

MOLECULAR REGULATION OF LUNG DEVELOPMENT

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■ **Abstract** There is increasing evidence suggesting that formation of the tracheo-bronchial tree and alveoli results from heterogeneity of the epithelial-mesenchymal interactions along the developing respiratory tract. Recent genetic data support this idea and show that this heterogeneity is likely the result of activation of distinct networks of signaling molecules along the proximal-distal axis. Among these signals, fibroblast growth factors, retinoids, Sonic hedgehog, and transforming growth factors appear to play prominent roles. We discuss how these and other pattern regulators may be involved in initiation, branching, and differentiation of the respiratory system.

INTRODUCTION

The mammalian lung evolved as a system of branched conduits for air and blood coupled to a vast network of honeycomb-like alveolar structures designed for gas exchange. In the developing respiratory system, while airway branching is eminently a prenatal event, formation of the alveoli spans pre- and postnatal life, and in many species such as the rat and mouse occurs only postnatally (93, 109).

Primordial lung buds originate as outpouchings of the primitive foregut endoderm, and the airway tree is generated by reiterated budding and branching of these tubules. Blood vessels are formed in situ from mesodermal cells around the tips of the branching tubules (vasculogenesis), or migrate to the lung by sprouting from the pulmonary artery (angiogenesis). As the lung develops, vascular and airway components intermingle at the distal end of this tree to form the future alveolar-capillary barrier. The complex branching pattern of the airways ensures that during postnatal life air is properly cleared of particulates, humidified, and distributed evenly to all alveolar units.

The questions of how lung morphogenesis occurs and which molecules direct lung development have attracted the interest of developmental biologists for

decades and recently have been much explored. We review these aspects from the events that precede lung formation to late developmental stages. Because the vast majority of the genetic studies have been performed in the mouse, this species is used as the model system throughout most of the text.

BEFORE LUNG MORPHOGENESIS

Formation of the Foregut

In the mouse, gut morphogenesis is initiated around gestational day 7.5, when a single sheet of endodermal cells located in the external surface of the embryo undergoes anterior-posterior (A-P) specification and expresses specific A-P marker genes (reviewed in 118). Fate map analysis has shown that anterior endodermal cells generate the ventral foregut that will subsequently form lungs, liver, ventral pancreas, and stomach (52). From day 7.5–8.5, complex morphogenetic movements of the endodermal layer and looping of the embryo lead to invagination and closure of this layer to form the primitive gut tube. Endodermal cells form the epithelial lining of the tube. Lateral plate mesodermal cells migrate and condense around the endoderm to form the mesenchyme (splanchnic mesoderm) and the serosa (somatic mesoderm) of the gut. Mesothelial cells (serosa) surrounding the gut in contact with the coelomic cavity form pleural, pericardial, and peritoneal membranes.

Among the earliest endodermal signals essential for gut morphogenesis are the GATA (zinc finger proteins that recognize GATA DNA sequences) and hepatocyte nuclear factors (HNF) transcription factors. Targeted deletion of *GATA-4* in mice is lethal by day 7–9.5, resulting in impaired folding of the embryo and disruption of endoderm morphogenesis (50). *GATA-6*^{-/-} mice die even earlier (at day 6.5–7.5) from disruption of endodermal differentiation, with down-regulation of *GATA-4* and *HNF-4* (75). Interestingly, once the lung forms, *GATA-6* but not *GATA-4* continues to be expressed in the lung epithelium. There is genetic evidence suggesting that *GATA-6* may be required for activation of the lung developmental program in the foregut endoderm (75).

Expression of *HNF3 α* and β is also found in the foregut endoderm and is maintained in the lung epithelium throughout embryonic to adult life (16, 74). *HNF3 β* null mutant mice die by day 7–9.5, with multiple defects that include failure of the foregut endoderm to invaginate and form a closed tube. This results in severe disruption of gut morphogenesis (1, 119). In these mutants, preserved expression of *HNF3 β* suggests that endodermal differentiation has been initiated. However, the increasing number of dying cells in embryos that survive until day 9.5 suggests that *HNF3 β* is a survival factor for the endoderm (119). Several studies implicate *HNF3 β* in lung differentiation and regulation of surfactant protein gene expression (reviewed in 89).

