Compensatory Roles of Foxa1 and Foxa2 During Lung Morphogenesis

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Running Title: Foxa1 and Foxa2 in Pulmonary Morphogenesis

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Foxa1 and Foxa2 are closely related family members of the Foxa group of transcription factors that are co-expressed in subsets of respiratory epithelial cells throughout lung morphogenesis. Shared patterns of expression, conservation of DNA binding and transcriptional activation domains indicate that they may serve complementary functions in the regulation of gene expression during lung morphogenesis. While branching morphogenesis of the fetal lung occurs normally in the $\text{Foxa}2^{-/-}$ and $\text{Foxa}1^{-/-}$ mice, deletion of both Foxa1 and Foxa2 (in $\text{Foxa}2^{-/-}$, $\text{Foxa}1^{-/-}$ mice) inhibited cell proliferation, epithelial cell differentiation and branching. Dilation of terminal lung tubules and decreased branching were observed as early as E12.5. Foxa1 and Foxa2 regulated Shh and Shh dependent genes in the respiratory epithelial cells that influenced expression of genes in the pulmonary mesenchyme that are required for branching morphogenesis. Epithelial cell differentiation, as indicated by lack of expression of SP-B, SP-C, CCSP and Foxj1, was inhibited. Foxa family members regulate signaling and transcriptional programs required for morphogenesis and cell differentiation during formation of the lung.

Lung morphogenesis begins with a ventral out-pouching of endodermally derived cells from the anterior foregut into the surrounding mesenchyme at E9-9.5 in the mouse. Lung tubules are formed by branching morphogenesis as respiratory epithelial cells proliferate and differentiate to form the conducting airways. Thereafter, terminal airways sacculate and separte to form the alveoli typical of the peripheral lung. The ordered process mediating branching morphogenesis and the formation of the alveoli are regulated by the precise temporal-spatial expression of many transcription factors, including Gata6, Ttf1 and forkhead transcription factors, including Foxa1, Foxa2, Foxj1, Foxf1, Foxp1 and Foxp2 that regulate gene expression and influence cell differentiation in the lung (1-6).

$\text{Foxa}$ (previously termed $\text{HNF3}$) transcription factors comprise a subfamily of forkhead transcription factors that share more than 90% homology in the winged helix DNA binding domain. Likewise, the transactivation domains in the C-terminal and N-terminal regions of $\text{Foxa}1$ and $\text{Foxa}2$ share structural similarity (7,8). Foxa proteins are expressed primarily in endodermally derived tissues where they influence embryonic patterning, cell differentiation and function (9). The Foxa family includes Foxa1, Foxa2 and Foxa3 (previously termed HNF3$\alpha$, HNF3$\beta$ and HNF3$\gamma$ respectively). Only Foxa1 and Foxa2 are expressed in the lung (10). In mouse, Foxa2 is first expressed in the primitive streak at E6.5, shortly after the onset of gastrulation (11). Thereafter, Foxa2 is present in the notochord, gut endoderm, and in the ventral midline of the central nervous system. Later in embryonic development, Foxa2 is detected in organs derived from foregut and hindgut endoderm (10-12), in tissues of ectodermal and mesodermal origins and in endodermally derived epithelial cells in the lung (13). Foxa1 mRNA is detected in the endoderm...
during the late primitive streak stage of development in the mice. Later in development, Foxa1 mRNA is observed in the ventral floorplate, notochord, and gut in a pattern similar to Foxa2 (10-12,14,15). In the lung, the temporal-spatial expression patterns of Foxa1 and Foxa2 are similar. Foxa1 and Foxa2 are present in the nuclei of epithelial cells from the onset of lung bud formation and in respiratory epithelial cells lining conducting and peripheral airways of the mouse and human fetus (13,16,17).

Null mutation of the Foxa2 gene in the mouse embryo inhibits formation of the notochord and endoderm before the onset of lung morphogenesis (18,19). Thus the potential role of Foxa2 in lung formation and function could not be studied. Ectopic expression of Foxa2 in distal respiratory epithelial cells in the lung of transgenic mice disrupts branching morphogenesis and arrested differentiation of peripheral epithelial cells, suggesting its potential role in lung formation, (20). Recent studies in which Foxa2 was conditionally deleted in the lung epithelial cells showed that Foxa2 is required for normal alveolarization and postnatal lung homeostasis, but is not required for branching morphogenesis (21). In spite of the expression of Foxa1 throughout lung development, alterations in branching morphogenesis were not observed in Foxa1<sup>-/-</sup> mice. Foxa1<sup>-/-</sup> mice survive after birth but generally die between postnatal day (PN) 2 and PN14, exhibiting growth retardation and hypoglycemia (22,23).

