Lung epithelial branching program antagonizes alveolar differentiation

Daniel R. Chang, Denise Martinez Alanis, Rachel K. Miller, Hong Ji, Haruhiko Akiyama, Pierre D. McCreery, and Jichao Chen

*Department of Pulmonary Medicine and Biochemistry and Molecular Biology, University of Texas MD Anderson Cancer Center, Houston, TX 77030; and Department of Orthopedics, Kyoto University, Sakyo, Kyoto 606-8507, Japan

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Mammalian organs, including the lung and kidney, often adopt a branched structure to achieve high efficiency and capacity of their physiological functions. Formation of a functional lung requires two developmental processes: branching morphogenesis, which builds a tree-like tubular network, and alveolar differentiation, which generates specialized epithelial cells for gas exchange. Much progress has been made to understand each of the two processes individually; however, it is not clear whether the two processes are coordinated and how they are deployed at the correct time and location. Here we show that an epithelial branching morphogenesis program antagonizes alveolar differentiation in the mouse lung. We find a negative correlation between branching morphogenesis and alveolar differentiation temporally, spatially, and evolutionarily. Gain-of-function experiments show that hyperactive small GTPase Kras expands the branching program and also suppresses molecular and cellular differentiation of alveolar cells. Loss-of-function experiments show that SRY-box containing gene 9 (Sox9) functions downstream of Fibroblast growth factor (Fgf)/Kras to promote branching and also suppresses premature initiation of alveolar differentiation. We thus propose that lung epithelial progenitors continuously balance between branching morphogenesis and alveolar differentiation, and such a balance is mediated by dual-function regulators, including Kras and Sox9. The resulting spatial delay of differentiation by the branching program may provide new insights to lung immaturity in preterm neonates and the increase in organ complexity during evolution.

An optimal organ design maximizes not only the number of its basic functional units, for example alveoli in the lung and glomeruli in the kidney, but also the efficiency of transporting gas and liquid to and from these functional units. This design is often achieved by building a branched, tree-like structure with functional units attached at the end, as seen in the lungs, kidneys, and mammary glands. Compared with a beads-on-a-string design, where all functional units are connected sequentially on a linear duct, a branching design not only packs the maximal number of functional units within a given 3D organ space without entanglement of the ductal system, but also increases transport efficiency by minimizing the average distance between each functional unit to the entry point of an organ.

Two developmental processes underlie a branching design: branching morphogenesis, a process that builds the branched ducts, and cellular differentiation, a process that builds the functional units. These two processes have to be somehow coordinated to ensure a fundamental feature of a branching design: all functional units are only located at the termini of the branched ducts.

Branching morphogenesis has been well studied both morphologically and genetically in the mouse lung. The entire airway lumen is bordered by epithelial cells that originate from the ventral foregut around embryonic day 10 (E10) (1). It has been shown that epithelial progenitors undergo a highly organized process of branching morphogenesis to give rise to the first 5,000 or so airway branches within 5 d of development (E10 to E15) (2). Branching morphogenesis involves reciprocal interactions between the lung epithelium and the surrounding mesenchyme, and requires secretion of a fibroblast growth factor (FGF10) by the mesenchymal cells, activating its membrane receptor (FGFR2) and subsequently a small GTPase (KRAS) in the epithelial cells (3–5). Additional signaling pathways, including Wnt, Sonic hedgehog (Shh), and Tgf signaling pathways, are also essential for normal branching (1, 6).

Lung epithelial progenitors not only build a branched duct system via branching morphogenesis, but also differentiate into specialized alveolar cells required for gas exchange, a process named alveolar differentiation. There are two alveolar cell types: type I cells that are flat and cover more than 90% of the alveolar surface, across which gases diffuse; and type II cells that are cuboidal and synthesize pulmonary surfactants, lipoprotein complexes that hydrate the alveolar surface and prevent alveolar collapsing by reducing surface tension (7). Genetic studies in mice have identified several transcription regulators specifically required for alveolar differentiation (8–12).

Although both branching morphogenesis and alveolar differentiation have been extensively studied, much less is known about whether and how they are coordinated. Each process is generally viewed as independently controlled and occurring during early versus late lung development, respectively. Although defects in alveolar differentiation are not expected to

Significance

Mammalian organs, including the lung and the kidney, often adopt a branched design to maximize their functional capacity and efficiency. Lung formation requires two developmental processes: branching morphogenesis to build a treelike tubular network, and alveolar differentiation to generate specialized epithelial cells for gas exchange. Although each process has been extensively studied, much less is known about whether and how the two processes are coordinated. We show that an epithelial branching morphogenesis program antagonizes alveolar differentiation in the mouse lung. Our findings may provide fresh insights to lung immaturity in preterm neonates and the increase in organ complexity during evolution.
We examined how this branching process in late-stage lungs was spatially related to alveolar differentiation and found that SOX9+ cells at the branch tips were the least differentiated. We sustained sections from E17 to E19 lungs for SOX9 and a panel of alveolar markers for both alveolar type I [receptor for advanced glycosylation of end products (RAGE), AQP1] and type II (ABCAL3, LAMP3, SFTPB) cells. Because branching occurs in a 3D space and new branches can grow internally away from the surface of the lung, tissues in the center of a physical section can be at the topologically distal edge of the airway tree; therefore, we assessed the spatial distributions of these alveolar markers only in regions where the airway lumen extended continuously from the proximal conducting airways to the distal branch tips. Consistent with our time-course transcriptome analysis (Fig. 1A), expression of alveolar markers increased over time. Notably, this increase mostly occurred in the nonbranch tip regions of the distal epithelium, whereas SOX9+ cells at the branch tips expressed a very low level of alveolar markers (Fig. 1B and Fig. S3). Alveolar differentiation expanded distally over time and at E19 most epithelial cells, except for a few SOX9+ cells at the branch tips, were differentiated.

This difference in alveolar differentiation in branch tip versus nonbranch tip regions was also supported by cell morphology analysis: ECAD staining showed that undifferentiated cells at the branch tips were clustered and cuboidal, whereas differentiated alveolar type I and type II cells at the nonbranch tip regions were flat and isolated cuboidal cells, respectively (Fig. 1B and Fig. S2). Our analyses revealed an unexpected negative correlation between branching and alveolar differentiation along the proximal/distal axis of the airway tree, suggesting that in late-stage lungs, branching continues at the distal edge of the airway tree and leaves behind cells undergoing alveolar differentiation.