Formation of Foregut Derivatives: The Role of Local and Diffusible Signals

Thyroid and liver buds are first seen at day 8.5; lung and pancreatic buds form one day later. Several transcription factors are locally expressed in overlapping domains along the endoderm in a pattern that suggests that they may be responsible for induction of organ-specific developmental programs. For example, *Nkx 2.1* (or thyroid transcription factor 1, *TTF-1*) is found in the anterior endoderm near the prospective sites of thyroid and lung bud formation (48), whereas *Pdx-1* is expressed at a more posterior location, in the area of the prospective pancreatic buds (83). Nevertheless, genetic studies have shown that these factors individually are not necessarily critical for initiation of organ-specific morphogenesis. *Nkx 2.1*^{-/-} mice do not have thyroid; however, they form lungs that are highly abnormal (48, 72). Although pancreas development is dramatically disrupted in *Pdx-1*^{-/-} mice, the initial steps of pancreas morphogenesis and expression of glucagon and insulin do take place (83).

While expressing their own repertoire of pattern regulators, endodermal cells are also exposed to signals that diffuse from developing structures that neighbor the gut. For example, posterior endoderm is exposed to retinoic acid (RA) from the node and to fibroblast growth factor (FGF)-4 from the posterior mesoderm (118). Importantly, FGFs secreted from the adjacent cardiac mesoderm activate signaling by FGFR-1 and -4 in the ventral foregut endoderm to induce the initial steps of hepatogenesis and liver-specific gene expression (43). During pancreatic development, the day 7.5 endoderm receives patterning signals from the neighboring notochord. The notochord secretes activin- β B and FGF-2, which in turn inhibit Sonic hedgehog (Shh) signaling in the pre-pancreatic dorsal endoderm, and initiate a program of pancreatic gene expression (35). Surgical deletion of the notochord in chicks at a stage when this structure still touches the foregut endoderm prevents expression of pancreatic genes (47). Whether notochord deletion also affects lung bud morphogenesis has not been reported. Interestingly, delayed separation of the notochord from the gut endoderm has been associated with disruption of tracheal development leading to tracheoesophageal fistula and tracheal atresia in rats (70).

Prespecification of Patterns in Lung Morphogenesis

The genetic programs that determine axis and branching patterns are defined at an early developmental stage. For example, the left-right (L-R) axis of the lung appears to be specified well before there is any sign of lung. L-R differences in pattern are first seen when secondary buds form; these differences continue to develop as airways undergo branching and are assembled into lobes by the visceral pleura (lobation), which result in more lobes in the right lung (mouse, four; human, three) than in the left (mouse, one; human, two).

The basis for L-R asymmetry in viscera lies in genes such as *Lefty-1* and *-2*, *nodal*, and *Pitx-2*, whose inactivation in mice reveals striking lung phenotypes (67). *Lefty-1*^{-/-} mice show left pulmonary isomerism, a condition where single-lobed lungs are found bilaterally (67). In these mice, the lobation pattern typically seen on the right side (four lobes) is substituted for left-side pattern (one lobe). Interestingly, *Lefty-1*, *-2*, and *nodal* are only transiently expressed in the embryo at around day 8–8.5, on the left side of the prospective floor plate and lateral plate mesoderm, and are no longer detected at later stages (67 and references therein). Thus specification of the branching pattern and of lobation of the lung is linked to the general body plan and has started at least 24–36 h prior to the appearance of the primordial lung bud (day 9.5).

L-R-determining genes are targets of RA. Expression of *Lefty*, *nodal*, and *Pitx* genes occurs at a stage when RA synthesis and utilization are highly active in the foregut and its surroundings (discussed further below). Experiments where RA signaling is antagonized in whole-embryo cultures show that RA is necessary for expression of genes such as *Lefty-1* and its targets (14). Although no L-R asymmetry in retinoid receptor distribution has been reported in the lung (21, 22), compound *RAR* α/β 2 knockout mice do not have the left lung (65). The requirement of RA signaling activation for expression of L-R genes, or for a gene that is asymmetrically distributed in the embryo prior to lung development, may be one of the reasons for this defect.

