Foxa2 regulates alveolarization and goblet cell hyperplasia

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Summary

The airways are lined by several distinct epithelial cells that play unique roles in pulmonary homeostasis; however, the mechanisms controlling their differentiation in health and disease are poorly understood. The winged helix transcription factor, FOXA2, is expressed in the foregut endoderm and in subsets of respiratory epithelial cells in the fetal and adult lung. Because targeted mutagenesis of the *Foxa2* gene in mice is lethal before formation of the lung, its potential role in lung morphogenesis and homeostasis has not been determined. We selectively deleted *Foxa2* in respiratory epithelial cells in the developing mouse lung. Airspace enlargement, goblet cell hyperplasia, increased mucin and neutrophilic infiltration were observed in lungs of the *Foxa2*-deleted mice. Experimental goblet cell hyperplasia caused by ovalbumin sensitization, interleukin 4 (IL4), IL13 and targeted deletion of the gene encoding surfactant protein C (SP-C), was associated with either absent or decreased expression of *Foxa2* in airway epithelial cells. Analysis of lung tissue from patients with a variety of pulmonary diseases revealed a strong inverse correlation between FOXA2 and goblet cell hyperplasia. FOXA2 is required for alveolarization and regulates airway epithelial cell differentiation in the postnatal lung.

Supplemental data available online

Key words: Lung, Forkhead, Transcription factor, Development, Inflammation, Winged helix

Introduction

The respiratory tract is lined by a complex epithelium that mediates mucociliary clearance, host defense, fluid and electrolyte transport, and surfactant homeostasis, which is required for gas exchange. The diverse epithelial cell types lining the lung vary during development and spatially along the cephalo-caudal axis of the airways. Cell types and functions are strongly influenced by injury and inflammation. Marked changes in epithelial cell differentiation accompany common pulmonary disorders, including asthma, chronic obstructive lung disease (COPD), bronchopulmonary dysplasia, infection and other inflammatory conditions that cause airway remodeling, emphysema and respiratory dysfunction. However, the factors that mediate the stereotypic changes in respiratory epithelial cells associated with inflammatory lung diseases, including squamous metaplasia and goblet cell hyperplasia, remain poorly understood.

Lung morphogenesis begins with a ventral out-pouching of endodermally derived cells from the anterior foregut into the surrounding mesenchyme at embryonic day (E) 9-9.5 in the mouse. Lung tubules undergo branching morphogenesis associated with proliferation and differentiation of pulmonary cells, which leads to the formation of the alveolar-capillary gas exchange region that is required for postnatal survival. Lung formation and epithelial-cell differentiation are regulated by several transcription factors, including thyroid transcription factor-1 (TTF-1), GATA-6, and forkhead transcription factors FOXA1, FOXA2, FOXF1 and FOXJ1 (Cardoso, 1995; Costa et al., 2001; Perl and Whitsett, 1999). FOXA2 (previously termed HNF-3β) is a member of the winged helix nuclear factor gene family. In mice, *Foxa2* is expressed first in the primitive streak at E6.5, shortly after the onset of gastrulation (Sasaki and Hogan, 1993). Thereafter, *Foxa2* is expressed in the notochord, gut endoderm and ventral midline of the CNS. Later in embryonic development, *Foxa2* is expressed in endodermally derived tissues including liver, lung, pancreas and intestine (Ang et al., 1993; Monaghan et al., 1993). The temporal-spatial patterning of *Foxa2* expression is regulated precisely during lung development, being restricted to subsets of respiratory epithelial cells. In the mouse lung, *Foxa2* is expressed in endodermally derived cells and is generally present at higher levels in epithelial cells that line conducting

airways compared to peripheral airways. After birth, FOXA2 is detected in subsets of conducting airway cells and in type II epithelial cells in the alveoli of mice and humans (Stahlman et al., 1998; Zhou et al., 1996). Functional analyses of the regulatory regions of several lung-specific genes have demonstrated a role of *Foxa2* in regulating the transcription of several genes that play important roles in lung morphogenesis and homeostasis, including *Titf1* (Ikeda et al., 1996), *Sftpb* (which encodes SP-B) (Bohinski et al., 1994) and *Scgblal* (Bingle and Gitlin, 1993; Bingle et al., 1995).

Null mutation of the *Foxa2* gene in the mouse embryo inhibits formation of the notochord and endoderm before the onset of lung morphogenesis (Ang and Rossant, 1994; Weinstein et al., 1994). Ectopic expression of *Foxa2* in distal respiratory epithelial cells in the lungs of transgenic mice disrupts branching morphogenesis and arrests differentiation of peripheral respiratory epithelial cells at the late pseudoglandular stage of development. These effects indicate a strict requirement for precise temporal-spatial control of *Foxa2* expression during normal lung morphogenesis (Zhou et al., 1997). Because Foxa2$^{-/-}$ embryos die before lung morphogenesis, its potential role in pulmonary formation and function has not been elucidated. We therefore utilized a conditional Cre/loxP recombination system to delete *Foxa2* in respiratory epithelial cells of the developing lung.

Materials and methods

Animals and transgene genotype

Foxa2$^{loxP/loxP}$ mice were generated at the University of Pennsylvania (Sund et al., 2000) and maintained as homozygotes. Homologous recombination between loxP sites was accomplished utilizing (tetO)$_7$CMV-Cre mice (Sauer, 1998), kindly provided by Dr Corrinne Lobe, University of Toronto. For lung-specific, doxycycline-induced recombination, either SP-C-rtTA$^{-/tg}$ or CCSP-rtTA$^{-/tg}$ transgenic lines were used (Perl et al., 2002a; Tichelaar et al., 2000). Triple transgenic mice, termed *Foxa2*$^{\Delta/\Delta}$, were generated by crossing (tetO)$_7$-Cre$^{-/tg}$/*Foxa2*$^{loxP/loxP}$ and SP-C-rtTA$^{-/tg}$/*Foxa2*$^{loxP/loxP}$ or CCSP-rtTA$^{-/tg}$/*Foxa2*$^{loxP/loxP}$. *Foxa2*$^{loxP/loxP}$ littermates lacking either rtTA or Cre alleles served as controls. Transgenic mice were identified by PCR using genomic DNA from the tails of fetal and postnatal mice, as previously described (Perl et al., 2002a).

Animal husbandry and doxycycline administration

Animals were maintained in pathogen-free conditions according to protocols approved by Institutional Animal Care and Use Committee at Cincinnati Children's Hospital Research Foundation. All animals were housed in humidity- and temperature-controlled rooms on a 12:12 hour light:dark cycle and were allowed food and water ad libitum. There was no serologic evidence of either pulmonary pathogens or bacterial infections in sentinel mice maintained within the colony. No serological evidence of viral infection or histological evidence of bacterial infection was detected in representative mice. Gestation was dated by detection of the vaginal plug. Dams bearing double- and triple-transgenic pups were maintained on doxycycline in food (25 mg g^{-1}; Harlan Teklad, Wisconsin) for various time spans. The mice were killed by injection of anesthetic and exsanguinated.

Mouse models with goblet cell hyperplasia

Lung samples from each of the following mice and controls were fixed with 4% paraformaldehyde and embedded in paraffin for Alcian Blue and FOXA2 staining.

Ovalbumin challenge model: BALB/c mice were obtained from the National Cancer Institute (Frederick, Maryland) and housed under specific pathogen-free conditions. Mice were treated twice by intraperitoneal injection with 100 μg ovalbumin (OVA, Sigma, grade V) and 1 mg aluminum hydroxide (alum) followed by two intranasal treatments, 3 days apart, with either 50 μg OVA or saline, starting at least 10 days after the second sensitization, as previously described (Mishra et al., 2001). Mice were killed 18 hours after the second intranasal administration.

Conditional expression of IL13: Four-week-old bitransgenic mice bearing CCSP-rtTA and (tetO)$_7$CMV-IL13 transgenes, identified by Southern blot analysis, were fed doxycycline in their food for two weeks to induce expression of IL13 in the lung. Histological analysis of the lung from double transgenic mice revealed marked perivascular and peribronchial inflammatory lesions, thickened basement membranes, smooth muscle hyperplasia, deposition of collagen and production of mucus.

IL4 treatment of *Stat6*$^{-/-}$ mice: Signal transducer and activator of transcription-6-deficient (*Stat6*$^{-/-}$) mice on a BALB/c background were obtained originally from Michael Grusby (Harvard, Massachusetts) and bred at the University of Cincinnati College of Medicine. Briefly, control and *Stat6*$^{-/-}$ adult mice were treated daily for 4 days with either 2 μg IL4 (in 40 μl) or 40 μl normal saline intratracheally. Mice were killed 1 day after the last intratracheal inoculation and the tissues stained for FOXA2 with Eosin as counterstain.