The DNA binding domains of Foxa1 and Foxa2 are highly conserved and bind similar cis-acting elements. Since activation domains are also conserved, Foxa1 and Foxa2 may either compete or serve complementary roles at transcriptional targets (24-26). Since the pattern of their expression in the developing lung is similar, we hypothesized that Foxa1 and Foxa2 may play partially overlapping roles in the lung morphogenesis (13,16,17). To assess the potential roles of Foxa proteins in lung formation, Foxa1<sup>-/-</sup>/Foxa2<sup>ΔΔ</sup> mice were produced in which both genes were deleted in respiratory epithelial cells in the mouse fetus.

### EXPERIMENTAL PROCEDURES

#### Transgenic Constructs

Triple transgenic mice, SPC-rtTA<sup>ΔΔ</sup>(tetO)7/Cre<sup>ΔΔ</sup>/Foxa2<sup>LoxP/LoxP</sup>, were generated by crossing SPC-rtTA<sup>ΔΔ</sup>(tetO)7/Foxa2<sup>LoxP/LoxP</sup> and (tetO)<sup>ΔΔ</sup>/Cre<sup>ΔΔ</sup> mice as reported previously (21). Doxycycline food was administered to the pregnant dam to induce recombination in epithelial cells of the fetal lung resulting in deletion of the Foxa2<sup>ΔΔ</sup> gene (SPC-rtTA<sup>ΔΔ</sup>(tetO)-Cre<sup>ΔΔ</sup>/Foxa2<sup>LoxP/LoxP</sup>) referred to as Foxa2<sup>ΔΔ</sup>. Transgenic mice, SPC-rtTA<sup>ΔΔ</sup>/Foxa1<sup>-/-</sup>/Foxa2<sup>LoxP/LoxP</sup>/Foxa1<sup>-/-</sup> (termed Foxa1<sup>-/-</sup>/Foxa2<sup>ΔΔ</sup>), were generated by crossing SPC-rtTA<sup>ΔΔ</sup>(tetO)-Cre<sup>ΔΔ</sup>/Foxa2<sup>LoxP/LoxP</sup> and Foxa1<sup>-/-</sup>. After doxycycline food was administered to the pregnant dam, the Foxa2<sup>ΔΔ</sup> gene was selectively deleted in subsets of epithelial cells in the Foxa1<sup>-/-</sup> lung. These mice are termed Foxa1<sup>-/-</sup>/Foxa2<sup>ΔΔ</sup>. Genotypes were verified by PCR (21,22).

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### Animal Use and Doxycycline Administration

Mice were housed in pathogen free conditions in accordance with IACUC guidelines. Gestational age was determined by detection of the vaginal plug (as E0.5) and correlated with length and weight of each pup at the time of sacrifice. Dams bearing transgenic pups were maintained on food containing doxycycline (625 mg/kg; Harlan Teklad, Madison, WI) from E0.

### Histology, Immunohistochemistry, In Situ Hybridization and Electron Microscopy

Fetal lungs were prepared for histology as previously described (27). Antibodies used for immunohistochemistry were: pro-SP-C (1:1000, rabbit polyclonal, AB3428, Chemicon), CCSP (1:7500, rabbit polyclonal, kindly provided by Dr. Barry Stripp, University of Pittsburgh), TTF-1 (1:1000, rabbit polyclonal AB3426, Chemicon), TTF-1 (1:7500, rabbit polyclonal, generated in this lab), platelet endothelial cell adhesion molecule-1 (PECAM-1) (1:500, rat...
polyclonal, clone CD31, Pharmingen), α-SMA (alpha-smooth muscle actin) (1:15K, mouse monoclonal, clone 1A4, Sigma-Aldrich), Foxa2 (1:5000, rabbit polyclonal anti-rat Foxa2, generated in this laboratory) (13), Foxa1 (1:2000, guinea pig polyclonal antibody, generated in this laboratory), alpha-smooth muscle actin (1:15K, mouse monoclonal) and alpha-phospho-histone H3 (1:400, rabbit polyclonal, US Biological). Specificity of these Foxa1 and Foxa2 antibodies was recently discussed (13). 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. In situ hybridization for Shh mRNA was performed by using 35S-UTP and 35S-mouse monoclonal (laboratory), alpha-smooth muscle actin (1:10K, guinea pig polyclonal antibody, generated in this laboratory), Foxa1 (1:2000, rabbit polyclonal anti-rat Foxa1, generated in this laboratory) (13), Foxa2 (1:5000, rabbit polyclonal anti-rat Foxa2, generated in this laboratory), Foxa2 (alpha-smooth muscle actin) (1:15K, mouse monoclonal, clone 1A4, Sigma-Aldrich), Foxa2 (1:400, rabbit polyclonal, US Biological). Relative abundance of smooth muscle mRNAs was assessed by MOE430A RNA chips by hybridizing to lung mRNAs from control and Foxa1−/−/Foxa2Δ/Δ mice at E14.5 as previously discussed.