LUNG BUD INITIATION

The molecular mechanisms responsible for primary lung bud induction are little understood. Lung buds originate from the ventral foregut endoderm, which fuse in the midline as they grow to form the lung and the tracheal primordia (104). This process is fully blocked in mice that lack both *Gli2* and *Gli3* zinc-finger transcription factors. Gli 1, 2, and 3 (vertebrate homologs of the *Drosophila* gene *cubitus interruptus*) are expressed in the foregut mesoderm and have been identified as downstream components of the Shh signaling cascade (38). *Gli2*^{-/-}; *Gli3*^{-/-} embryos have multiple defects in endodermal derivatives, including agenesis of lung and trachea, and do not survive beyond day 10.5 (77). However, it is unclear how the effects of mesenchymal Gli expression are transduced to the foregut endoderm at the sites of lung bud initiation. In this process, Gli 2 and 3 seem to act independently of Shh because lung agenesis is not a feature of Shh knock-out mice (88). Glis may ultimately be necessary to maintain expression of genes that promote endodermal survival such as hepatocyte nuclear factor, *HNF3* β (1, 119). *Gli2*^{-/-}; *Gli3*^{-/-} mice have decreased levels of *HNF3* β compared with that of wild-type animals (77).

Results from genetically altered mice and vitamin A-deprived rats have implicated signaling by FGF and RA in lung bud initiation. These regulators are discussed in greater detail in subsequent sections.

Foregut cells likely start to acquire specialized lung features before lung bud formation is initiated. Perhaps the first sign of lung differentiation is expression of the lung-specific surfactant protein gene *SP-C*. Expression of *SP-C* mRNA has been reported at the tips of epithelial tubules from embryonic day 11 and localizes to type II alveolar cells in the adult lung (120). However, studies in a transgenic mouse bearing a reporter *CAT* (chloramphenicol acetyl transferase) gene driven by 3.7-kb human *SP-C* promoter suggest that endogenous *SP-C* might start at least one day earlier and become restricted to the tips of secondary buds at day 11 (120). Other surfactant protein genes, *SP-A*, *SP-B*, and *SP-D*, appear later, and their expression in the adult is restricted to the distal lung in alveolar type II and bronchiolar epithelial cells (66).

LUNG MORPHOGENESIS: Role of Fibroblast Growth Factors

The FGF family of polypeptides currently consists of 23 members. FGF signaling is transduced by four transmembrane tyrosine kinase receptors (FGFRs). FGF-FGFR interactions are modulated by heparan sulfate proteoglycans (HSPG), such as syndecans and perlecan. Upon ligand binding, receptors dimerize and autophosphorylate, leading to activation of a variety of intracellular pathways that are fundamental to control cell proliferation, differentiation, and pattern formation (reviewed in 107).

FGFs are widely expressed throughout animal phyla and are found in species as diverse as worms, insects, and humans. A remarkable feature of FGF signaling is its conserved functional role in branching morphogenesis during evolution (reviewed in 68). The branching pattern of the *Drosophila* trachea is established by expression of the FGF *branchless* (*Bnl*) in clustered cells near tracheal epithelial tubules at prospective sites of budding. *Bnl* activates an FGFR, *breathless* (*btl*), in the epithelium. This results in epithelial bud migration and elongation toward the *Bnl*-expressing cells (106). Thus *Drosophila* FGF acts as a chemotactic factor for the epithelium. Homogeneous expression of the *FGFR* may ensure that all epithelial cells are capable of responding to the local chemotactic stimulus by budding.

FGF-10 and Budding

In the developing mouse lung, a mechanism involving FGF-10 and FGFR-2, reminiscent of that described above in flies, regulates airway branching. *FGFR-2* is expressed throughout the respiratory tract epithelium from the earliest stages of lung development (embryonic day 9.5) and during branching morphogenesis (12, 90). The mesenchyme expresses *FGF-10* in a localized fashion in close association with distal epithelial tubules. In situ hybridization analysis of day 11–12 embryonic lungs has shown that local expression of *FGF-10* is dynamic; it appears to precede distal bud formation, and signals are down-regulated once the bud is formed (6).