We extended the temporal and spatial negative correlations between branching and alveolar differentiation to the Xenopus lung, one of most primitive lungs in evolution. Similar to the mouse lung, the Xenopus lung arises from the foregut, expresses the lung lineage factor Nkx2.1 (27), and initially forms one tube on each side of the embryo (Fig. 1C and Fig. S4). Unlike the mouse lung, which branches extensively after its specification, the initial two tubes of the Xenopus lung simply grew posteriorly and the entire lung epithelium differentiated to express alveolar markers, including SftpB and SftpC (Fig. 1C and Fig. S4) (28). Importantly, although Sox9 was present in the Xenopus genome and was detected in the pharyngeal arches, it was not expressed in the Xenopus lungs at all stages examined (Fig. 1C and Fig. S4). Therefore, the Xenopus lung lacks the branching program and Sox9 expression, and initiates alveolar differentiation immediately after lung specification.

### Hypervactive Kras Suppresses Alveolar Differentiation
To examine whether there is a causal relationship in the observed negative correlations between branching and alveolar differentiation, we augmented the branching program in epithelial progenitors by expressing a hyperactive Kras allele (29) at the time of alveolar differentiation and found that hypervactive Kras suppressed molecular and cellular differentiation of the progenitors. We used a Sox9CreERT2 allele (30) to target lung progenitors late in development to avoid difficulty in interpreting potentially secondary phenotypes by targeting the early lung epithelium, for example, with a ShhCre allele. Expressing the hypervactive Kras in lung progenitors after E15 resulted in an increase in the branching program with SOX9 expression, normally restricted to a few cells at the branch tips of E19 lungs, expanded to half of the distal lung epithelium (Fig. 2A). This phenotype was similar to that in lungs expressing the hyperactive Kras throughout the epithelium at an earlier stage (see, for example, Fig. 4B). This expansion in the branching program was accompanied by a restriction of alveolar differentiation to regions near the conducting
airways, as assessed by expression of alveolar type I (AQP1 and RAGE) and type II (ABCA3 and LAMP3) markers (Fig. 2B and Fig. S5). We note that expression of SFTPB was more uniform throughout the distal epithelium in the \( Kras \) mutant lung, although cells in the expanded branching regions were clustered, a similar arrangement to that of cells undergoing branching morphogenesis (Fig. 2C and Fig. S5).

On the cellular level, hyperactive \( Kras \) suppressed cell flattening, a morphological hallmark of alveolar type I cells. ECAD staining showed that although the control lung epithelium at E19 mostly consisted of isolated cuboidal type II cells intermingled with presumable flat type I cells, the expanded branching regions of the \( Kras \) mutant lung consisted of closely packed epithelial cells, an organization reminiscent of that in undifferentiated branch tips of the early lungs (Fig. 2C). ECAD is a cell-junction protein and does not outline the entire cell; therefore, to visualize the morphology of individual epithelial cells, we used a Cre reporter (\( Rosm\text{mTmG} \)) that expresses GFP throughout the cell membrane upon recombination (31). Under our experimental conditions, the \( Rosm\text{mTmG} \) allele was less sensitive to the Cre recombinase than the hyperactive \( Kras \) allele, and therefore labeled a subset of \( Kras \) mutant cells. This process allowed visualization of cell morphology at a single-cell resolution and revealed that the expanded branching regions of the \( Kras \) mutant lung lacked flat alveolar type I cells (Fig. 2C).

Although \( Kras \) is well known as an oncogene for many cancers (32), it is also an essential component of receptor tyrosine kinase signalings during development, including the \( Fgf \) signaling in lung morphogenesis (5, 33). Therefore, the \( Kras \) gain-of-function experiments suggested that an augmented branching program in late developing lungs suppresses alveolar differentiation and alters the observed spatial negative correlation between branching and alveolar differentiation (Fig. 1B).

Fig. 1. Temporal (A), spatial (B), and evolutionary (C) negative correlations between branching morphogenesis and alveolar differentiation. (A) Time-course microarray expression profiling of FACs-purified distal lung epithelial cells. Gene expression values (log2) in E14 samples are used as a baseline for comparison. Horizontal histograms for E15–E19 samples are the frequency distributions of average fold-changes for all genes; the units are shown as the number of genes (# genes). Alveolar markers are up-regulated (red), and branching (green) and cell cycle (blue) related genes are down-regulated over time. Down-regulated genes appear to have a smaller fold-change than up-regulated genes, possibly because of perdurance of transcripts. (B) Confocal images of immunostained E18 lung sections in areas where the airway lumen can be continuously traced from the proximal conducting airways (black/white long dashed lines) to the distal nonbranch tip (magenta) and branch tip (green) regions, as illustrated in the schematics. Branch tips are outlined with dashed lines. The boxed regions are enlarged as insets outlined in corresponding colors, showing a spatial negative correlation between branching and alveolar differentiation. AQP1 labels alveolar type I cells, the vasculature and the mesothelium, which are separated by dashed lines (Insets). There is a very low level of SOX9 expression in nonbranch tip epithelium, possibly because of protein perdurance. (Scale bars, 20 \( \mu m \).

(C) Whole-mount in situ hybridization and immunostaining of \( Xenopus \) embryos of indicated stages (St) showing the absence of Sox9 expression and branching in the \( Xenopus \) lung. The lungs are indicated with dashed lines if stained or arrowheads if unstained. Open arrowheads indicate Sox9 expression in the pharyngeal arches. The two \( Xenopus \) Sox9 homologs (Sox9a and Sox9b) are 95% identical on the nucleotide level. OPT images of immunostained embryos (Left, Bottom) are shown as maximal intensity projection (Left) and sectional view (Right). (Scale bar, 200 \( \mu m \).