**Statistical Analysis of α-Phospho-histone Stained Cell Counting**

Sections of lungs from control and Foxa1−/−/Foxa2Δ/Δ mice were immunostained for αPH3 and the number of mitotic nuclei was assessed (stained cells/µm²) using Metamorph Imaging software (Universal Imaging Corporation). T-test analysis was carried out using Excel software.

**RESULTS**

**Deletion of Both Foxa1 and Foxa2**

To assess the efficiency of Cre-mediated Foxa2 gene deletion in the Foxa1−/−/Foxa2Δ/Δ mice, Foxa2 immunostaining was assessed in the fetal mouse lung at E12.5, E15.5 and E18.5 (Fig. 1). In control (Fig. 1A,E,I) and Foxa1−/− mice (Fig. 1B,F,J), Foxa2 was detected in the nuclei of most pulmonary epithelial cells, including those lining the airways and alveolar type II cells in peripheral saccules. In Foxa2Δ/Δ and Foxa1−/−/Foxa2Δ/Δ mice, Foxa2 was absent in subsets of respiratory epithelial cells. As shown previously (21), Foxa2 staining was absent in most epithelial cells in both conducting airways and peripheral saccules in Foxa2Δ/Δ mice at E18.5 (Fig. 1K). The extent of loss of Foxa2 staining in Foxa1−/−/Foxa2Δ/Δ mice was less than previously observed in the Foxa1−/−/Foxa2Δ/Δ mice (21), indicating that the lack of Foxa1 may decrease the efficiency of Foxa2 gene targeting directed by the SP-C-rtTA, (tetO)-Cre system. Foxa1 was detected in nuclei of respiratory epithelial cells in both proximal and peripheral regions of lungs of control (Fig. 3A) and Foxa2Δ/Δ mice (Fig. 3C). As expected, Foxa1 staining was absent in lungs from Foxa1−/− (Fig.
3B) and Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice (Fig. 3D). In Foxa1\textsuperscript{−/−} mice, deletion of Foxa1 resulted in increased level of Foxa2 mRNA (1.5 fold, n=3, p<0.05) at E18.5, suggesting that Foxa2 may compensate for the loss of Foxa1 in lung from the Foxa1\textsuperscript{−/−} mice. Similarly, Foxa1 mRNA was increased after conditional deletion of Foxa2 in the lung (21).

**Deletion of Both Foxa1 and Foxa2 Inhibits Branching Morphogenesis of the Lung**

At the light microscopic level, lung morphology was similar in Foxa1\textsuperscript{−/−} (Fig. 1D,F,J), Foxa2\textsuperscript{ΔΔ} (Fig. 1C,G,K) and control mice (Fig. 1A,E,I) at E12.5, E15.5 and E18.5. At E12.5, branching of lung tubules was similar in mice of each genotype (Fig. 1A,B,C,D). However, by E15.5, enlarged tubules were readily observed at sites in which both Foxa1 and Foxa2 were deleted (Fig. 1H). At E18.5, enlarged cystic, bronchial tubules were noted in the lungs of Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice. Small terminal lung sacculles typical of the normal lung at E18.5 failed to form in the regions lacking both Foxa1 and Foxa2. Lung morphology was maintained in the regions of the lung parenchyma in which Foxa2 staining was present. (Fig. 1I,J,K,L). The sites and extent of lung malformation correlated well with the sites and extent of deletion of Foxa1 and Foxa2.

In order to assess the effect of deletion of both Foxa1 and Foxa2 on branching morphogenesis, lungs from control and Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice were excised and imaged at E12.5, E13.5, E14.5 and E18.5. Differences in the extent of branching were observed in Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice as early as E12.5 and thereafter, less branching was detected in lungs of Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice compared to controls (Fig. 2A-H). Lobulation was not disturbed in the Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice. Abnormal dilated peripheral tubules were observed as early as E13.5 (Fig. 2B). Lungs of Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice were visibly smaller than those from Foxa1\textsuperscript{−/−}, Foxa2\textsuperscript{ΔΔ} mice (Fig. 2B,D,F,H) and controls (Fig. 2A,C,E,F).