IL4 overexpression mouse model: The generation of IL4 expressing mice was described previously using the CCSP promoter, which is selective for conducting airway epithelial cells (Rankin et al., 1996). Sections from CCSP-IL4 and control adult mice (n=4 per group) were prepared for Alcian Blue and FOXA2 staining.

SP-C deficient mouse model: Adult *Sftpc*$^{-/-}$ mice (129/Sv) strain spontaneously develop goblet cell hyperplasia and enhanced MUC5AC staining in the conducting airways (Glasser et al., 2003). Adult *Sftpc*$^{-/-}$ mice and littermate controls (kindly provided by Dr Stephan Glasser, Cincinnati Children's Hospital Medical Center) were prepared for Alcian Blue and FOXA2 double staining (n=4 per group).

Histology and immunohistochemistry

Tissues from fetal and neonatal lungs were prepared as described previously (Wert et al., 2000). Antibodies used were generated to: pro-SP-C (1:1000, rabbit polyclonal, AB3428, Chemicon); CCSP (1:7500, rabbit polyclonal, kindly provided by Dr Barry Stripp, University of Pittsburgh); SP-B (1:1000, rabbit polyclonal, generated in this lab); TTF-1 (1:1000, rabbit polyclonal, kindly provided by Dr Roberto DiLauro); platelet endothelial cell adhesion molecule-1 (PECAM-1) (1:500, rat polyclonal, clone CD31, Pharmingen); FOXA2 (1:800, sheep immunoaffinity purified IgG, Upstate Biotechnology); MUC5AC (1:500, chicken polyclonal antibody, kindly provided by Dr Samuel Ho); and phosphohistone H3 (1:100, rabbit polyclonal, United States Biological). Immunostaining was performed as described previously (Zhou et al., 1996) using a Foxa1 monoclonal anti-mouse antibody generated in our laboratory (1:50) using a Mom-kit (Vector Laboratories Inc). After staining for FOXA2, lung sections were counterstained for neutral or acidic mucins cells with periodic acid Schiff (PAS) reaction or Alcian Blue PH2.5 method (Poly Scientific R&D Corp). Elastin staining was performed using orcein as directed (Poly Scientific R&D Corp). All experiments shown are representative of findings from at least two independent dams, generating at least four triple transgenic offspring that were compared with littermates.

Lung morphometry

Morphometric measurements were performed on inflation-fixed lungs on postnatal day 16 (PN16) (n=3-5 for each genotype). At least five representative fields were studied in each mouse. Slides were viewed by using a 20× objective and the images transferred by video camera to computer screen using METAMORPH imaging software

(Universal Imaging). Percent fractional airspace areas and percent fractional areas of lung parenchyma were determined as previously described (Liu et al., 2003; Wert et al., 2000). Percent fractional area of respiratory airspace was determined by airspace surface area divided by total area. The pairwise *t*-test was used to determine significant changes at $P<0.05$.

RNA analysis

S1 nuclease protection and RNAse protection assays were performed as described previously (Jobe et al., 2000; Rausa et al., 2000). mRNAs encoding SP-A, SP-B, SP-C, CCSP and FOXA2 were quantified by either S1 nuclease protection assay or RNAse protection assay with ribosomal protein L32 as an internal control. A rat FOXA2 probe was kindly provided by Dr R. Costa, University of Illinois.

Protein measurements

Mice were anesthetized, exsanguinated and BALF collected as described previously (Wert et al., 2000). IL13, IL4, interferon γ (IFNγ), IL5, GM-CSF, MIP2 and KC were measured in the supernatant of lung homogenates using ELISA Kits (R&D Systems) according to the manufacturer's protocol. Western blot analysis for SP-B and SP-C were performed on lung homogenates from *Foxa2*$^{\Delta/\Delta}$ and control littermates at PN16, as previously described (Melton et al., 2003).

Pulmonary function studies

Lung mechanics were assessed in adult CCSP-rtTA, *Foxa2*$^{\Delta/\Delta}$ and control mice at 7 weeks of age by a computerized Flexi Vent system (SCIREQ), as previously described (Liu et al., 2003; Schuessler and Bates 1995).

Transcription of the MUC5AC promoter in vitro

The MUC5AC promoter-luciferase construct, consisting of 3.7 kb of the mouse gene was kindly provided by Dr Carol Basbaum, University of San Francisco (Li et al., 1998). The construct was transfected in H292 cells, a pulmonary cell line that produces MUC5AC in vitro. Fugene 6 (Roche Molecular) was used for transfection according to the manufacturer's directions. Trans-retinoic acid (3 ng ml) was added to H292 cells 24 hours after transfection for a positive control. Forty-eight hours after transfection, luciferase activity was assessed and normalized for co-transfection efficiency by β-galactosidase activity. All transfections were performed in triplicate and data are expressed as mean±s.e.m.