Sox9 Is Required for Branching Morphogenesis. Suppression of alveolar differentiation by the gain-of-function Kras-induced branching program led us to hypothesize that there are positive regulators of branching morphogenesis, the loss-of-function mutations of which will cause premature alveolar differentiation. One intriguing candidate is Sox9, which is highly expressed in distal epithelial cells undergoing active branching (21) (Fig. 3) and required for the development of two other branched organs, the kidney and the pancreas (34, 35). To test the role of Sox9 in mouse lung development, we deleted it specifically in the epithelium with a ShhCre allele (16, 17). Sox9 expression was abolished in the mutant lung epithelium before E12, but was not affected in the cartilages surrounding the tracheal epithelium (Fig. 3A). In contrast to a previous study (21), in which deletion of Sox9 did not affect lung development and function, epithelial deletion of Sox9 using ShhCre resulted in a smaller lung with fewer and dilated airway branches. This branching phenotype was apparent as early as E13 and persisted throughout embryonic development (Fig. 3A and Fig. S6). No viable Sox9 mutant mice were obtained postnatally. Despite significant disruption of branching, the coalignment between the epithelial branches and the arterial vasculature was not affected (Fig. S6).

Besides branch number and morphology, Sox9 regulates a subset of branching-related genes in a cell-autonomous manner. In a microarray expression profiling experiment comparing control and Sox9 mutant lungs (Dataset S2; described in detail below), we identified several genes down-regulated in the Sox9 mutant, including Clu and Mia, which have been implicated in lung development and cancer (25, 36). In situ hybridization and immunostaining experiments showed that these genes were expressed at the branch tips and down-regulated in the Sox9 mutant as early as E11 (Fig. 3B and Fig. S7A and B). Using an Nkx2.1CreER allele (37), we deleted SOX9 in a subset of epithelial cells and observed a mosaic expression pattern of Clusterin that matched perfectly with that of SOX9 (Fig. 3C and Fig. S7E), demonstrating a cell-autonomous regulation of Clu by Sox9.

The expression of many other genes that are normally restricted to the branch tips, including Bmp4, Spry2, Id2, Thbs1, and Dlk1, were not affected in the Sox9 mutant as assayed via both microarray and in situ hybridization experiments (Fig. S7C).
In addition, there are no significant changes in cell polarity in the Sox9 mutant lung as assessed by apical [Zonula occluden 1 (ZO1) and Podocalyxin-like (POD)] (17), basement membrane [Entactin (Ent) and Perlecan (Perl)] and cytoskeleton [acetylated-Tubulin (AcT)] marker expression (Fig. S8). These results suggested that these genes are regulated by
Sox9-independent signals and may be responsible for the remaining branching program in the Sox9 mutant lung (Fig. 3A).

Because the Sox9 mutant lung was smaller, we examined molecules and cellular processes related to cell proliferation and apoptosis. One candidate was Cyclin D1 (CCND1), which is known to be transcriptionally activated by growth factor-induced Ras signaling (38). We found a high level of CCND1 expression in the branching regions of the epithelium, and significantly fewer cells expressing a normal level of CCND1 in the Sox9 mutant lung ($P < 0.001$, $\chi^2$ test) (Fig. S9A). However, the percentage of proliferating cells measured by the incorporation of a nucleotide analog (EdU, 5-ethyl-2'-deoxyuridine) was not significantly lower in the Sox9 mutant branch tips, compared with their matched branch tips in littermate control lungs ($P > 0.05$, Student $t$ test) (Fig. S9B), suggesting either compensation by other Cyclin D proteins or a small proliferation defect that was undetectable using the current method but accumulated over time to affect the size of the lung.

**Fig. 4.** Sox9 functions downstream of the Fgf/Kras branching signal. (A) OPT images of E13 whole-mount immunostained lungs from littermate control and Fgfr2$^{cko}$;Shh$^{Cre+}$ mutant embryos. The boxed areas are shown in a cross-section view. Arrowheads indicate that SOX9 expression in the branching epithelium is lost in the Fgfr2 mutant lung. Arrows indicate that SOX9 expression in the cartilage precursors is not affected. (Scale bar, 200 μm.) (B) OPT images of E13 whole-mount immunostained lungs from control, Sox9$^{cko}$;Shh$^{Cre+}$ mutant, Kras$^{LSL-G12D}$;Shh$^{Cre+}$ mutant, and Kras$^{LSL-G12D}$;Sox9$^{cko}$;Shh$^{Cre+}$ mutant embryos. Regions of the tracheas (brackets) are shown in a cross-section view. Epithelial Sox9 deletion suppresses the overgrowth and ectopic tracheal branch phenotypes in the Kras mutant lung. We note that Sox9 and Kras double-mutant branches remain cystic, possibly because of the presence of the Sox9-independent branching program (Fig. S7C and D). (Scale bars, 200 μm.)
In addition to its requirement in the epithelium, Sox9 indirectly regulates mesenchymal cell survival and Fgf10 expression, key components of the epithelial-mesenchymal interactions required for branching morphogenesis (1). When examining expression of cleaved Caspase-3 (Casp3), an apoptotic marker, we found that the branching regions of both control and mutant lungs had no apoptotic cells. However, apoptotic cells were scattered throughout the distal mesenchyme of the mutant epithelia. Dysregulation of Fgf10 expression might contribute to the cystic airway branch phenotype in the Sox9 mutant (Fig. 3 A). Based on all of these data, Sox9 is a positive mediator of the Fgf/Kras branching program.

To determine whether Sox9 functionally mediates the Fgf/Kras branching program, we examined the genetic interaction between Sox9 and Kras. When we activated the hyperactive Kras allele throughout the lung epithelium, the Kras mutant lung was larger and most of the epithelium was actively branching (Fig. 4 B). We noted that overactivation of Kras increased the size but not the number of branch tips, suggesting that normal branch formation requires precise regulation of Kras activity. Nevertheless, ectopic branches formed from the normally nonbranching tracheal epithelium, comparable to the ectopic tracheal branches induced by the Fgf-expressing lung mesenchyme in a tissue graft experiment (40). Genetic deletion of Sox9 in the hyperactive Kras background reduced the size of the lung and suppressed the formation of ectopic tracheal branches (Fig. 4 B and Fig. S11). The genetic interaction between Sox9 and Kras, together with the Sox9 mutant phenotypes, showed that Sox9 is a positive mediator of the Fgf/Kras branching program.