**Deletion of Foxa1 and Foxa2 Disrupts Epithelial Cell Differentiation in the Lung**

At E15.5 and E18.5, the dilated lung tubules and peripheral lung sacculles seen in the lung of Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice were lined primarily by immature cuboidal epithelial cells. At the ultrastructural level, epithelial cells lining the abnormal lung tubules of Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice at E18.5 were glycogen rich, lacked microvilli, cilia and granules typical of various fully differentiated cell types seen in control mice (Fig. 4). In control mice, squamous type I and cuboidal type II cells (the latter with lamellar bodies) were observed throughout peripheral regions of the lungs. Ciliated and nonciliated columnar epithelial cells were observed in conducting airways of control littermates. At E15.5, the respiratory epithelium of both control and Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice consisted of undifferentiated cuboidal and columnar epithelial cells that lacked secretory granules, cilia or lamellar bodies (data not shown). Type I and differentiated type II cells were not observed in the abnormal tubules seen in Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice at E18.5. Cuboidal cells lining the abnormal lung tubules and many of the terminal sacculles had the ultrastructural features typical of respiratory epithelial cells seen in normal mice at E15.5 (Fig. 4). The pulmonary vascular bed was intact and there was no evidence of hemorrhage. PECAM staining demonstrated that the lungs of Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice were well-vascularized (Supplemental Fig. 1).

To assess whether deletion of Foxa1 and Foxa2 altered expression of differentiation markers, serial sections were immunostained for proSP-C, SP-B and TTF1 at E18.5 and correlated with sites of Foxa2 deletion. Although TTF1 staining was not altered following deletion of both Foxa1 and Foxa2 (Fig. 5E,F), SP-B and pro-SPC staining was absent. Cells lacking SP-B and pro-SP-C were correlated with the absence of Foxa2 staining in the Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice (Fig. 5C,D), supporting a cell autonomous role for the Foxa proteins in epithelial cell differentiation. Likewise, staining for CCSP and Foxj1 was absent in conducting airway epithelial cells lacking both Foxa1 and Foxa2 (Fig. 6). Thus Foxa1 and Foxa2 are required for expression of epithelial cell differentiation markers typical of normal peripheral and conducting airways.

**Deletion of Foxa1 and Foxa2 Affects Cell Proliferation**

Lungs of Foxa1\textsuperscript{−/−}/Foxa2\textsuperscript{ΔΔ} mice were visibly smaller than control mice. In order to discern whether deletion of Foxa1 and Foxa2 altered cell proliferation, serial sections of lung tissue were stained for alpha-phospho-histone H3 ([\textsuperscript{\textcircled{P}}PH3]) and Foxa2 (Supplemental Fig. 2) at E15.5.
Numbers of cells stained for PH3 were significantly decreased in the lungs of Foxa1<sup>−/−</sup>/Foxa2<sup>Δ/Δ</sup> mice, compared with controls (P<0.05). In serial sections, PH3 staining was observed in epithelial cells that lacked both Foxa1 and Foxa2 staining, indicating their mitotic activity. In addition, there was no evidence of cell death, nuclear fragmentation or abnormal mitotic activity in the respiratory epithelium after deletion of Foxa1 and Foxa2 as assessed by light and electron-microscopy at E15.5 and E18.5 (Fig. 4 and data not shown).