Interestingly, in day 11.5 lung epithelial explants growing in the absence of mesenchyme, recombinant FGF-10 can substitute for the mesenchyme and induce generalized budding when dissolved in the culture medium (6, 85). Moreover, a gradient of FGF-10 protein can be established in these cultures by implanting a heparin bead soaked in FGF-10 near the explant. Under this condition, epithelial buds grow toward the FGF-10 source and engulf the bead within 48 h (85, 117). This is also seen when FGF-10 beads are grafted onto intact day 11.5 mouse lungs and grown in culture, simulating a situation where high levels of FGF-10 are artificially fixed in time and space (85). By overriding the dynamic pattern of endogenous *FGF-10*, the FGF bead appears to redirect airway growth to an ectopic position and distorts the normal pattern of branching. Results from these assays also show that, when compared with another FGF such as FGF-7, FGF-10 acts at relatively short-range distances in the developing lung. In explants cultured in the absence of mesenchyme, the chemotactic effect is observed in epithelial buds located as far as 150 μm from an FGF-10 bead (117), and within 50 to 75 μm distant from the FGF-10 bead when it is grafted onto intact lung explants (85). This difference could be accounted for by FGF-10 binding to matrix components in the mesenchyme such as HSPGs (40), which may locally restrict the FGF-10 effects in intact lungs in vitro or in vivo. This effect and the mechanisms that transcriptionally regulate *FGF-10* expression (see below) contribute to fine tuning of the patterning effects of FGF-10 in the developing lung.

In addition to being a source of FGF-10, the mesenchyme also modulates the response of the epithelium to FGF-10. When FGF-10 beads are grafted onto proximal and distal sites of day 11.5 lungs, effects are elicited only in distal epithelium. Since at this time proximal and distal epithelia express *FGFR-2* at apparently similar levels (12, 90), the lack of effects in proximal airways suggests that the mesenchyme contains factors that interfere with FGF-10 activation and cellular activities in the epithelium. Indeed, epithelial explants isolated from proximal airways cultured in the absence of mesenchyme are able to migrate toward an FGF-10 bead (117).

Collectively the data are compatible with the idea that FGF-10 locally induces and guides bud outgrowths to proper positions during lung branching morphogenesis. The critical role that FGF-10 plays in lung pattern formation has been confirmed by genetic studies. In *FGF-10* knock-out mice, induction of primary lung buds is disrupted. As a result, lungs do not form and mice have a blunt-ended tracheal tube (71, 96). There is also genetic evidence that the chemotactic response and lung bud induction elicited in the epithelium by FGF-10 result from local activation of *FGFR-2*. Lung agenesis is similarly found when *FGFR-2* is inactivated either by excision of the *IIIb* exon (19) or by disruption of the *IIIc* exon and the transmembrane domain (2). Thus signaling by FGF-10-*FGFR-2* is prototypical of epithelial-mesenchymal interactions classically described in grafting experiments, where mesenchymal FGF-10 is an inducer of distal epithelial buds.

FGF-10 Effects: Similarities and Differences with Other FGFs

Because FGFR-2 is also a high-affinity binding receptor for FGF-1 and FGF-7 (107), these ligands could theoretically have the same bud-inducing potential as FGF-10. In reality only FGF-1 has this potential. FGF-1 can mimic the FGF-10 effects (6, 81, 85) likely because of its ability to bind to all FGFR, including FGFR-2. Nevertheless, experiments using FGF-1 beads show that its chemotactic effect is less marked than that elicited by FGF-10 (85). Furthermore, the late onset of expression and spatial distribution of endogenous FGF-1 do not seem to be relevant for early branching (see below).