**Sox9 Functions Downstream of the Fgf/Kras Branching Signal.** The Sox9 expression pattern and mutant phenotypes (Fig. 3) led us to examine the role of Sox9 in the known Fgf/Kras branching morphogenesis program. We examined expression of Sox9 in the absence of the Fgf/Kras branching signal by genetically deleting the Fgf receptor (Fgfr2) from the epithelium. The Fgfr2 mutant lung failed to branch and notably, epithelial expression of SOX9 was lost and the entire epithelium expressed SOX2 (Fig. 4 A), a marker and regulator of the proximal conducting airways (19, 39). Conversely, expression of the hyperactive Kras allele throughout the epithelium led to an overgrown lung with expanded Sox9 expression domain (Fig. 4 B). These data suggested that Sox9 is a downstream component of the Fgf/Kras branching program.

**Sox9 Suppresses Premature Initiation of Alveolar Differentiation.** A microarray expression profiling experiment provided the initial clue that Sox9 is a dual-function regulator of both branching morphogenesis and alveolar differentiation (Fig. 5 A). In addition to the down-regulated genes (Fig. 3 and Fig. S7), the Sox9 mutant lung also up-regulated a number of genes, including SftpB, SftpC, Lamp3, Napsa, and Cth, that are known or
were shown (Fig. S12A) to be expressed by differentiated alveolar cells and involved in the production and processing of surfactant proteins (41, 42).

We confirmed the microarray results by in situ hybridization and immunostaining experiments, showing that several alveolar markers increased dramatically in Sox9 mutant lungs compared with control lungs (Fig. 5 B and C, and Fig. S12 B and C). Strikingly, this premature alveolar differentiation occurred at the earliest stage of branching morphogenesis (E11), when the lung consisted of only two tubes and before any morphological difference between Sox9 control and mutant lungs (Fig. 5B). This aspect showed that the alveolar differentiation phenotype is not secondary to the branching defects in the Sox9 mutant lung. We note that precocious activation of alveolar genes was restricted to the distal branching epithelium, suggesting the presence of negative regulators (e.g., Sox2) of alveolar differentiation in the proximal conducting airways.

Although a number of alveolar genes were expressed prematurely in Sox9 mutant lungs, the full alveolar differentiation program were not activated, suggesting the presence of additional negative regulators or lack of the complete set of positive regulators of alveolar differentiation in early developing lungs. Surfactant proteins involved in immune defense (Sftpα and Sftpδ) (43) and markers for alveolar type I cells (RAGE and AQP1) were not precociously expressed in the Sox9 mutant lung (Dataset S2). An alternative possibility for this incomplete derepression of alveolar differentiation is that the most ancient alveolar differentiation program during lung evolution is to synthesize surfactants to reduce alveolar surface tension and this basic program is suppressed by Sox9, whereas immune defense-related surfactant proteins and alveolar type I cells are functional improvements that arise later in evolution and are controlled by Sox9-independent mechanisms. Taking these data together, we concluded that Sox9 promotes branching morphogenesis and also suppresses premature initiation of alveolar differentiation.

Discussion

The experiments presented herein reveal a previously unappreciated link between branching morphogenesis and alveolar differentiation in lung epithelial progenitors (Fig. 5D): the branching program antagonizes the alveolar differentiation program when specific dual-function regulators including Kras and Sox9 promote branching and suppress alveolar differentiation. This model does not exclude the possibility that Kras and Sox9 function to maintain cell shape and proliferation, cellular processes that are necessary for branching morphogenesis and affected in the Kras and Sox9 mutant lungs. The cellular mechanisms underlying the balance between branching and alveolar differentiation require further investigation.

Such a model suggests a new area of research in lung development in which the two developmental processes are studied not only individually, but also as a balanced choice of the progenitors. Our data imply that genes other than Kras and Sox9 that are typically regarded as branching-related based on expression patterns and mutant phenotypes (e.g., Id2, Bmp4, and Sry2) need to be investigated for their potential roles in alveolar differentiation in gain and loss-of-function experiments similar to those in this study. Conversely, we speculate that genes controlling alveolar differentiation (e.g., Cebpa, Cebi, and Carml) may suppress branching morphogenesis if overexpressed during early lung development. Indeed, the Crebi mutant lung has excessive SOX9+ epithelial cells (9), although it is unknown whether these cells form extra branches.

The spatiotemporal transition from branching morphogenesis to alveolar differentiation (Fig. 1) may provide new insights to mechanisms and therapies of lung defects in premature birth. This transition is interrupted by premature birth, especially in very/extremely low birth-weight infants born at the cananlicular stage of lung development (gestation week 24 in humans and E17 in mice) (44, 45). Interruption to this transition may lead to not only cellular immaturity as a result of insufficient alveolar differentiation, but also structural immaturity as a result of incomplete branching morphogenesis. Both cellular and structural immatures need to be considered when optimizing current therapies, such as antenatal glucocorticoids and mechanical ventilation, and designing new therapies.

The balance between branching morphogenesis and alveolar differentiation (Fig. 5D) may be modulated during evolution to increase the complexity and capacity of the lung. Although all vertebrate lungs mediate gas exchange with specialized alveolar cells, their complexity ranges from the two-tube amphibian lung to the tree-like mammalian lung. The Xenopus lung does not branch and expresses alveolar genes at the beginning of lung development, whereas the mouse lung starts by branching extensively and shifts to alveolar differentiation later in development (Fig. 1). We speculate that branching morphogenesis is an evolutionary addition to the ancestral alveolar differentiation program, and that the complex tree-like structure arises via a developmental delay of alveolar differentiation to allow integration of the branching morphogenesis program. Such a temporal coordination will also ensure positioning the alveolar region at the termini of the airway. We further speculate that the integration between the two developmental processes is achieved by dual-function regulators, including Kras and Sox9, and that modulation of the amount/activity of these dual-function regulators in conjunction of developmental time will generate a balance between varying complexity in different species.

Similar mechanisms may be applicable to the development and evolution of other brachted organs. The metanephros develops in a centrifugal manner analogous to the lung: the proximal/medullary epithelia are older and more differentiated than the distal/cortical epithelia, and the cortical epithelia continue to branch, whereas the medullary epithelia differentiate and become physiologically active. Interestingly, loss of β-catenin in the ureteric bud is shown to cause both branching defects and premature differentiation, reminiscent of the lung phenotype in the Sox9 mutant (46, 47).