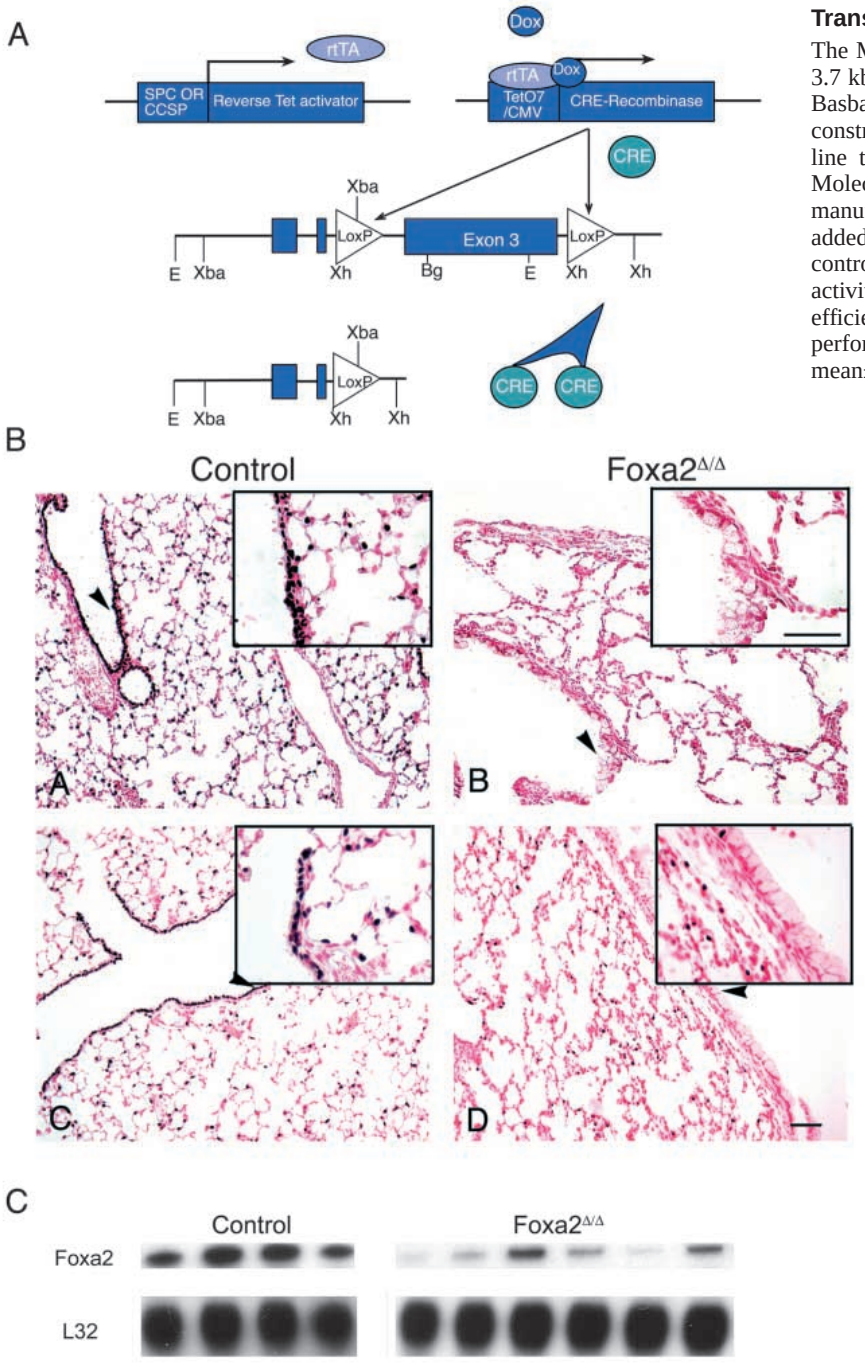

Fig. 1. (A) Conditional control of *Foxa2* gene targeting. In triple transgenic mice (SP-C-rtTA$^{-/tg}$, (tetO)$_7$CMV-Cre$^{-/tg}$, *Foxa2*$^{loxP/loxP}$ and CCSP-rtTA$^{-/tg}$, (tetO)$_7$CMV-Cre$^{-/tg}$, *Foxa2*$^{loxP/loxP}$), rtTA is expressed in epithelial cells under the control of either the human SP-C or CCSP promoters. In the presence of doxycycline, rtTA binds to the (tetO)$_7$CMV promoter and activates the expression of Cre-recombinase, causing recombination and deletion of exon 3 in *Foxa2*$^{loxP/loxP}$ mice, and producing *Foxa2*$^{\Delta/\Delta}$ mice. (B) Immunohistochemistry demonstrates *Foxa2* deletion. Lung sections were prepared on PN16 for immunohistochemistry using anti-FOXA2 antibody. Triple transgenic mice, SP-C-rtTA$^{-/tg}$, (tetO)$_7$CMV-Cre$^{-/tg}$, *Foxa2*$^{loxP/loxP}$ (B part B) and CCSP-rtTA$^{-/tg}$, (tetO)$_7$CMV-Cre$^{-/tg}$, *Foxa2*$^{loxP/loxP}$ (B part D), and littermate control mice (B part A, B part C) were maintained on doxycycline from E0. Inserts are higher magnifications (×40) of the regions indicated by arrowheads. Nuclear FOXA2 staining was observed in epithelial cells of conducting and peripheral airways and alveoli, and was absent or decreased in *Foxa2*$^{\Delta/\Delta}$ mice. Goblet cell hyperplasia was observed in both SP-C-rtTA and CCSP-rtTA *Foxa2*$^{\Delta/\Delta}$ mice (insets). Figures are representative of at least four individual mice. Scale bar, 50 μm. (C) RNA protection assay for estimation of *Foxa2* mRNA. RNA protection assays were used to quantitate Foxa2 mRNA in lungs from SP-C-rtTA, *Foxa2*$^{\Delta/\Delta}$ and control littermates at E18.5 and compared with L32 mRNA. Dams were treated with doxycycline from E0 to E18.5.

Statistical analysis

Either ANOVA or Student's *t*-test were used to determine the levels of difference between groups. Values for all measurements were expressed as the mean±s.e.m. and *P* values for significance were 0.05.

Human tissues

Human lung tissue was obtained at either autopsy or lobectomy under protocols approved by the Committee on Human Research, Vanderbilt University.

Results

Conditional deletion of FOXA2 in the lung

Triple transgenic *Foxa2*$^{loxP/loxP}$, SP-C-rtTA$^{-/tg}$, (tetO)$_7$-Cre$^{-/tg}$ (SP-C-rtTA compound mice) and *Foxa2*$^{loxP/loxP}$, CCSP-rtTA$^{-/tg}$, (tetO)$_7$-Cre$^{-/tg}$ (CCSP-rtTA compound mice) mice were produced in which *Foxa2* was selectively deleted in subsets of respiratory epithelial cells in the developing lung. In these mice, the reverse tetracycline responsive transactivator (rtTA) was expressed in lung epithelial cells under the control of either SP-C or CCSP promoter elements. In the presence of doxycycline, rtTA binds to the (tetO)$_7$CMV promoter, activating expression of Cre-recombinase, which deletes exon 3 of the *Foxa2* gene (Fig. 1A). When SP-C-rtTA compound and CCSP-rtTA compound mice were maintained on doxycycline from E0, pups were born at the expected Mendelian frequency. At birth, body and lung weights of triple transgenic mice were the same as their controls.

Immunohistochemistry demonstrates deletion of *Foxa2*

To assess the efficiency of Cre-mediated gene deletion in SP-C-rtTA compound and CCSP-rtTA compound mice, dams were maintained on doxycycline from E0. FOXA2 staining was assessed at PN16. In wild-type mice, FOXA2 was detected in epithelial cells of conducting airways and in alveolar epithelial type II cells, which is consistent with previous studies (Zhou et al., 1996). FOXA2 staining was absent in most epithelial cells of peripheral conducting airways and alveoli in SP-C-rtTA compound mice (Fig. 1B). Under control of CCSP-rtTA, *Foxa2* was deleted primarily in conducting airways and in restricted subsets of peripheral respiratory epithelial cells. The extent of deletion of *Foxa2* was variable in both SP-C and CCSP-rtTA compound mice, ranging from complete absence of FOXA2 staining to heterogeneous persistence of staining. *Foxa2* deletion was assessed by RNAase protection analysis (Fig. 1C), demonstrating the variable, but marked decrease in FOXA2 mRNA in the lungs of *Foxa2*$^{\Delta/\Delta}$ mice.

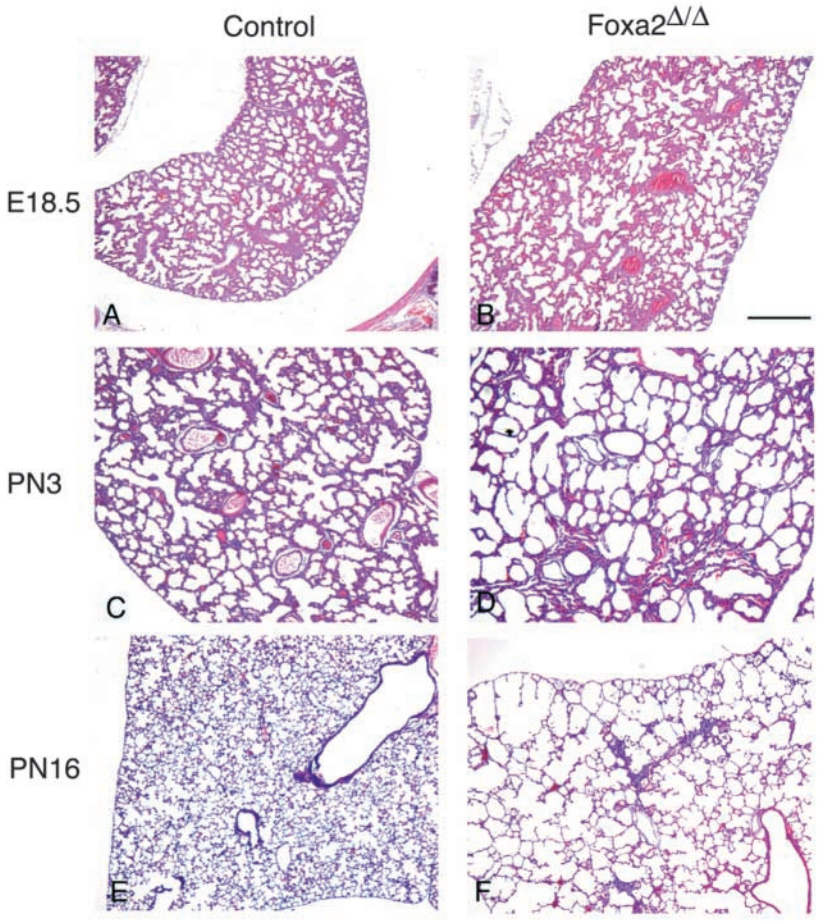

Fig. 2. Effects of *Foxa2*-deletion on lung morphogenesis. Triple transgenic mice (SP-C-rtTA$^{-/tg}$, (tetO)$_7$CMV-Cre$^{-/tg}$, *Foxa2*$^{loxP/loxP}$) and littermate controls were maintained on doxycycline from E0. (A-F) Lung sections of triple transgenic mice and littermate controls were prepared on E18.5 (A,B), PN3 (C,D) and PN16 (E,F) and stained with Hematoxylin and Eosin to assess lung morphology. Figures are representative of at least four individual mice. Scale bar: 300 μm.