**Foxa Activity is Required for Expression of Shh**

Shh is expressed by epithelial cells of the developing lung, providing a paracrine signal that is required for specification of smooth muscle in the lung mesenchyme and branching morphogenesis (31,32). In order to determine whether deletion of Foxa1 and Foxa2 altered Shh signaling, in situ hybridization was used on lung sections prepared at E15.5. Shh mRNA was decreased or absent in the dilated tubules of the Foxa1<sup>−/−</sup>/Foxa2<sup>Δ/Δ</sup> mice (Fig. 7B) compared with littermate controls (Fig. 7A), indicating that the normal pattern of Shh expression was dependent on the presence of Foxa family members. Likewise, the content of Shh mRNA was significantly decreased at E14.5 (Fig. 7C). α-SMA (α-smooth muscle actin), a smooth muscle cell marker and known Shh target, is normally expressed in the smooth muscle cells around the bronchi and bronchioles of the developing lung. Consistent with previous data that α-SMA was absent in the peripheral lung mesenchyme in Shh<sup>−/−</sup> mice (28,32), α-SMA immunostaining of lungs from Foxa1<sup>−/−</sup>/Foxa2<sup>Δ/Δ</sup> mice demonstrated that α-SMA was decreased or absent around bronchioles lacking Foxa1 and Foxa2. Thus, the combined deletion of Foxa1 and Foxa2 inhibited mesenchymal smooth muscle cell differentiation, consistent with decreased expression of Shh (Fig. 7E,F,G). Consistent with the observed decreased Shh expression, Hedgehog-interacting protein (Hhip), Myocardin (Myocd) and a number of mRNAs related to smooth muscle cell differentiation were decreased in the Foxa1<sup>−/−</sup>/Foxa2<sup>Δ/Δ</sup> mice at E14.5, including Thrombospondin 3 (Thbs3), Calponin 1 (Cnn1), Myosin heavy polypeptide 11 (Myh11), Myosin light polypeptide 9 (Myl9), Actin gamma2 smooth muscle enteric (Actg2), Actin associated protein SM22 alpha (Tagln) (Table I). Foxa1 and Foxa2 immunostaining was assessed in lungs from Shh<sup>−/−</sup> mice. Nuclear staining for Foxa1 and Foxa2 was observed in epithelial cells of respiratory tubules in Shh<sup>−/−</sup> mice at E18.5, demonstrating that Shh is not required for expression of Foxa proteins in the lung (data not shown). Wnt7b mRNA was significantly decreased (Fig. 7D), perhaps consistent with previous studies demonstrating that Foxa2 regulated Wnt7b gene transcription in vitro (33).

**DISCUSSION**

The formation of the fetal lung occurs by a process of branching morphogenesis and depends upon complex paracrine signaling between epithelium and mesenchyme that in turn controls transcriptional programs regulating cell behavior during development. In the present study, branching and epithelial cell differentiations were perturbed after deletion of Foxa1 and Foxa2 in lung epithelial cells, indicating that Foxa transcription factors are required for lung morphogenesis and function. Deletion of Foxa1 and Foxa2 activity inhibited expression of Shh and Wnt7b, providing potential mechanisms by which the lack of Foxa1 and Foxa2 activity influences branching morphogenesis and differentiation of the lung.

**Foxa1 and Foxa2 Play Redundant Roles During Branching Morphogenesis of the Fetal Lung**

Expression of Foxa1 and Foxa2 is first detected at the onset of lung bud formation. Thereafter, Foxa1 and Foxa2 are expressed in a similar temporal-spatial pattern in respiratory epithelial cells of the conducting and peripheral airways (13). Branching morphogenesis was not perturbed in single knockout Foxa1<sup>−/−</sup> or Foxa2<sup>Δ/Δ</sup> mice. In the Foxa1<sup>−/−</sup>/Foxa2<sup>Δ/Δ</sup> mice, formation of small peripheral acinar tubules and terminal saccules was markedly disrupted in regions where deletion of both Foxa1 and Foxa2 had occurred, supporting the hypothesis that the transcription factors Foxa1 and Foxa2 act in a complementary manner during branching morphogenesis.

The timing and extent of recombination with the SP-C-rtTA/tetO-Cre system were variable and dependent upon the varying efficiency of targeted recombination of each floxed gene (21,34). Gene targeting occurs in epithelial cells
Depends Upon Foxa Transcription Factors

Pulmonary Epithelial Cell Differentiation Depends Upon Foxa Transcription Factors

Foxa1 transcription factors were previously shown to regulate cell differentiation in many organs, including lung, central nervous system, pancreas and liver (20,21,45-48). Targeted disruption of Foxa2 in the mouse lung inhibited maturation of type II cells and caused goblet cell hyperplasia in the conducting airways, suggesting an important role of Foxa2 in the regulation of epithelial cell differentiation and function in both conducting airways and alveoli. Expression of epithelial cell markers, including SP-A, SP-B, and CCSP was decreased, but their expression was not entirely dependent on Foxa2 (21). In contrast, immunostaining for the epithelial cell differentiation markers, SP-B, SP-C, CCSP, and Foxj1, was absent in the relatively immature columnar and cuboidal cells lining the enlarged tubules in the Foxa1+/Foxa2Δ/Δ mice, indicating that their expression required Foxa activity.