Materials and Methods

Mice. Mice carrying Rosa26+; Sox9-CreER, ShhCre, Sox9FLOX, Fgf21FLOX, Rosa26tm6, Sox9flox, Nkx2.1CreER, and Kra1−/−;G12D alleles have been previously described (5, 16, 20, 29–33, 40–48, 59). The same alleles were generated using the following primers: 5′-GAGACACCTCTGCCCAGAT-3′; 5′-CGCTCCTCTCGTGCTTTACAG-3′; and 5′-TGTGAATGATCGTACATAACGAT-3′. The day in which a vaginal plug was observed was denoted as E1. For Sox9CreERTm, Kra1−/−;G12D experiments, 2 mg tamoxifen (T6548; Sigma) dissolved in corn oil (C8267; Sigma) was given intraperitoneally to timed pregnant mice at E15. To activate the Rosa26tm6 allele, 0.5 mg tamoxifen was injected to label epithelial cells at a low density. For Sox9CreERTm;Nkx2.1CreERTm experiments, 3.5 mg tamoxifen was injected at E10. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Texas A&M Health Science Center Institute of Biosciences and Technology (protocol no. 11004).

Xenopus Embryos. Xenopus laevis embryos were obtained and grown using standard methods (51). At Nieuwkoop and Faber stages 36, 38, 40, and 42, embryos were fixed as previously described (52) and kept in methanol at −20 °C.

Antibodies. The following antibodies were used for immunostaining: rabbit anti-SOX9 (1:1,000; AB5535, Millipore; also recognizes the Xenopus SOX9), goat anti- SOX9 (1:1,000, AF3075, R&D Systems), goat anti-SOX2 (1:250; sc-17320; Santa Cruz Biotechnology), rat anti- ECAD (1:1,000; 131900, Invitrogen), rabbit anti-AQP1 (1:1,000; AB2219, Millipore), rabbit anti- CCND1 (1:500; RM9104S0, ThermoFisher), rabbit anti-AQP1 (1:1,000; AB2219, Millipore), rabbit anti- AQP4 (1:500; 9661, Cell Signaling), rabbit anti-ePtk (1:500; 4370, Cell Signaling), rabbit anti- SFTPC (1:1,000; AB3768, Millipore), rabbit anti-SFTPβ (1:1,000; WRA8B5552, Seven Hills Bioreagents), rabbit anti-Abca3 (1:1,000; WRA8B7056S, Seven Hills Bioreagents).
Tissue Harvest and Immunostaining. Harvested embryos were fixed in PBS (pH 7.4) with 0.5% paraformaldehyde (PFA; P6148, Sigma) for 3 h at room temperature. Adult lungs were inflated with 0.5% PFA in PBS at 25 cm H₂O pressure, and then dissected and fixed with 0.5% PFA in PBS at room temperature for 3-6 h. Samples prepared for section immunostaining were cryoprotected in PBS with 20% (vol/vol) sucrose and 33% (vol/vol) Optimal Cutting Temperature Compound (OCT; 4583; Tissue-Tek) at 4 °C overnight and then embedded in OCT. All section and whole-mount stainings were carried out essentially as previously described (17, 53). Sections 10 μm in thickness were blocked in PBS + 5% (vol/vol) normal goat serum (017-000-121; Jackson ImmunoResearch) for 1 h followed by incubation with primary antibodies diluted in PBS + 0.3% Triton X-100 overnight at 4 °C. The next day, samples were rinsed and removed slides with PBS followed by incubation with secondary antibodies and DAPI diluted in PBS + 0.1% Triton X-100 + 0.1% Tween-20 for 90 min at room temperature. Secondary antibodies were removed by washing with PBS for 1 h followed by final mounting with Aquamount mounting medium (18606, Polysciences).

For whole-mount immunostaining, fixed samples were placed in PBS overnight at 4 °C, then dehydrated through a PBS/methanol gradient and bleached with 6% (vol/vol) hydrogen peroxide (H2O2, sigma) in methanol overnight at 4 °C. The next day, samples were rehydrated in a methanol/PBS gradient, then blocked in PBS + 0.3% Triton X-100 + 5% normal donkey serum for 2 h, followed by addition of primary antibodies into blocking solution and incubated overnight at 4 °C. Samples were then washed for 6 h with PBS + 0.1% Triton X-100 + 0.1% Tween-20, followed by addition of secondary antibodies and DAPI diluted in PBS + 0.1% Triton X-100 + 0.1% Tween-20 for 90 min at room temperature. Secondary antibodies were removed by washing with PBS for 1 h followed by final mounting with Aquamount mounting medium (18606, Polysciences).

OPT Microscopy. Following whole-mount immunostaining, samples were embedded in 1% (vol/vol) low-melting agarose (16520-100, Invitrogen). Embedded samples were then dehydrated in three passages of ethanol and cleared in two passages of BABB: one part benzyl alcohol (24112, Sigma) and two parts benzyl benzoate (B6630, Sigma). Samples were imaged using the Biomics 3001 OPT scanner with GFP, TXR, and Cy5 filters at high resolution (1.0 μm thickness). Serial sections from the same samples were reconstructed using the NRecon Reconstruction software (Bruker-microCT, BE) and visualized using maximal intensity projection or section view with the Biomics Viewier software (Biomics).

