells relative to the total number of bronchial epithelial cells. Values are expressed as mean ± SD (n=5–6 animals/group). (C–H) CC10 IHC combined to AB staining in wt and \textit{Hoxa5}^{+/+} mice after vehicle (C–E) or GSI (F–H) treatments. Arrowheads indicate goblet cells. (I–K) qPCR analysis for \textit{Agr2}, \textit{Clca3}, and \textit{Scgb1a1} expression in lungs from wt and \textit{Hoxa5}^{+/+} mice treated with vehicle or GSI. Values are expressed as mean ± SEM (n=5–6 animals/group). *p<0.05, **p<0.01, ***p<0.001. Scale bars, 100 μm (C,D,F,G), 50 μm (E,H).
Fig. 9. Decreased Notch signaling activity in Hoxa5−/− mice after GSI treatment. Immunostaining for N1ICD (A–F) and HEY2 (G–L) was performed in lungs from wt and Hoxa5−/− mice treated with vehicle (A–C,G–I) or GSI (D–F,J–L). Reduced N1ICD and HEY2 staining was observed along the epithelium of GSI-treated Hoxa5−/− mice. Arrowheads indicate goblet cells. (M) qPCR analysis of Hey2 expression in lungs from wt and Hoxa5−/− mice treated with vehicle or GSI. Values are expressed as mean ± SEM (n=5 animals/group). **p<0.01. Scale bars, 100 μm (A,B,D,E,G,H,J,K), 50 μm (C,F,I,L).
In Hoxa5−/− lung specimens on day 84 after naphthalene injection, most likely resulting from the stress due to the naphthalene-induced injury. Although inflammation was observed for both genotypes, goblet cell metaplasia was solely seen in mutants with no effect on FOXA2 expression (supplementary material Fig. S1A–E). Altogether, our data indicate that in normal and regenerative conditions, the loss of Hoxa5 function causes goblet cell metaplasia that develops independently of inflammation and Foxa2-driven mechanisms.

Goblet cell metaplasia was detectable after birth in Hoxa5−/− specimens, but abnormal Clara cell differentiation was already observed prior to birth as revealed by the reduced CC10 expression levels in E18.5 Hoxa5−/− embryos. SSEA1 staining was similar between wt and E18.5 Hoxa5−/− embryos. According to the widespread SSEA1 expression along nascent airways epithelium during embryogenesis, it was proposed that SSEA1-positive cells might serve as progenitors for the differentiated airway epithelial cells, including Clara cells (Xing et al., 2010). The reduced CC10 staining observed in E18.5 Hoxa5−/− specimens may thus reflect abnormal terminal differentiation, contributing to the impaired Clara cell identity observed in the postnatal period, rather than impaired cell fate specification. This is further supported by our observations that except for Clara and goblet cells, all airway cell types analyzed were not affected by the Hoxa5 mutation indicating that Hoxa5 does not act on multipotent progenitors but may specifically instruct the secretory cell lineage.

The characterization of the lung airway epithelium of Hoxa5−/− mice following naphthalene-induced injury revealed cellular and molecular similarities with the abnormal epithelial cell differentiation phenotype seen in Hoxa5−/− mice during normal development. This supports the concept that lung morphogenesis, repair, and regeneration are tightly coordinated by conserved transcriptional networks (Whitsett et al., 2011). Evidences accumulated herein also demonstrate that the mesenchymal expression of Hoxa5 is essential for the correct cell fate of the secretory cell lineage in lung airway epithelium during development and regeneration.

Molecular mechanisms and goblet cell differentiation

The SPDEF, FOXA2 and NKX2.1 transcription factors participate in molecular networks governing goblet cell differentiation (Wan et al., 2004; Chen et al., 2009; Maeda et al., 2011). In Hoxa5−/− mice, SPDEF expression was augmented at sites of goblet cell metaplasia whereas the NKX2.1 protein, normally expressed in Clara cells, was not detected in goblet cells. These results indicate that in the Hoxa5−/− environment, SPDEF and NKX2.1 may contribute to the regulatory pathway leading to the transdifferentiation process. As well, Hoxa5 may indirectly act on Spdef and Nkx2.1 gene expression in airway epithelium to determine the secretory cell fate. However FOXA2 expression, reported to be repressed by SPDEF in a mouse model in which Spdef expression is specifically targeted to Clara cells, remained unchanged in goblet cells from Hoxa5−/− specimens indicating the involvement of alternative transcriptional networks (Park et al., 2007).