Overlapping and Distinct Roles Foxa1 and Foxa2 in Regulating Pulmonary Gene Expression

Since Foxa1 and Foxa2 may bind to the same DNA motif (24-26), Foxa1 and Foxa2 may either compete or serve complementary roles at specific transcriptional targets. Ectopic expression of Foxa1 in Foxa2- ES cells partially restored the Foxa2 target gene expression (45). Foxa1 and

...not consistent with lack of Shh alone. Wnt7b, a signaling molecule expressed in epithelial cells of the lung, plays a role in lung morphogenesis (44). Foxa2 trans-activates the Wnt7b promoter in NIH-3T3 cells (39). Deletion of Wnt7B caused lung hypoplasia associated with defects in smooth muscle in the major pulmonary vessels (44). These findings are consistent with present observations in Foxa1+/Foxa2Δ/Δ mice, indicating that effects of their deletion may be, in part, mediated by altered Wnt7b signaling. Consistent with these in vitro studies, Wnt7b mRNA was significantly decreased in the lung of Foxa1+/Foxa2Δ/Δ mice. Thus, Foxa1 and Foxa2 are required for precise expression of Wnt7b that in turn may influence β-catenin dependent signaling to influence lung morphogenesis or cell differentiation (33). Mechanisms by which Foxa1 and Foxa2 influence epithelial cell differentiation during lung morphogenesis are likely complex and remain to be identified.

Pulmonary Epithelial Cell Differentiation

Branching morphogenesis of the lung requires precise signaling from both epithelial and mesenchymal compartments to regulate cell proliferation, migration and subsequent lung specific gene expression. Many genes and associated signaling pathways influence the process of branching morphogenesis, including β-catenin, Rar/Rxr, Tgfβ, Shh, Fgf10 and Bmp4 (1,3,35-38). Although the mechanisms by which Foxa1 and Foxa2 regulate branching morphogenesis are not understood completely, Foxa1 and Foxa2 have been implicated in several of these pathways. Foxa2 regulates Shh during early embryogenesis and CNS formation (39).

Foa2 positively regulated Shh transcription within the CNS and axial mesoderm. Foxa1, Foxa2 and Shh are co-expressed in subsets of epithelial cell during early lung morphogenesis (39-43). In the present study, decreased or absent of Shh mRNA was observed after deletion of Foxa1 and Foxa2. Similarities in the disruption of branching morphogenesis seen in the lungs of ShhΔ/Δ and Foxa1+/Foxa2Δ/Δ mice support the concept that loss of Foxa activity influences lung branching morphogenesis and mesenchymal cell differentiation, at least in part, by inhibiting Shh expression. The absence of smooth muscle differentiation as indicated by α-SMA staining at site of Foxa1 and Foxa2 deletion is consistent with decreased Shh that is required for normal expression of myocardin and its downstream targets, including Thbs3, Myh11, Myl9, Cnn1, Actg2, Tagln, α-SMA (28). While similar branching abnormalities are observed in Shh deficient and Foxa1+/Foxa2Δ/Δ mice, changes in cell differentiation in the two models are distinct. The differentiation markers, Foxj1, CCSP, SP-C and SP-B were expressed in ShhΔ/Δ but not in Foxa1+/Foxa2Δ/Δ mice, suggesting that the phenotypic changes seen in the latter model are...
F Foxa2 were shown to transcriptionally activate expression of many of the same genes, including Tif1 (49), Cfr (50), Sftpb (51) and Scgblal (21,52,53). A number of genes which are expressed in the lung are regulated by both Foxa1 and Foxa2, including Sftpb (51), Scgblal (52,53), lysozyme M and lysozyme P (54). However, the distinct phenotypes in Foxa1-/− and Foxa2∆/∆ mice (21-23) and the distinct expression patterns of Foxa1 and Foxa2 in many organs (13) suggest that Foxa1 and Foxa2 may also differentially regulate gene expression during embryogenesis and lung development.

F Foxa1 and Foxa2 may regulate gene transcription directly or indirectly by stabilizing nucleosome positioning (55,56). Foxa also modulates gene expression by interacting with other transcription factors, including SP1, C/EBP and RAR/RXR (57-59). Foxa1 and Foxa2 are co-expressed with Ttf1 and share transcriptional targets in the respiratory epithelium (54,60). It is therefore likely that other transcription factors interact and cooperate with Foxa1 and Foxa2 in a complex transcriptional program that regulates gene expression in the developing lung.