Effects of *Foxa2* deletion on lung morphogenesis

When SP-C-rtTA compound mice were maintained on doxycycline from E0, approximately 50% of the pups died between PN1 and PN30 (*n*=24 litters). At E16.5-18.5, lung morphology was not perturbed in *Foxa2*$^{\Delta/\Delta}$ pups (Fig. 2A,B). However, by PN3, fewer peripheral lung saccules and decreased alveolar septation were observed, indicating an abnormality in postnatal alveolarization (Fig. 2C,D). Airspace enlargement, focal neutrophilic infiltrations and goblet-cell hyperplasia were observed at PN16 and later (Fig. 2E,F). Morphometric analysis of fractional airspace and fractional respiratory parenchyma supported the histological assessment of alveolar abnormalities in SP-C- but not in CCSP-rtTA-deleted mice (Fig. 3). Increased numbers of neutrophils and macrophages were observed in bronchoalveolar lavage fluid of 1-month-old mice after deletion of *Foxa2*. Differential cell counts showed a significant increase in neutrophils compared to littermate controls (10±4.2% compared to 0.25±0.5%, $P<0.05$ by ANOVA). Some neutrophils stained for Ly-6 and the alveolar macrophages were generally MAC-3 positive (data not shown). Repeated bacterial cultures of the lung indicated no pulmonary infection. Likewise, sentinel mice did not indicate bacteria or viral pathogens in the colony. No bacteria were found on lung

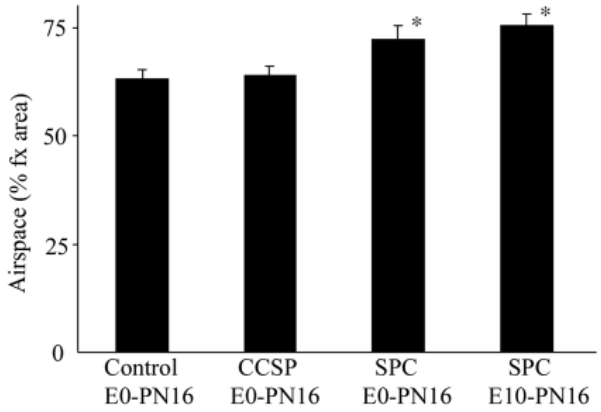

Fig. 3. Morphometric analysis. Fractional airspace and fractional lung parenchyma were calculated in lungs of mice from each genotype after exposure to doxycycline for the defined times. Fractional airspace ratio was significantly increased in SP-C-rtTA but not CCSP-rtTA, *Fox2*$^{\Delta/\Delta}$ mice. *P<0.05 vs control mice (Student's *t*-test).

sections or were observed on cytospins of BALF (data not shown).

Goblet cell hyperplasia after deletion of *Foxa2*

Goblet cell hyperplasia was observed in bronchi and bronchioles after deletion of *Foxa2* in both SP-C-rtTA and CCSP-rtTA compound mice (Fig. 1B, inserts). In lungs of control mice, goblet cells, indicated by either Alcian Blue or MUC5AC staining, were observed rarely. In contrast, staining for acidic and neutral mucins was observed in conducting airways of *Foxa2*$^{\Delta/\Delta}$ mice, assessed by Alcian Blue and periodic acid Schiff staining, respectively (Fig. 4C-F). Likewise, extensive MUC5AC staining was detected at the sites of goblet cell hyperplasia (Fig. 4G,H). CCSP-rtTA compound mice maintained on doxycycline from E0 survived postnatally. Airspace enlargement and neutrophilic infiltrates were not detected in the peripheral lung of CCSP-rtTA triple-transgenic mice. Loss of FOXA2 staining was less extensive in alveolar regions and more extensive in the bronchi and proximal bronchioles in the CCSP-rtTA compared to SP-C-rtTA *Foxa2*$^{\Delta/\Delta}$ mice, consistent with the activity of the promoters. Goblet cell hyperplasia was more prominent in larger airways in the CCSP-rtTA-deleted than the SP-C-rtTA-deleted mice, consistent with sites of gene targeting in the two models (see Fig. S1 at http://dev.biologists.org/supplemental/).

Timed deletion of *Foxa2* during lung morphogenesis

In order to determine the temporal requirements for *Foxa2* during lung morphogenesis, dams and pups were treated with doxycycline at various times during development. Airspace enlargement, variable neutrophilic infiltration and goblet cell hyperplasia were detected in the SP-C-rtTA, *Foxa2*$^{\Delta/\Delta}$ mice, whereas no pulmonary abnormalities were observed in the littermate controls. When SP-C-rtTA compound mice were treated postnatally with doxycycline, from PN1 to PN16, the extent of *Foxa2* deletion was less and airspace enlargement was decreased compared to those maintained on doxycycline prenatally (Fig. 5). Airspace abnormalities were not observed in CCSP-rtTA-deleted mice (Figs 1, 3).

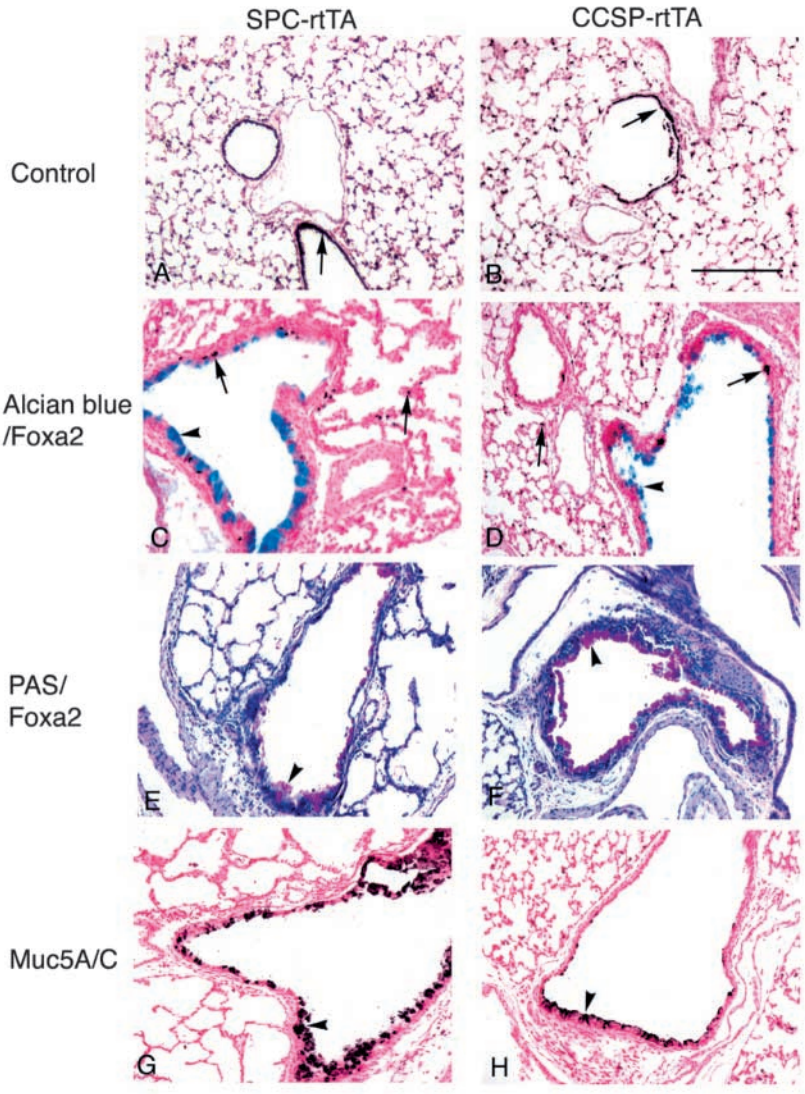

Fig. 4. Goblet cell hyperplasia after *Foxa2* deletion. (A-H) Compound transgenic mice, either SP-C-rtTA$^{-/tg}$, (tetO)$_7$CMV-Cre$^{-/tg}$, *Foxa2*$^{loxP/loxP}$ (C,E,G) or CCSP-rtTA$^{-/tg}$, (tetO)$_7$CMV-Cre$^{-/tg}$, *Foxa2*$^{loxP/loxP}$ (D,F,H), and controls (non-Foxa2 deleted) (A,B) were treated with doxycycline from E0. At PN16, lung sections were stained for FOXA2 (arrows) and either Alcian Blue or periodic acid Schiff (PAS). Staining by Alcian Blue was observed in cells with goblet cell morphology (A-D, arrowheads). Similarly, PAS (E,F, arrowheads) and MUC5AC staining (G,H, arrowheads) were observed in goblet cells in conducting airways in *Foxa2*-deleted mice. FOXA2 staining was absent in nuclei of the goblet cells (C-H). Figures are representative of at least four individual mice of each genotype. Scale bar: 200 μm.