Our study on the possible participation of Wnt signaling in goblet cell metaplasia in Hoxa5−/− mice demonstrated that the expression of Wnt ligands in the lung was altered by the Hoxa5 mutation. Activation of the canonical Wnt pathway, when assessed with a Wnt responsive reporter mouse line, appeared also more pronounced in lungs from Hoxa5−/− specimens. However, lacZ expression did not convincingly coincide with areas of goblet cell metaplasia, reducing the potential of the Wnt canonical pathway to be a major player in goblet cell fate in Hoxa5−/− mutants. This is supported by the lack of change in FOXA2 expression in Hoxa5−/− mutants. Indeed, mice expressing an activated form of β-catenin in airway epithelial cells develop goblet cell metaplasia associated with reduced FOXA2 expression (Mucenski et al., 2005). Nevertheless, the variations in expression of several members of the Wnt pathway raise the possibility that Wnt signaling may be involved in other aspects of the Hoxa5 lung phenotype.

Several studies have highlighted the key role of Notch signaling in controlling the delicate balance between ciliated and secretory cell fates (Tsao et al., 2009; Morimoto et al., 2010). N1ICD misexpression in lung epithelium resulted in goblet cell metaplasia without change in FOXA2 expression, a situation reminiscent to what we observed in Hoxa5 mutants (Gushe et al., 2009). Moreover, administration of a Notch inhibitor reduces goblet cell metaplasia in an ovalbumin-induced asthma model (Kang et al., 2009). Apparent discrepancies between the data were reported, as selective inhibition of the Notch pathway can also result in airway goblet cell metaplasia (Tsao et al., 2011). However, all these results suggest that different thresholds of Notch activation may influence airway epithelial cell fate depending on the developmental timing and the cell population it acts upon. In Hoxa5−/− mice and in patients suffering from COPD, activation of the Notch signaling pathway in airway goblet cells occurred. The contribution of the Notch pathway to goblet cell metaplasia was further confirmed in Hoxa5−/− mice by partially rescuing the phenotype via administration of a GSI. Although promising, these data did not allow concluding whether the decreased number of goblet cells in airways from GSI-treated Hoxa5−/− mice results in the hampering or the reversion of the phenotype.

In Hoxa5−/− mice, goblet cell metaplasia was associated with increased expression of Rbpjk, Jag2 and Hey2. Whereas the conditional removal of Rbpjk in lung endoderm promotes ciliated cell expansion at the expense of Clara cells, the augmented Rbpjk expression levels in Hoxa5−/− specimens support the notion that quantitative regulation of Notch signaling is crucial for airway epithelial cell fate (Tsao et al., 2009). The ligand Jag2 is expressed in lung mesenchyme and epithelium but its role in lung development still remains vague since Jag2−/− mice die at birth from cleft palate (Jiang et al., 1998; Kong et al., 2004).

We described for the first time Hey2 expression in airway goblet cells. Hey2 transcripts were previously detected in the distal epithelium of primary lung buds and Hey2 downregulation was reported in lung buds from GSI-treated explants, a result in agreement with our data (Tsao et al., 2008). These observations indicate that Hey2 is a putative mediator of the Notch pathway in airway epithelium differentiation. However, the exact role of Hey2 in goblet cell differentiation remains an issue.

Collectively, our observations expand the in vivo data reported so far and confirm that modulation of Notch signaling represents a potential therapeutic approach to restrain goblet cell differentiation and mucus hyperproduction in airways from patients with chronic lung diseases. As misregulated Hoxa5 gene expression is associated with emphysema, the alveolar component of COPD, one can hypothesize that Hoxa5 downregulation may confer susceptibility to develop COPD by
impaired alveolar formation and homeostasis, and by promoting goblet cell metaplasia (Golpon et al., 2001; Mandeville et al., 2006; Boucherat et al., 2007). Even though the nature of the factors influenced by the loss of Hoxa5 function in lung mesenchyme and impacting on Notch signaling in the airway epithelium remains to be defined, our findings unveil Hoxa5 as a micromanager controlling not only organ formation and axial patterning but also details of cell morphogenesis and function.

Acknowledgements
We thank Dr. J. Charron for helpful comments on the manuscript; Dr. D. Dufort for the TCF/Lef-fLuc reporter mouse line; Drs. S. Ho; G. Singh and J. Whitsett for antibodies; M. Lemieux for technical assistance; H. Crépeau and Dr. J. Aubin for statistical analyses, and S. Biardel and the “Banque de Tissus du Réseau En Santé Respiratoire du FRSQ” at IUCPQ for providing human specimens. This work was supported by a grant from the Canadian Institutes of Health Research (MOP-15139 to O.B.) and by a postdoctoral fellowship from the Fonds de la Recherche en Santé du Québec-Institut National de la Santé et de la Recherche Médicale (to O.B.).

Competing Interests
The authors declare that there are no competing interests.

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