Although FGF-7 does not show chemotactic properties when applied to lung explants, it has a potent effect on epithelial cell proliferation. Mesenchyme-free lung epithelial explants treated with FGF-7 show generalized growth that results in formation of cyst-like structures instead of budding (12). This effect is also seen in lungs of transgenic mice carrying an *SP-C* or *CC10* promoter-driven *FGF-7* transgene (101, 112). FGF-7, in contrast to FGF-10, appears to act as a long-range signal in the developing lung. When grafted onto day 11.5 embryonic lungs and cultured, the effect of FGF-7-loaded beads in the epithelium is seen over 500 μm from the source (85). It is intriguing that *FGF-7* and *FGF-10* share a high degree of homology and have high-affinity binding to FGFR-2 but exert very different effects in the developing lung epithelium.

How does FGF signaling exert its effects on budding? The studies in *Drosophila* have shown that *branchless* acts purely as a chemotactic factor (106). By contrast, lung bud induction by FGF-10 involves both chemotaxis and epithelial proliferation, although FGF-10 is less potent than FGF-7 as a mitogen in lung epithelial cells (85, 117). Interestingly, at least in keratinocytes, heparin enhances the mitogenic activity of FGF-10 while inhibiting that of FGF-7 (40).

Whether proliferation is the primary driving force for lung epithelial budding has been debated. Experiments using mesenchyme-free lung epithelial cultures suggest that the appearance of differential cell proliferation within the epithelium is not the initial event that triggers lung bud induction. In these cultures, FGF-1 induces budding within 18 h in culture, but regional differences in BrDU incorporation are evident only after budding has initiated (82). Alternatively, FGF signaling could initially induce changes in cell-cell adhesion and cell motility, thus leading to cell rearrangement and trigger budding, as shown in other systems (reviewed in 31).

FGFs as Differentiation Signals

Studies in organ cultures have shown that grafting distal lung mesenchyme onto trachea results in induction of tracheal epithelial buds with cellular features of lung type II cells, such as lamellar bodies (organelles that store surfactant), and *SP-C* expression (98, 104). Interestingly, a mixture of soluble factors that includes FGF-7 and FGF-1 can substitute for distal mesenchyme inducing type II cell differentiation in tracheal epithelial cultures (*trans*-differentiation). By deleting individual

components of this mixture, Shannon and collaborators (99) show that FGF-7 and FGF-1 are necessary in the medium for *trans*-differentiation; however, none of the factors in the mixture can individually induce *SP-C* expression in trachea. In contrast, there is evidence that in lung epithelial cultures, exogenous FGF-7 by itself is able to induce at least a partial program of type II cell differentiation. In the absence of mesenchyme and serum, FGF-7 induces precocious expression of high levels of the differentiation markers *SP-A* and *-B* and lamellar body formation. This effect cannot be reproduced by FGF-1 or FGF-10 treatment at any concentration, even though both factors also bind the FGF-7 receptor (FGFR2-IIIb) with high affinity (12). FGF-7 likely continues to be a proliferation and differentiation factor for the distal lung throughout life; in the adult lung, FGF-7 acts as an alveolar type II cell mitogen and increases *SP-A* and *-B* mRNA expression (105, 114). Surprisingly, lungs from *FGF-7*^{-/-} mice appear normal, suggesting that the role of FGF-7 in the lung overlaps with that of other growth factors (32). In fetal rat lung epithelial cells cultured in EHS matrix, expression of *SP-A*, *-B*, and *-C* has been shown to be induced by FGF-2 through a MAPK-independent pathway (62).

Modulation of FGF Signaling: FGF Interactions

An important observation from the studies above is that uncontrolled availability of FGF-10 (recombinant protein in beads) leads to distortions of the branching pattern. Thus fine-tuning of branching might require that FGF-10 levels in time and space be precisely controlled by other pattern regulators expressed in both mesenchyme and the epithelium. Dynamic expression of *FGF-10* might result from a combination of local induction and restriction of expression by inhibitors.

Although inhibitors of *FGF-10* gene expression have been identified (see below), to date *FGF-10* inducers have not yet been reported in the lung. Interestingly, if embryonic lung mesenchymal cells are cultured in the absence of the epithelium, *FGF-10* mRNA levels markedly increase within 24 to 48 h. This contrasts with the decrease in levels of FGF-7 mRNA, assessed within the same period (53, 92). The data suggest that the developing lung epithelium has diffusible factors that are inhibitory for *FGF-10* and stimulatory for *FGF-7*. There is evidence that mesenchymal factors also influence *FGF-10* expression and its effects in the epithelium.