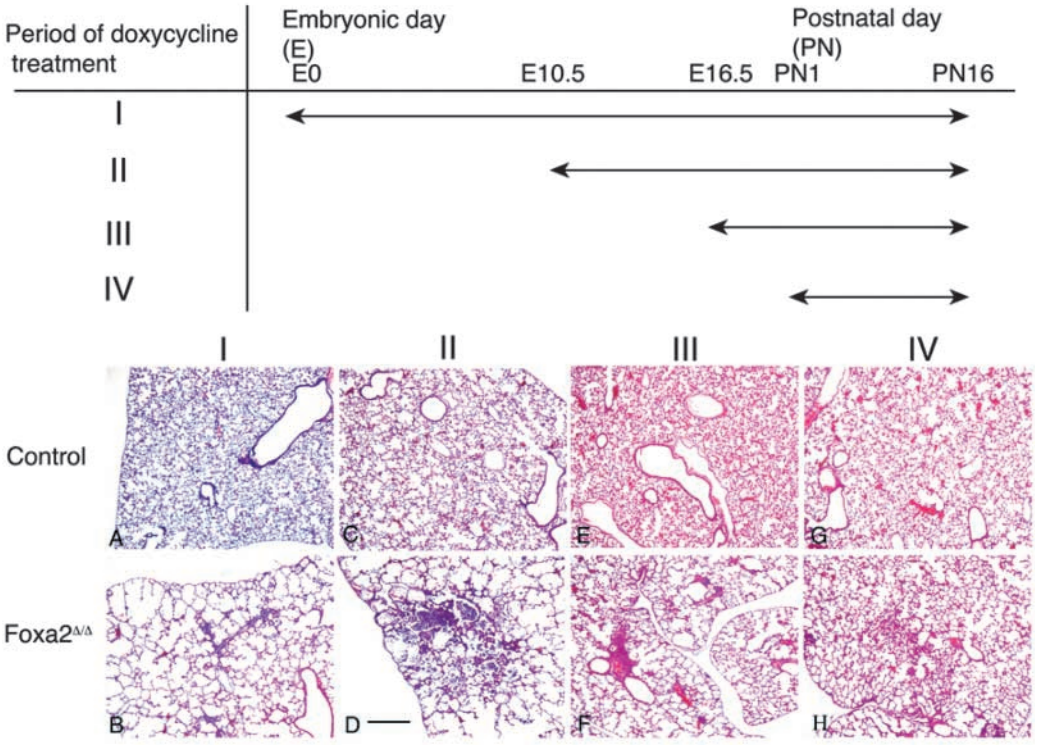

Fig. 5. Timed deletion of *Foxa2* during lung morphogenesis. The period of doxycycline treatment is shown in the top panel. Lung sections from SP-C-rtTA$^{-/tg}$, (tetO)$_7$CMV-Cre$^{-/tg}$, *Foxa2*$^{loxP/loxP}$ (B,D,F,H) and littermate controls (A,C,E,G) were prepared on PN16 and stained with hematoxylin-eosin. Airspace enlargement and neutrophilic infiltrations were observed in the *Foxa2*-deleted mice. Figures are representative of at least four individual mice of each genotype. Scale bar: 300 μm.

Effects of *Foxa2* deletion on epithelial cell gene expression and PECAM

Because FOXA2 influences the transcription of the *Titf1*, *Sftpb*, and *Scgb1a1* genes in vitro (Bingle and Gitlin, 1993; Bingle et al., 1995; Bohinski et al., 1994; Ikeda et al., 1996), S1-nuclease protection assays were utilized to quantitate mRNA encoding surfactant protein A (SP-A), SP-B, SP-C and Clara cell secretory protein (CCSP) at E17.5. When SP-C-rtTA compound mice were maintained on doxycycline from E0 to E17.5-18, mRNAs encoding CCSP, SP-A and SP-B were decreased significantly (data not shown). Likewise, the content of SP-B in lung homogenates from surviving mice at PN16 was decreased significantly to 52.9±6% of control values (n=4) (see Fig. S2 at http://dev.biologists.org/supplemental/). Because SP-B is crucial for surfactant function, this decrease in SP-B may render the SP-C-rtTA, *Foxa2*$^{\Delta/\Delta}$ mice more susceptible to respiratory dysfunction and death. Recent work from this laboratory demonstrates that reduction of SP-B to 20-30% of normal levels causes respiratory failure in adult mice (Melton et al., 2003). Immunohistochemical staining for CCSP was decreased in non-ciliated respiratory epithelial cells in the *Foxa2*$^{\Delta/\Delta}$ mice (see Fig. S3 at http://dev.biologists.org/supplemental/). By contrast, SP-C protein content and immunostaining were unchanged (supplemental Figs S2 and S3). At E18.5, SP-A mRNA was reduced 12.7-fold and CCSP mRNA reduced 3-fold in SP-C-rtTA *Foxa2*$^{\Delta/\Delta}$ mice compared to controls. Immunostaining for TTF-1, mature SP-B, pro-SP-C, T1α (a type I cell marker), FOXJ1 (a ciliated cell marker) and FOXA1 was unaltered (Fig. 6, and see Fig. S3 at http://dev.biologists.org/supplemental/). PECAM staining indicated normal distribution of pulmonary capillaries in the enlarged alveoli. Elastin staining was present in alveolar septa, however fewer septae were detected after *Foxa2* deletion, indicating a primary abnormality in alveolarization-septation (Fig. 6). Elastin fibers were neither fragmented nor shortened, indicating that alterations in alveolar size in *Foxa2*$^{\Delta/\Delta}$ mice were not associated with elastin destruction. The extent and distribution of phosphohistone-3 staining, an indicator of cell proliferation, were unchanged in the *Foxa2* deleted mice at E18.5 and PN2 (data not shown).

Pulmonary mechanics

Because most SP-C-rtTA, *Foxa2*$^{\Delta/\Delta}$ mice either died or were ill by maturity, lung mechanics were assessed on 7-week-old CCSP compound mice during forced oscillatory ventilation. Airway and tissue resistance, and elastance were increased significantly and compliance decreased, indicating abnormalities in both conducting airways and alveolar regions of the *Foxa2*$^{\Delta/\Delta}$ mice (Fig. 7).

Decreased FOXA2 staining in mouse models with goblet cell hyperplasia

Increased expression of either IL4 or IL13, deletion of SP-C (Glasser et al., 2003; Jain-Vora et al., 1997; Kuperman et al., 2002; Rankin et al., 1996) and allergen challenge (Tomkinson et al., 2001) each cause pulmonary inflammation and goblet cell hyperplasia in vivo. We hypothesized that decreased expression of *Foxa2* may contribute to the pathogenesis of goblet cell hyperplasia. FOXA2 staining was either decreased or absent in goblet cells in the airways in each of these mouse models, supporting the concept that decreased FOXA2 is either associated with or required for goblet cell hyperplasia (Fig. 8). Nuclear staining of FOXA2 was decreased or absent in the surface cells with characteristics of goblet cells

indicated by mucin, PAS and Alcian Blue staining. *Foxa2* staining was maintained in non-goblet bronchiolar epithelial cells and in basal cells that serve as precursors to goblet cells. Neither deletion of *Foxa2* nor treatment with IL13 altered phosphohistone-3 staining in the airways undergoing goblet cell hyperplasia, indicating that goblet cells were derived by differentiation of precursor cells (basal and Clara cells) rather than from proliferation (data not shown).

Effects of IL4 on goblet cell hyperplasia and *Foxa2* are *Stat6* dependent

Intratracheal administration of TH_2 cytokines and IL4 causes goblet cell hyperplasia in wild-type but not *Stat6*$^{-/-}$ mice (Kuperman et al., 1998). In control mice, FOXA2 staining was either decreased or absent in goblet cells after intratracheal administration of IL4. In contrast, neither decreased *Foxa2* staining nor goblet cell hyperplasia were observed in *Stat6*$^{-/-}$ mice (Fig. 9).

Cytokine expression

To assess whether *Foxa2* altered expression of cytokines that are known to cause lung inflammation and goblet cell hyperplasia, IL4, IL13, IL5, IFNγ, MIP2, KC and GM-CSF were measured by ELISA in lung homogenates from SP-C-rtTA compound mice at PN2 or PN9. Whereas KC content was modestly but significantly increased (from 40±3 pg ml^{-1} to 74±4 pg ml^{-1}, P=0.01, n=6) in the *Foxa2*-deleted mice, no statistically significant differences were detected in the other cytokines. Expression of mRNA encoding cytokines that are known to be associated with lung inflammation and airway remodeling, including IL4, IL5, IL6, IL9, IL10, IL11, IL13, IL17 and tissue necrosis factor α (Chen et al., 2003; Han et al., 1987; Matheson et al., 2002; Mishra et al., 2001; Rankin et al., 1996), were not altered in the SP-C-rtTA, *Foxa2*$^{\Delta/\Delta}$ transgenic mice (data not shown).