Section and Whole-Mount in Situ Hybridization. In situ hybridization was performed essentially as described previously (17, 53). To generate digoxigenin-labeled riboprobes (1127703910, Roche), cDNAs for target genes were isolated as PCR products derived from mouse embryonic lung RNAs and Xenopus stage 42 embryonic RNAs. Riboprobes were then transcribed with a T7 RNA polymerase (18033-019, Invitrogen) from the cDNAs. For section in situ hybridization, fixed adult lungs were incubated in PBS with 20% sucrose, 33% OCT, and 0.4% PFA overnight at 4 °C, followed by embedding in OCT. Embryonic lungs were quickly dissected and frozen in OCT. Ten-micrometer sections were fixed in 4% PFA in PBS for 10 min at room temperature, followed by treatment with fresh acetylation solution (diethylpyrocarbonate-treated H₂O with 0.0133% triethylamine and 0.0025% acetic anhydride) for 10 min. Sections were then incubated in hybridization solution [50% (vol/vol) formamide, 5x SSC (sodium chloride and sodium citrate), 5x Denhardt’s solution, 500 μg/ml salmon sperm DNA, 250 μg/ml yeast RNA] for 1 h at 62 °C, followed by overnight incubation in 1 μg/ml riboprobes diluted in hybridization solution at 62 °C under a coverslip in a slide oven (24100, Boeckel). The next day, slides were incubated in 2x SSC for 30 min at room temperature, followed by 0.2x SSC for 2 h at 65 °C. Slides were then blocked with TBST (100 mM Tris, 150 mM sodium chloride, 0.1% Tween-20, pH 7.5) with 5% normal sheep serum (013-000-121; Jackson ImmunoResearch) followed by application of antidigoxigenin alkaline phosphatase Fab fragment (11091274910, Roche) diluted in TBST (1:100) at 4 °C overnight. The next day, slides were washed in TBST for 2 h at room temperature followed by detection of alkaline phosphatase activity with nitro-blue tetrazolium (N6639, Sigma) and 5-bromo-4-chloro-3'-indolylphosphate (B6149, Sigma).

For whole-mount in situ hybridization, fixed lungs were dehydrated and stored in methanol at −20 °C. For E11 and E13 lungs, the whole lung was stained intact. For E14 and E15 lungs, branches were isolated and stained individually. Samples were rehydrated in a methanol/DEPC-PBS (diethylpyrocarbonate-treated PBS) gradient at room temperature and permeabilized in DEPC-PBS with 10 μg/ml protease K for 10 min. Samples were then fixed in PBS with 4% PFA and 0.25% (vol/vol) glutaraldehyde for 20 min and washed with DEPC-PBS afterward. Prehybridization solution (50% formamide, 4x SSC, 1X Denhardt’s solution, 250 μg/ml salmon sperm DNA, 250 μg/ml yeast RNA, 50 μg/ml heparin, 0.1% Tween-20) was then applied and samples were incubated at 62 °C for 1 h in a slide oven. Subsequently, 1 μg/ml riboprobes diluted in 1 ml of hybridization solution (prehybridization solution with 10% (wt/vol) >500 kDa dextran sulfate) was added to samples followed by incubation at 62 °C in a slide oven overnight. The next day, samples were washed in 50% formamide with 4x SSC and 0.1% Tween-20 at 65 °C for 1 h, incubated in 100 μg/ml RNase A diluted in 100 mM Tris (pH 7.5) with 1% (vol/vol) SDS for 30 min. The next day, samples were washed in 50% formamide with 2x SSC and 0.1% Tween-20 at 65 °C overnight in a water bath. The following day, samples were washed in TBST for 20 min followed by blocking with TBST + 5% normal sheep serum for 2 h. An antidigoxigenin alkaline phosphatase Fab fragment was then added to the blocking solution for 3 h followed by TBST washes for 3 h and an overnight wash with 0.5% (vol/vol) TBST. The next day, samples were washed with TBST for 2 h followed by detection of alkaline phosphatase activity with nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate. Following the alkaline phosphatase reaction, samples were fixed in 4% PFA in PBS overnight, cleared in 75% (vol/vol) glycerol and mounted on a depression-well slide with coverslip for imaging. Whole-mount in situ hybridization of Xenopus embryos was performed using the same protocol as for mouse lungs. For the staining of Xenopus embryos, samples were washed with 4% PFA in PBS and bleached in 0.5x SSC with 5% formamide and 1.2% hydrogen peroxide for 2 h on a light box. Samples were then washed in 100% methanol three times in 1 d, followed by two washes of BABB in 1 d. To minimize experimental variation, littermate control and mutant embryos from approximately 30 eggs from the same mating were used. Littermates were identified by toe-tip FACS phenotypes. After injection, Edu staining was performed as described above. Following removal of secondary antibodies, Edu reaction was developed on slides for 30 min according to the manufacturer’s instructions. Confocal images were taken of matching branch tips (RCd lobar and RCd.L1 branches (2)) of littermate control and mutant embryos. For each branch tip, branches of the L.L1-L5 branch lineages (2) from E13 littermate control and Sox9CKO/CKO;ShhCre/+ mutant lungs, or Xenopus embryos of different stages were analyzed on the FLUOVIEW Viewer software (Olympus). Images of sections and whole-mount stainings were taken on an upright microscope (BX60, Olympus), or a fluorescence stereoscope with a 2× Plan Apochromat objective (M205C, Leica), a OPT microscope (Bioptics), or a confocal microscope (FV1000, Olympus).

Edu Staining and CCND1 Cell Count. Edu staining was performed using a Click-IT Edu imaging kit (C10337, Invitrogen). E14 pregnant mice were given intraperitoneal injection of 5-bromo-2-deoxyuridine and embryos were harvested 1.5 h after injection. Edu staining was incorporated with the section immunostaining protocol described above. After removing secondary antibodies, Edu reaction was developed on slides for 30 min according to the manufacturer’s instructions. Confocal images were taken of matching branch tips (RCd lobar and RCd.L1 branches (2)) of littermate control and Sox9CKO/CKO;ShhCre/+ mutant lungs. At least 200 cells from five sections were counted for each branch tip. Branches of the L.L1-L5 branch lineages (2) from E13 littermate control and Sox9CKO/CKO;ShhCre/+ mutant lungs were used for CCND1 cell count. At least 250 cells from seven branch tips were counted. Cell counting was performed on epithelial cells of the distal half of each branch tip, where SOX9 was normally expressed at a high level. Images were analyzed on the FLUOVIEW Viewer software (Olympus).