Foxa1 mRNA was induced after deletion of Foxa2 in respiratory epithelial cells. Likewise, Foxa2 mRNA was increased in lungs of Foxa1−/− mice, supporting the concept that these Foxa family members play complementary roles in the regulation of branching morphogenesis and expression of genes required in lung function after birth. Foxa proteins are utilized recurrently and at discrete times during foregut differentiation. Foxa expression is required for A-P axis formation in the early embryo, branching morphogenesis and epithelial cell differentiation prior to birth and for epithelial cell function in the postnatal lung.

ACKNOWLEDGEMENTS
Funding was made possible by National Institutes of Health grants: by HL56387, HL75770 and HL38859. We thank Anne Perl, Dave Loudy, Jean Clark. and Ann Maher for their contribution.

REFERENCES
TABLE I  Genes related to smooth muscle cell differentiation which were decreased in Foxa1^{-/-}/Foxa2^{∆/∆} mice at E14.5

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Lung RNAs from Foxa1^{-/-}/Foxa2^{∆/∆} and age-matched control mice were prepared at E14.5. Lung cRNA was hybridized to the murine genome MOE430A chips and analyzed as previously described. Genes in the Shh pathway and associated with smooth muscle cell differentiation were significantly decreased in the Foxa1^{-/-}/Foxa2^{∆/∆} mice.

FIGURE LEGENDS

Fig. 1 Deletion of Foxa1 and Foxa2 disrupts branching morphogenesis. Lung sections of control (A,E,I), Foxa1^{-/-} (B,F,J), Foxa2^{∆/∆} (C,G,K) and Foxa1^{-/-}/Foxa2^{∆/∆} (D,H,L) mice were prepared on E12.5, E15.5 and E18.5 and stained with Foxa2 polyclonal antibody. While lung architecture was not altered in Foxa1^{-/-} and Foxa2^{∆/∆} mice at these different developmental stages, combined deletion of both Foxa1 and Foxa2 disrupted branching after E12.5, resulting in formation of large lung cysts by E15.5 and thereafter. Scale bar=300 μm.
Fig. 2 Abnormal branching morphogenesis after deletion of Foxa1 and Foxa2. Fetal lungs were imaged at E12.5 (A,B), E13.5 (C,D), E14.5 (E,F) and E18.5 (G,H). Abnormal branching was observed in Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> as early as E12.5 (J) compared to controls (A). At E13.5 and E14.5, dilated tubules were observed in lungs from Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> mice (D,F). At E18.5, cystic malformations were observed in lungs from Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> mice (H). Scale bar=1 mm.

Fig. 3 Deletion of Foxa1 in the fetal lung. Lung sections of E18.5 mice were prepared and stained with polyclonal Foxa1 antibody. Foxa1 was absent in all the lung cells of Foxa1<sup>−/−</sup> (B) and Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> (D) mice. In control (A) and Foxa2<sup>−/−</sup> (C) mice, Foxa1 was present in airway epithelial cells and subsets of alveolar type II cells. Expression of Foxa1 was most prominent in proximal airways. Scale bar=100 µm.

Fig. 4 Lung ultrastructure in Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> mice. Electron microscopy was performed on lungs from Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> mice (B) and control littermates (A) observed at E18.5. Squamous Type I cells (thick arrow) and cuboidal type II cells (arrowhead) containing lamellar bodies, apical microvilli and highly organized rosette glycogen were observed in lungs of control mice (A). The densely stained cell below the type I cell is a capillary endothelial cell creating the gas-exchange area (A). Type I cells were not observed in lung of Foxa2<sup>−/−</sup> mice (B). The pulmonary vascular bed in Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> mice was intact, but in the absence of type I cells, the thin walled alveolar-capillary interface seen in the control mice was not formed. Type II cells were immature. Lamellar bodies were absent and apical microvilli were sparse, in Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> mice (B).

Fig. 5 Cell autonomous effects of Foxa1 and Foxa2 deletion on gene expression in respiratory epithelial cells. Serial sections of lung from E18.5 embryos from Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> mice were stained for Foxa2 (A,C,E), mature SP-B (B), proSP-C (D) and TTF1 (F). Expression of TTF1 was not affected by deletion of Foxa1 and Foxa2. ProSP-C and SP-B were detected in cells expressing Foxa2 (arrow). Cells lacking both Foxa1 and Foxa2 did not express either SP-B or ProSP-C (arrowhead). ProSP-C and SP-B were correlated with Foxa2 staining. Scale bar=100 µm.

Fig. 6 Deletion of Foxa1 and Foxa2 alters gene expression in conducting airway epithelial cells. Serial sections were prepared from Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> mice at E18.5 and stained for Foxa2 (A,C) and airway epithelial cell differentiation markers, Foxj1, a basal-ciliated cell marker (B) and CCSP, a non-ciliated bronchial cell marker (D). Foxj1 and CCSP were detected in cells expressing Foxa2 (arrow). Cells lacking both Foxa1 and Foxa2 did not express either Foxj1 or CCSP (arrowhead). Scale bar=100 µm.