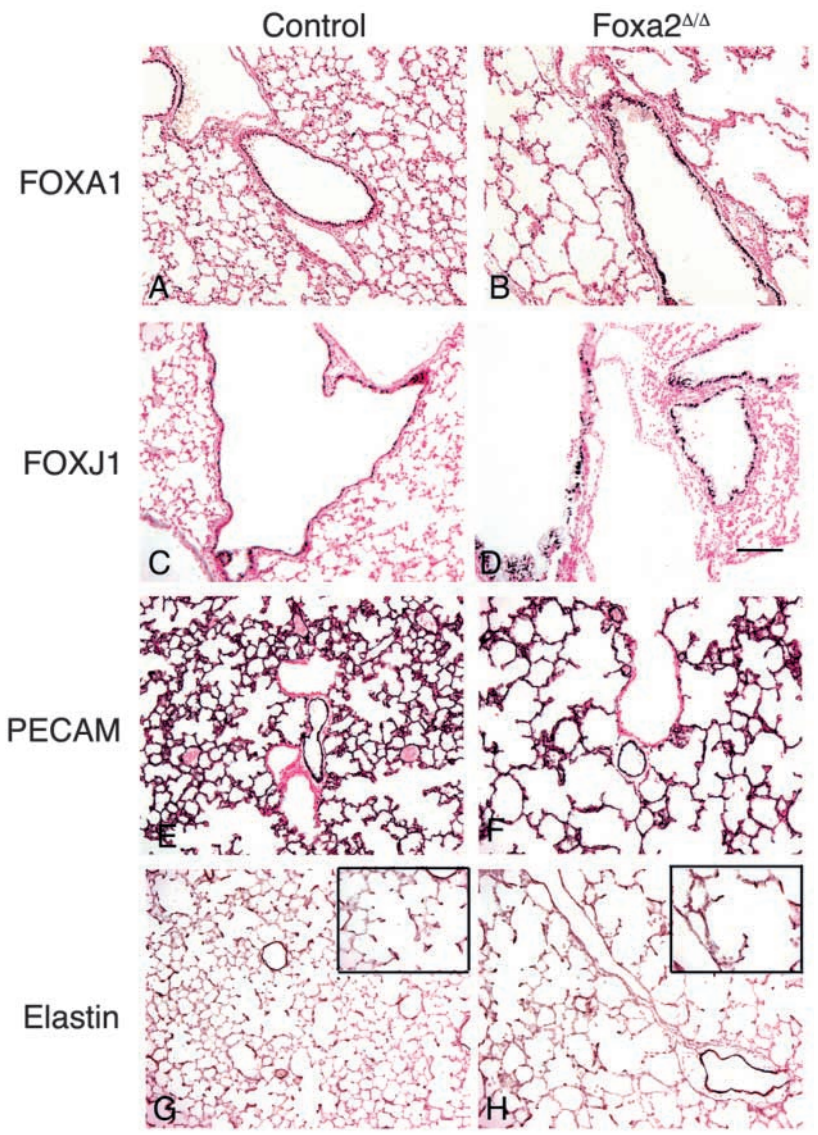

Fig. 6. FOXA1, FOXJ1, PECAM and elastin staining. Lung sections were prepared on PN16. Triple transgenic mice, SP-C-rtTA$^{-/tg}$, (tetO)$_7$CMV-Cre$^{-/tg}$, *Foxa2*$^{loxP/loxP}$ and littermate controls were maintained on doxycycline from E0. (A-H) Lung sections were stained for FOXA1 (A,B), FOXJ1 (C,D), PECAM (E,F), and elastin (G,H). Inserts are higher magnifications (×40). Figures are representative of at least four individual mice of each genotype. Scale bar: 100 μm.

FOXA2 inhibits transcription of the *Muc5ac* gene in vitro

To assess whether FOXA2 directly regulated mucin expression in respiratory epithelial cells, a luciferase reporter construct containing 3.7 kb regulatory region of the mouse *Muc5ac* gene was transfected with *Foxa2* into H292 cells. FOXA2 significantly inhibited the activity of the MUC5AC-luciferase construct in a dose-dependent manner (Fig. 10), indicating that FOXA2 inhibits the expression of genes associated with goblet cell phenotype.

Decreased FOXA2 associated with goblet cell hyperplasia in human lung disease

In order to determine the relationship between the loss of FOXA2 and goblet cell hyperplasia in humans, lung sections were obtained at either autopsy or at lobectomy from 10 patients with chronic lung disease. Tissue was immunostained for FOXA2 and counterstained with Alcian Blue. Five of the subjects were adults, four with cystic fibrosis and one with chronic pulmonary infection and bronchiectasis. Five subjects were infants dying in the first 6 months after birth with bronchopulmonary dysplasia. In all subjects, Alcian Blue-reactive, mucus-producing cells lacked FOXA2 staining (Fig. 11A-C) whereas most cells lining normal airways stained for FOXA2. FOXA2 was readily detected in nuclei of adjacent, non-goblet, Alcian Blue-negative epithelial cells lining both conducting and terminal airways (Fig. 11D). Loss of *FOXA2* was sufficient to cause goblet cell hyperplasia in the absence of inflammatory stimuli.

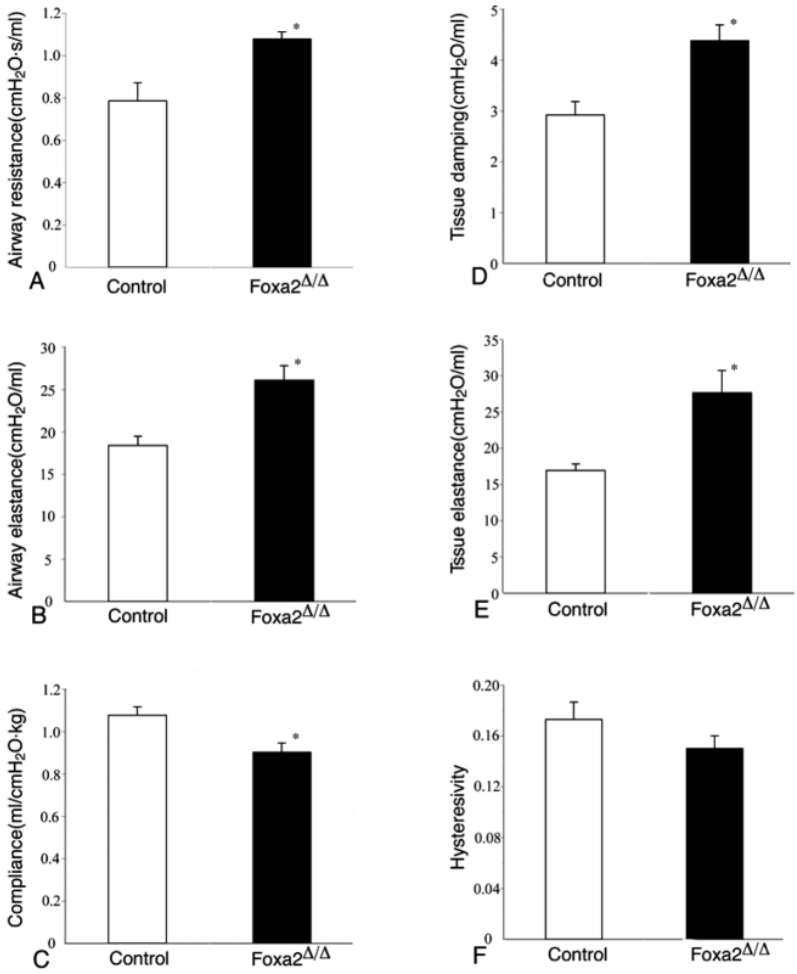

Fig. 7. Pulmonary mechanics**.** (A-F) Airway resistance (A), airway elastance (B), compliance (C), tissue damping (D), tissue elastance (E) and hysteresivity (F) were measured in 7-week-old CCSP-rtTA, *Foxa2*$^{\Delta/\Delta}$ (closed bar, *n*=6) and control mice (open bar, *n*=5). Increased airway resistance, airway elastance, tissue damping, tissue elastance and decreased compliance were observed. Values are mean±s.e.m. **P*<0.05 vs control mice (Student's *t*-test).

Discussion

The airways are lined by distinct cell types whose differentiation is precisely determined along the cephalo-caudal axis. Conducting airways are lined primarily by nonciliated columnar epithelial cells (Clara cells), and by ciliated and basal cells (Plopper et al., 1986). Generally, in the mouse, goblet cells are not observed unless the animals are exposed to inflammatory stimuli, including allergens. In humans, goblet cell hyperplasia is commonly associated with chronic lung diseases including asthma, cystic fibrosis, bronchopulmonary dysplasia and smoking-related chronic obstructive lung disease. In mice, Clara, goblet and ciliated cells lining conducting airways differentiate from basal cells and other non-ciliated progenitors during the perinatal and postnatal period (Ford and Terzaghi-Howe, 1992). In the present studies, abnormalities in alveolarization, goblet cell hyperplasia and mucus production were caused by deletion of *Foxa2* in airway epithelial cells.