FACS. Embryonic lungs were dissected free of extrapulmonary tissues, minced into 1-mm pieces with forceps, and digested with 2 mg/ml Collagenase type I ( Worthington, CLS-1) and 0.2 mg/ml DNase I ( Worthington, D) for 20 (E14 lungs) to 40 (E19 lungs) min at 37 °C with frequent agitation. An equal volume of solution containing 0.25% Trypsin/EDTA (Invitrogen, 25200-056) and 0.2 mg/ml DNase I ( Worthington, D) were added and incubated for another 8 min at 37 °C with frequent agitation. After digestion, FBS (Invitrogen, 10082-147) were added to a final concentration of 10% (vol/vol) and the samples were triturated by pipetting for 20–40 times. Dissociated cells were washed with PBS with 1% FBS and sorted on a BD Biosciences Influx sorter equipped with 488 and 561 lasers.
Microarray Expression Profiling. RNA was extracted from at least 2 x 10^5 FACS purified epithelial cells or lungs dissected free of extrapulmonary tissues from E13, E14, and E15 littermate control and Sox8-/-;Hsdm-/- mutant embryos using TRIzol reagents (15596018, Invitrogen) and an RNAeasy Mini kit (74104, Qiagen). Labeled cRNAs were generated using an Illumina TotalPrep RNA amplification kit (AM17191, Invitrogen) and hybridized to Illumina mouse WG-6 v2 expression beadchips (BD-201–0202, Illumina). The experiments were repeated with lungs from independent litters. The expression values from the microarrays were normalized using rank invariant normalization and analyzed using the R statistical software.

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Fig. S1. FACS purification of distal lung epithelial cells. (A) Stereoscope images of an embryonic day (E)16 SRY-box containing gene 2 (Sox2)EGFP+;RosatdT+/; Sonic hedgehog (Shh)Cre+ lung showing that distal epithelial cells express red (tdT), but not green (EGFP), fluorescent proteins. (Scale bar, 500 \( \mu \)m.) (B) A representative FACS graph of E17 Sox2EGFP+;RosaCre+/;ShhCre+ lungs showing separation of distal epithelial cells (P4 fraction) from proximal epithelial cells (P2 fraction) and nonepithelial cells (P5 fraction).
Fig. S2. Branching program in late lung development. (A) Confocal images of immunostained lung sections in areas where the airway lumen can be continuously traced from the proximal conducting airways (black/white long dashed lines) to the distal nonbranch tip (magenta) and branch tip (green) regions, as illustrated in the schematics. SOX9+ branch tips are present near the distal edges of E17–E19 lungs. Branch tips are outlined with dashed lines at E17–E18 and are too small to be completely captured on sections at E19. The boxed regions are enlarged (Insets) outlined in corresponding colors. Dashed magenta and green boxes indicate a low level of alveolar differentiation (Fig. S3) and branching, respectively. The images for E18 lungs are replicated from Fig. 1B. (Scale bar, 20 μm.) (B) Optical projection tomography (OPT) images of the distal edges of whole mount immunostained lungs showing an increase in the number of SOX9+ branch tips from E17 to E18 (arrowheads). Very few cells at the branch tips express SOX9. (Scale bar, 250 μm.)
Fig. S3. Temporal progression and proximal/distal distribution of the alveolar differentiation program. Confocal images of immunostained lung sections in areas where the airway lumen can be continuously traced from the proximal conducting airways (black/white long dashed lines) to the distal nonbranch tip (magenta) and branch tip (green) regions, as illustrated in the schematics. Differentiation of alveolar type I [Aquaporin 1 (AQP1) and receptor for advanced glycosylation of end products (RAGE) in A] and type II [ABCA3 and LAMP3 in B and Surfactant protein B (SFTPB) in C] cells occurs in nonbranch tip regions of the distal epithelium and expands distally at E19 when the branch tips consist of a few SOX9⁺ cells. Branch tips are outlined with dashed lines at E17–E18 and are too small to be completely captured on sections at E19. The boxed regions are enlarged as insets outlined in corresponding colors. Dashed magenta and green boxes indicate a low level of alveolar differentiation and branching, respectively. AQP1 labels alveolar type I cells, the vasculature, and the mesothelium, which are separated by dashed lines in the insets. The images for E18 lungs are replicated from Fig. 1B. Asterisks in B indicate autofluorescence from blood cells. (Scale bars, 20 μm.)
Xenopus lungs lack the branching program and Sox9 expression, and initiates alveolar differentiation immediately after lung specification. Whole mount in situ hybridization of Xenopus embryos at indicated stages (St). The lungs are indicated with dashed lines if stained or arrowheads if unstained. Open arrowheads indicate Sox9 expression in the pharyngeal arches. The onset of Sftpb expression is slightly later than that of Sftpc expression (Fig. 1C). (Scale bar, 200 μm.)
Fig. S5. Hyperactive Kras suppresses alveolar differentiation. Confocal images of immunostained lung sections in areas where the airway lumen can be continuously traced from the proximal conducting airways (black/white long dashed lines) to the distal nonbranch tip (magenta) and branch tip (green) regions, as illustrated in the schematics. Expression of alveolar type I (AQP1 and RAGE in A) and type II (ABCA3 and LAMP3 in B) markers is restricted to nonbranch tip regions closer to the conducting airways in the Kras mutant lung. Although SFTPB expression is more uniform in the Kras mutant, cells in the expanded branching regions are more clustered as shown by ECAD (E-cadherin) staining (C). The boxed regions are enlarged (Insets) outlined in corresponding colors. Dashed green boxes indicate a low level of branching with a few SOX9-positive cells. (Scale bars, 20 μm.)
Fig. S6. Persistent branching defects and normal co-alignment between airway branches and arteries in the Sox9 mutant lung. OPT images of whole-mount immunostained lungs from littermate control (Left) and Sox9^{CKO/CKO}; Shh^{Cre/+} mutant (Right) embryos. The RCd.L3 branch lineages (1) in the boxed areas are enlarged and traced with dashed lines. Only the SOX2^+ branches are traced for E19. The arteries in E19 lungs immunostained for smooth muscle actin (SMA) are identified by their connection to the main pulmonary arteries, traced with solid green lines and shown (Insets). (Scale bars, 250 μm.)