Fig. 7 Decreased Shh, []-SMA and Wnt7b. Lung sections from Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> and control littermates were prepared on E15.5 and used for in situ hybridization using SHH riboprobe. In controls (A), SHH mRNA was detected in the epithelial cells lining respiratory tubules. In Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> mice (B), SHH mRNA was absent or decreased in the epithelial cells lining the dilated respiratory tubules (arrow). Scale bar (white)=500 µm. Total lung RNA was prepared from fetal mouse lung at E14.5. Quantitative RT-PCR for Shh and Wnt7b mRNAs were performed. The relative concentration of each mRNA was normalized to the concentration of GADPH mRNA in each sample. The mean of the mRNA content in control littermates was set to 1. Relative values of Shh and Wnt7b mRNAs in Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> and control mice are shown as mean±s.d. (C,D). Statistics were done by 2-tailed t-test; n=4-5 animals/group, *P <0.05 vs. control. Lung sections from Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> and control littermates were prepared on E15.5 and immunostained for []-SMA. In controls (E), []-SMA was detected in the smooth muscle cells around the bronchioles (arrowhead). In Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup> mice (F), []-SMA was decreased or absent around the bronchioles (arrow). Serial sections were used for Foxa2 immunostaining to demonstrate the sites of deletion of Foxa1 and Foxa2. Scale bar (black)=100 µm.
Supplemental Fig 1  PECAM immunohistochemistry. Sections of lung from E15.5 and E18.5 embryos from Foxa1<sup>−/−</sup>/Foxa2<sup>Δ/Δ</sup> (B,D) and control (A,C) mice were stained for PECAM. Pulmonary vascular structures, as indicated by PECAM staining, were unchanged after deletion of Foxa1 and Foxa2 in the lung epithelial cells. Scale bar=100 μm.

Supplemental Fig 2  Phospho-histone H3 staining in Foxa1<sup>−/−</sup>/Foxa2<sup>Δ/Δ</sup> mice. Lung sections from control (A,B) and Foxa1<sup>−/−</sup>/Foxa2<sup>Δ/Δ</sup> (C,D) mice were prepared at E15.5. Serial sections were stained for Foxa2 (A,C) and alpha-phospho-histone H3 (a-PH3) (B,D). Numbers and cell distribution of phospho-histone H3 staining was significantly decreased in lungs of Foxa1<sup>−/−</sup>/Foxa2<sup>Δ/Δ</sup> mice (E). Phospho-histone stained cell counting shown as mean±s.d. (a-PH3 positive cells per μm²) and were compared with controls with the 2-tailed t-test; n=4-6 animals/group, *P<0.05 vs. control. Scale bar= 100 μm.
FIGURE 1

Control  Foxa1<sup>+/+</sup>  Foxa2<sup>−/−</sup>  Foxa1<sup>−/−</sup>/Foxa2<sup>−/−</sup>

E12.5

A  B  C  D

E15.5

E  F  G  H

E18.5

I  J  K  L
FIGURE 2

Control  \hspace{1cm} \text{Fo}xa_{1^{-/}}/\text{Fo}xa_{2^{A/A}}

E12.5

\hspace{1cm} A \hspace{1cm} B

E13.5

\hspace{1cm} C \hspace{1cm} D

E14.5

\hspace{1cm} E \hspace{1cm} F

E18.5

\hspace{1cm} G \hspace{1cm} H
FIGURE 4

Control  Foxa1⁻⁻/Foxa2ΔΔ

A  B

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FIGURE 5

Foa2 staining  Marker staining

A  B

pro-SP-C

Mature
SP-B

TTF1

E  F
FIGURE 6

Foca2 staining

Marker staining

CCSP

Foxj1

A

B

C

D
FIGURE 7

A) Control (Shh)  
B) Foxa1⁻/⁻/Foxa2ΔΔ (Shh)

C) Shh mRNA

D) Wnt7b mRNA

E) Control (α-SMA)  
F) Foxa1⁻/⁻/Foxa2ΔΔ (α-SMA)  
G) Foxa1⁻/⁻/Foxa2ΔΔ (Foxa2)
SUPPLEMENTAL FIGURE 1

Control  Foxa1^{-/-}/Foxa2^{Δ/Δ}

E15.5  
A

E18.5  
C

B

D