Goblet cell hyperplasia and decreased FOXA2 staining

Goblet cell hyperplasia in *Foxa2*$^{\Delta/\Delta}$ mice was associated with the accumulation of both neutral and acidic mucins, increased MUC5AC staining and decreased CCSP staining at cellular sites in which *Foxa2* was deleted in the conducting airways. In both mice and humans, goblet cells lacked FOXA2 staining but non-goblet columnar cells stained intensely, indicating that the effects of FOXA2 were cell autonomous rather than caused by secondary or reciprocal signaling among neighboring cells. Concentrations of lung

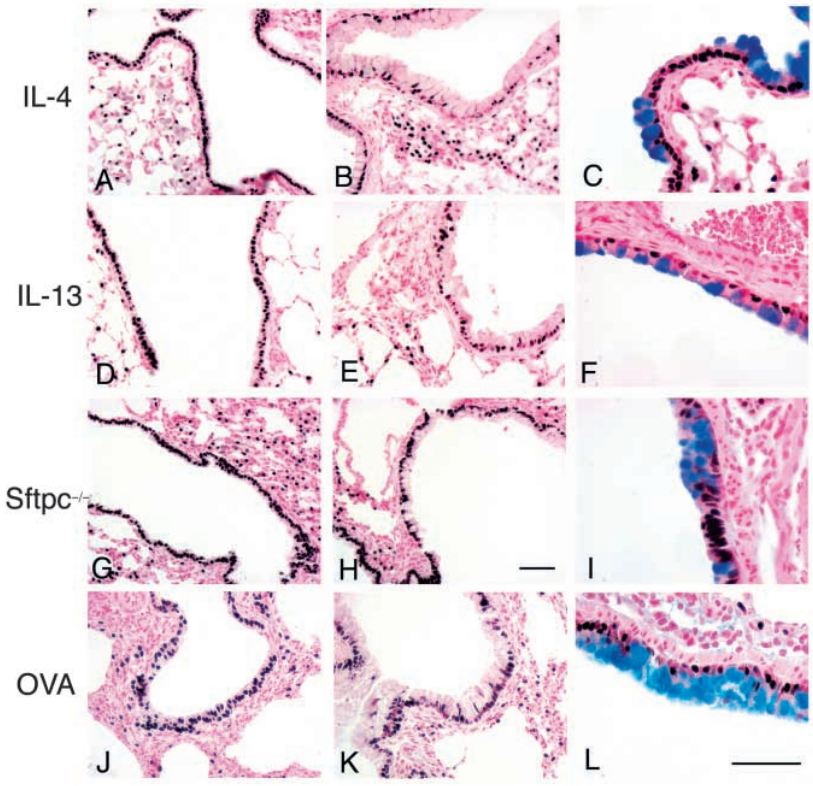

Fig. 8. FOXA2 staining of mouse models with goblet cell hyperplasia. (A-L) Lung sections were prepared from mouse models with goblet cell hyperplasia and stained for FOXA2. Models: overexpression of IL4 (A-C); overexpression of IL13 (D-F); deletion of SP-C (G-I); and ovalbumin challenged mice (J-L). Normal bronchi (A,D,G,J) and bronchi with goblet cell hyperplasia (B,E,H,K) are shown. FOXA2/Alcian Blue staining (C,F,I,L) indicates a close correlation between goblet cell hyperplasia and decreased or absent FOXA2 expression. Figures are representative of at least four individual mice from each model. Scale bars: 50 μm.

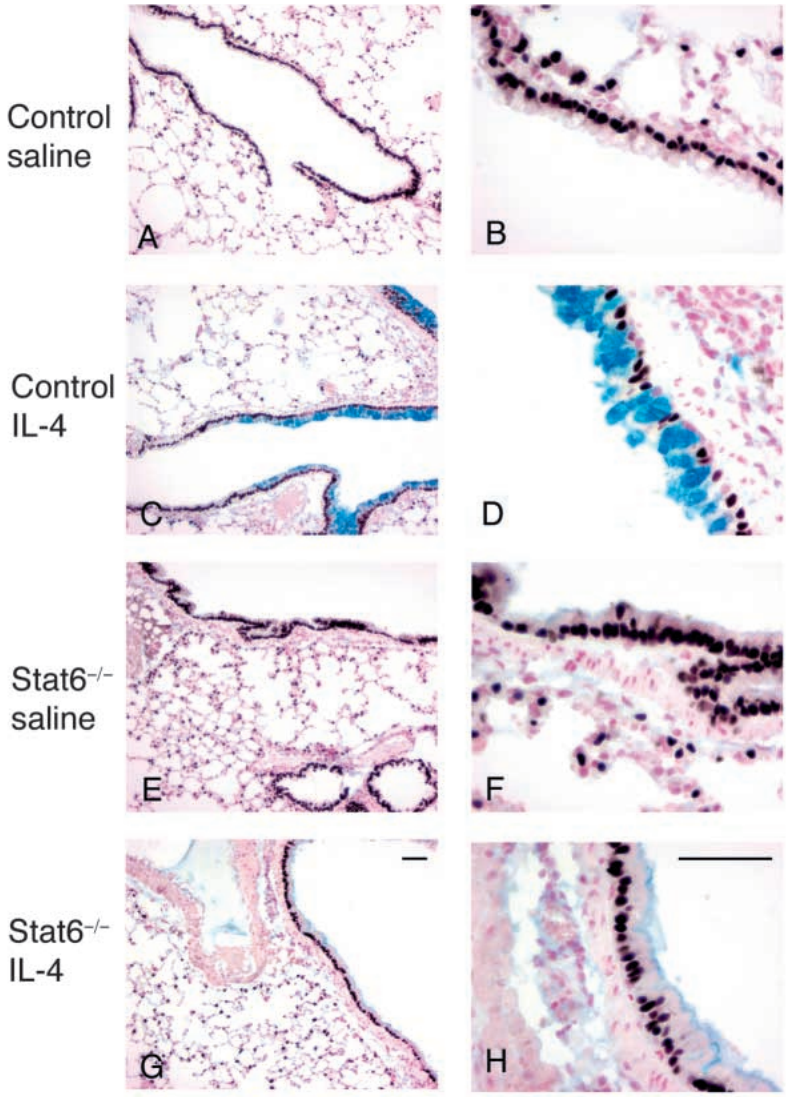

Fig. 9. Effects of IL4 on FOXA2 are STAT6 dependent. (A-H) Adult, control (A-D) and *Stat6*$^{-/-}$ (E-H) mice were treated intranasally with IL4. Lung sections were prepared and stained for FOXA2. The mice were treated with either saline (A,B,E,F) or IL4 (C,D,G,H). IL4 induced goblet cell hyperplasia in control but not *Stat6*$^{-/-}$ mice. Foxa2 staining decreased at sites of goblet cell hyperplasia in wild-type mice. Foxa2 staining and epithelial cell morphology were not perturbed in *Stat6*$^{-/-}$ mice. Figures are representative of four mice of each genotype. Scale bars: 50 μm.

cytokines that are associated with goblet cell hyperplasia (IL4, IL13 and IL5), and RNA levels of proinflammatory cytokines associated with goblet cell hyperplasia were not altered after deletion of *Foxa2*, indicating that the loss of FOXA2 in airway epithelial cells directly influenced goblet cell hyperplasia in these models. The finding that FOXA2 inhibited transcriptional activity of the *Muc5ac* gene in vitro, supports the concept that FOXA2 also directly inhibits mucin gene expression. Goblet cell hyperplasia following *Foxa2* deletion was more extensive in proximal conducting airways in the CCSP-rtTA compound mice, consistent with the distinct sites of expression of the CCSP promoter used to express the rtTA and the sites of gene targeting in the two models. Goblet cell hyperplasia was seen in peripheral conducting airways in both models, again consistent with the sites of gene expression and recombination in the models (Perl et al., 2002a; Perl et al., 2002b; Stripp et al., 1992; Wert et al., 1993). Despite extensive deletion of *Foxa2* in subsets of cells in conducting airways and alveolar regions, not all *Foxa2*$^{\Delta/\Delta}$ cells become goblet cells, supporting the concept that additional factors influence mucus cell differentiation. Alternatively, it is unlikely that loss of *Foxa2* influences goblet cell differentiation in distinct subsets of conducting airway cells.