Fig. S7. Sox9-dependent and -independent branching related genes. Whole-mount (A, B, and D) and section (C) in situ hybridization of littermate control and Sox9 CKO/CKO;Shh^{Cre/+} mutant lungs. Expression of Clusterin (Clu) and Melanoma inhibitory activity 1 (Mia1) is dependent on Sox9, whereas expression of Bmp4, Spry2, Id2, Thbs1, Dlk1, and Shh is independent of Sox9. Branch tips are traced with dashed lines. [Scale bars, 200 μm (A, B, D) and 50 μm (C).] (E) Projection images of an E15 whole-mount immunostained Sox9^{CKO/CKO};Nkx2.1^{CreER/+} mutant lung showing the absence of CLU in SOX9^- cells (mut). (Scale bar, 10 μm.)
Fig. S8. Normal cell polarity in the Sox9 mutant lung. Confocal images of whole mount immunostained E15 (A and B) and E12 (C) littermate control and Sox9CKO/CKO;ShhCre/+ mutant lungs. The Sox9 mutant lung has no significant change in the distribution of apical [Zonula occluden 1 (ZO1) and Podocalyxin-like (POD)], basement membrane [Entactin (ENT) and Perlecain (PERL)] and cytoskeleton [acetylated Tubulin (AcT)] markers. (Scale bars, 20 μm.)
Cell proliferation in the Sox9 mutant lung. (A) Confocal images of the L.L1 branch lineage (1) of E13 whole-mount lungs from littermate control and Sox9<sup>CKO/CKO</sup>;Shh<sup>Cre/+</sup> mutant embryos, immunostained for ECAD and Cyclin D1 (CCND1). The boxed areas are enlarged in subsequent panels. Arrowheads indicate cells expressing a very low level of CCND1 [2% in control lungs (n = 293) versus 11% in Sox9 mutant lungs (n = 252), P < 0.001, χ² test]. (Scale bar, 20 μm.) (B) The percentages of 5-ethynyl-2'-deoxyuridine (EdU) cells in the RCd lobar and RCd.L1 (1) branch tips are not significantly different between E14 littermate control and Sox9<sup>CKO/CKO</sup>;Shh<sup>Cre/+</sup> mutant lungs (P > 0.05, Student t test). Error bars represent SDs.

Sox9 regulates mesenchymal cell survival and Fgf10 signaling. (A) Stereoscope images of whole-mount lungs from littermate control and Sox9\textsuperscript{CKO/CKO}, Shh\textsuperscript{Cre+} mutant embryos, immunostained for ECAD, cleaved Caspase-3 (CASP3), and DAPI. The boxed areas are enlarged (Insets). The number of apoptotic cells in the mesenchyme of the Sox9\textsuperscript{CKO/CKO}, Shh\textsuperscript{Cre+} mutant lungs increases from E13 to E15 (arrowheads). Confocal images of the E15 lungs are shown in the rightmost column. Compared with the confocal images, the stereoscope images are a projection view of the whole lung and therefore have more background auto-fluorescence and more apoptotic cells. Double-headed arrows indicate the increased distance between the epithelium and the mesothelium in the Sox9 mutant lung. (Scale bars, 100 \(\mu\)m.) (B) Fgf10 whole-mount in situ hybridization of littermate control and Sox9\textsuperscript{CKO/CKO}, Shh\textsuperscript{Cre+} mutant lungs. The boxed areas are shown as insets. Square brackets indicate increased Fgf10 expression in the Sox9 mutant lung. Branch tips are traced with dashed lines. (Scale bar, 100 \(\mu\)m.) (C) Confocal images of E13 whole-mount littermate control and Sox9\textsuperscript{CKO/CKO}, Shh\textsuperscript{Cre+} mutant lungs, immunostained for phosphorylated extracellular signal-regulated kinase (pERK) and ECAD. pERK staining appears more diffuse in the Sox9 mutant lung. Branch tips are traced with dashed lines. (Scale bar, 50 \(\mu\)m.)
Fig. S11. Epithelial deletion of Sox9 suppresses ectopic branches in the Kras mutant lung. (A) OPT images of whole-mount immunostained E15 littermate Kras\(^{LSL-G12D+/+}\);Shh\(^{Cre/+}\) mutant and Kras\(^{LSL-G12D+/+}\);Sox9\(^{ΔΔCDK4+/−}\);Shh\(^{Cre/+}\) mutant lungs. Ectopic branches in the Kras\(^{LSL-G12D+/+}\);Shh\(^{Cre/+}\) mutant lung continue to grow from E13 (Fig. 4B) to E15 (arrowheads), but are suppressed by epithelial deletion of Sox9. (Scale bar, 500 μm.) (B) There is a significant difference (P < 0.0001, Wilcoxon test) in the frequency distributions of ectopic branches formed on tracheal and extrapulmonary left/right main bronchial epithelia between E13 Kras\(^{LSL-G12D+/+}\);Shh\(^{Cre/+}\) (open column) and Kras\(^{LSL-G12D+/+}\);Sox9\(^{ΔΔCDK4+/−}\);Shh\(^{Cre/+}\) (filled column) mutant lungs. No ectopic branches are present in either control or Sox9\(^{ΔΔCDK4+/−}\);Shh\(^{Cre/+}\) mutant lungs.

Fig. S12. Precocious initiation of alveolar differentiation in the Sox9 mutant lung. (A) Section in situ hybridization of an adult lung for indicated genes. Arrowheads indicate expression in alveolar type II cells. (Scale bar, 20 μm.) (B) Whole-mount (Sftpc) (Scale bar, 200 μm) and section (Lamp3) (Scale bar, 20 μm) in situ hybridization of littermate control and Sox9\(^{ΔΔCDK4+/−}\);Shh\(^{Cre/+}\) mutant lungs. Branch tips are traced with dashed lines. We note that Sftpc is expressed at a low level in the control lung and up-regulated in the Sox9 mutant lung. (C) Confocal images of whole-mount immunostained littermate control and Sox9\(^{ΔΔCDK4+/−}\);Shh\(^{Cre/+}\) mutant lungs. Branch tips are traced with dashed lines. (Scale bar, 20 μm.)
Dataset S1. Microarray expression profiling of FACS-purified distal lung epithelial cells from E14 through E19

The original expression values are converted to a log$_2$ scale when comparing with baseline expression at E14. The two experiments are biological repeats using lungs from independent litters.

Dataset S2. Microarray expression comparison between littermate control and Sox9$^{CKO/CKO};$Shh$^{Cre/+}$ mutant lungs at E13, E14, and E15

The original expression values are converted to a log$_2$ scale when comparing control and Sox9 mutant lungs. The two experiments are biological repeats using lungs from independent litters.