Timing and sites of *Foxa2* deletion influence airspace enlargement

Airspace enlargement was prominent in mice in which *Foxa2* was deleted with the SP-C-rtTA but was not seen in CCSP-rtTA transgenes. The timing and extent of recombination is distinct in these two models. Deletion of *Foxa2* was extensive in the lung periphery in the SP-C-rtTA transgenic mice treated with doxycycline before birth, consistent with previous studies (Perl et al., 2002b). In the SP-C-

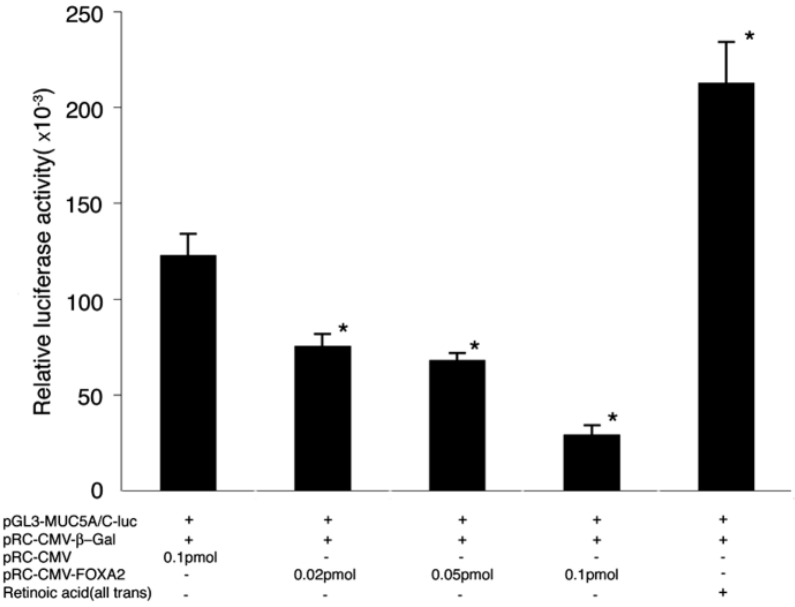

Fig. 10. FOXA2 inhibits *Muc5ac* transcription. H292 cells were transfected with promoter plasmid pGL3-MUC5AC-luc and increasing amounts of the expression plasmid pRC-CMV-FOXA2. MUC5AC promoter activity was determined by the relative luciferase activity normalized to β-galactosidase activity. As a positive control, all trans-retinoic acid (3 μg ml^{-1}) was added to induce MUC5AC expression. Plasmid pRC-CMV was used as empty vector control. *P<0.05 versus control (ANOVA).

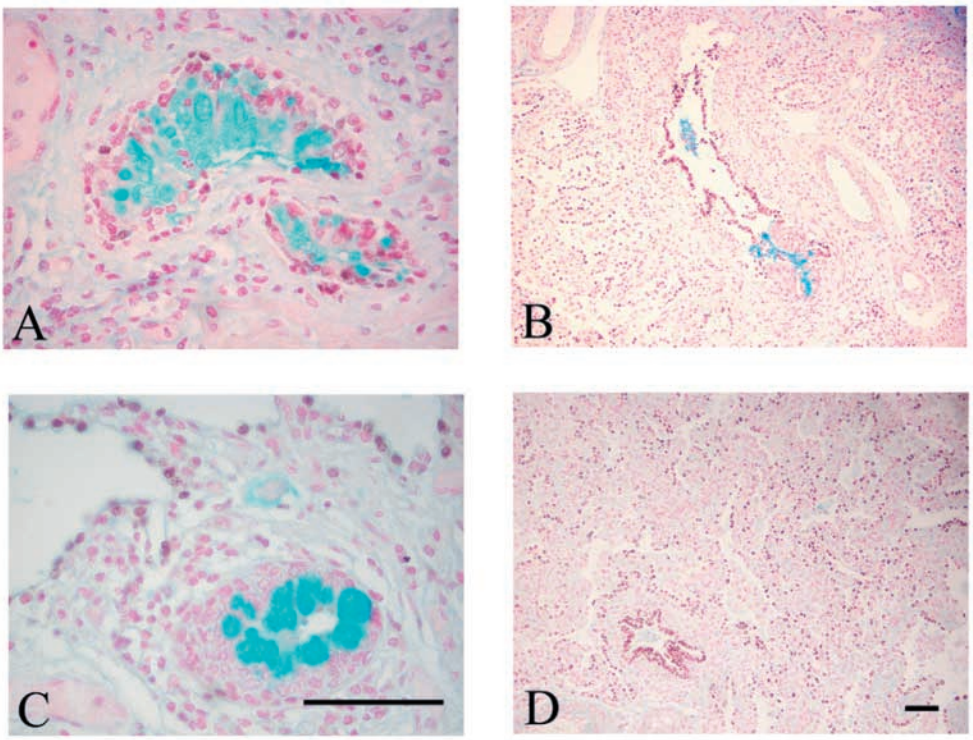

Fig. 11. FOXA2 staining of human lung tissue with goblet cell hyperplasia. (A) Photomicrograph (×40) of a small conducting airway of a 5-month-old infant who died with bronchopulmonary dysplasia. The nuclei of Alcian Blue-stained mucus cells are unstained by the FOXA2 antibody. A few cells with FOXA2-stained nuclei are observed in the basal layer of the conducting airways and a nearby terminal airway. (B) Photomicrograph (×10) of the lung from the patient in A, showing a cuboidal lined alveolar duct arising from the small columnar cell lined bronchus. The nuclei of the Alcian Blue-stained mucus cells in the bronchus lack FOXA2, but most of the nuclei of the cells lining the alveolar ducts are FOXA2 reactive. (C) Photomicrograph (×40) of the lung of a 27-year-old female who underwent lobectomy for bronchiectasis. This shows a small bronchus lined with Alcian Blue-stained mucus cells, the nuclei of which lack FOXA2 staining. Many nuclei of non-goblet cells lining terminal airways are FOXA2 reactive. (**D)**. Photomicrograph (×10) of the lung of a 6.5-month-old infant dying with bronchopulmonary dysplasia showing terminal airway cell nuclei immunostained for FOXA2. Nuclei of epithelial cells lining a small conducting airway lack Alcian Blue staining and are immunostained by the FOXA2 antibody. Scale bar: 50 μm.

rtTA compound mice, deletion of *Foxa2* occurs in lung progenitor cells and is extensive or complete as early as E6.5-8.5, which is prior to onset of branching morphogenesis. In contrast, the CCSP-rtTA transgene is not active until E14-15, and targeting occurs primarily in the conducting airways rather than in the peripheral lung before birth (Perl et al., 2002a). The size of the lungs and the morphology of peripheral lung saccules were unaltered before birth in the *Foxa2*$^{\Delta/\Delta}$ mice, whether deletion was induced with SP-C- rtTA or CCSP-rtTA. Thus, FOXA2 is not absolutely required for prenatal lung morphogenesis, cell differentiation and perinatal survival. Neither is it required for the expression of SP-B, a surfactant protein required for postnatal survival (Clark et al., 1995). Effects of *Foxa2* deletion on peripheral lung morphogenesis were apparent as early as PN3, and extensive airspace enlargement was observed during alveolarization (PN10-20). Most SP-C-rtTA, but not CCSP-rtTA, *Foxa2*$^{\Delta/\Delta}$ mice either died or were ill after PN28. The finding that SP-B was decreased significantly provides a potential basis for increased susceptibility to lung dysfunction. Reduction of SP-B to 20-30% of normal causes respiratory failure in mice (Melton et al., 2003). The increased postnatal mortality of SP-C-rtTA *Foxa2*$^{\Delta/\Delta}$ mice is likely to be related, at least in part, to the lack of SP-B. Most *Foxa2*$^{\Delta/\Delta}$ compound mice generated with SP-C-rtTA were dead by 1-2 months of age. By contrast, survival of CCSP-rtTA *Foxa2*$^{\Delta/\Delta}$ mice was unaltered during this time period. Elastin staining indicated deficient numbers of alveolar septae in the SP-C-rtTA *Foxa2*$^{\Delta/\Delta}$ mice, demonstrating that FOXA2 plays a crucial role in alveolarization. Abnormalities in peripheral airspaces generally occurred either before or in the absence of neutrophilic infiltrations and were not associated with fragmentation of elastin fibers.

Deletion of *Foxa2* caused abnormalities in alveolarization in the lung periphery and goblet cell hyperplasia in the conducting airways. The timing and extent of lung abnormalities in the two models indicates that these two processes may represent independent effects of FOXA2 at distinct cellular sites. For example, severe goblet cell hyperplasia was observed in large conducting airways where *Foxa2* deletion was most effective with the CCSP promoter, and occurred in the absence of detectable neutrophilic infiltration and airspace enlargement. Therefore, we propose that *Foxa2* plays important, distinct roles in the regulation and differentiation of subsets of conducting airway epithelial cells, which results in goblet cell hyperplasia, and in subsets of alveolar cells, in which the deletion of *Foxa2* perturbs alveolarization.

Summary

Goblet cell hyperplasia, mucin production, inflammation and airspace abnormalities are the hallmarks of asthma, chronic obstructive lung disease, cystic fibrosis and bronchopulmonary dysplasia. The present study identifies a role for *Foxa2* in a transcriptional pathway influencing features shared by these common pulmonary disorders.

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