The subsequent section reviews information about a selected number of pattern regulators that have been shown to interact with FGFs during lung morphogenesis.

Sonic hedgehog (Shh) Shh regulates pattern formation of a variety of developing structures including the lung (24). At day 9.5, when primary lung buds are forming, *Shh* is already expressed in the ventral foregut endoderm (55). *Shh* is subsequently expressed in the developing lung epithelium in a gradient fashion, with the highest levels in cells at the tips. In turn, most components of the Shh pathway, including Shh target genes and its receptor Ptc1, are found in the mesenchyme (5, 30). Although another receptor, Ptc2, has been identified in the epithelium (78), its role in transducing Shh signaling in the lung is unknown.

Shh signaling is initiated upon binding to Ptc1 (from now on referred to as Ptc) and results in activation of Shh target genes by Gli transcription factors (reviewed in 41). *Ptc* expression in the lung follows the proximal-distal gradient of *Shh* (5). *Glis* (1, 2, 3) are expressed in overlapping but distinct domains in the lung mesenchyme. The proximal-distal gradient is evident in *Gli1*, which together with *Ptc*, is transcriptionally up-regulated by Shh and is expressed in the subepithelial mesenchyme (30). The distinct phenotypes observed when *Gli* genes are inactivated individually or in combinations suggest that there are complex interactions between *Gli* members in regulating lung growth and pattern formation (30, 77, 86).

Shh knockout mice show that, while Shh is essential for branching morphogenesis, it is not required for primary bud induction as are Gli 2 and 3. Lungs from *Shh*^{-/-} mice develop as rudimentary sacs by failure of the epithelial tubules to branch properly. The defect seems to be primarily in the mesenchyme, as shown by an increase in mesenchymal cell death; decreased cell proliferation is also reported. Branching, but not proximal-distal differentiation, is affected (55, 88).

A paramount feature of *Shh*^{-/-} lungs is the widespread distribution of *FGF-10* signals in the mesenchyme, contrasting with the local pattern present in wild type (88). This supports the idea that, not only the presence of FGF-10, but its correct spatial distribution is necessary for patterning. If *FGF-10* signals are diffuse rather than localized, directional clues are lost and branching is disrupted. Importantly, data also suggest that under normal conditions Shh plays a role on controlling *FGF-10* expression in the distal lung. Two other models support this statement. When Shh is over-expressed in the lungs of transgenic mice, increased mesenchymal cell proliferation and down-regulation of *FGF-10* gene expression are observed (5, 6). Moreover, treatment of embryonic lung mesenchymal cells with recombinant Shh protein prevents the increase in *FGF-10* expression observed within 48 h when these cells are grown in control conditions (53). Thus Shh is part of an epithelial network of regulators that restricts *FGF-10* expression. Shh-FGF-10 interaction supports a model proposed by Bellusci and coworkers (6) in which the growing epithelial bud, which expresses high levels of Shh, interacts with a chemotactic source (FGF-10) in the distal mesenchyme to extinguish it (Figure 1).

Expression of *Shh* and *Ptc* do not seem to be influenced by FGF-10; however, both genes are down-regulated by FGF-7 in lung explant cultures (53). Interestingly, at early stages of lung development, high levels of *Shh* and *Ptc* but low *FGF-7* levels are found in the embryonic lung. Conversely, prior to birth, high levels of *FGF-7* and low levels of *Shh* and *Ptc* are detected (5, 6, 11, 85). Whether FGF-7 functions as an inhibitory factor of Shh signaling in vivo remains to be determined.

Bone Morphogenetic Protein-4 (BMP-4) BMPs belong to the TGF β superfamily of growth factors, with at least three members (BMP-4, -5, and -7) present in the developing lung (4, 49). BMP-4 is an important regulator of epithelial proliferation and proximal-distal cell fate during lung morphogenesis. Studies using a *BMP-4^{lacZ}* reporter mouse show mesenchymal signals as early as gestation day

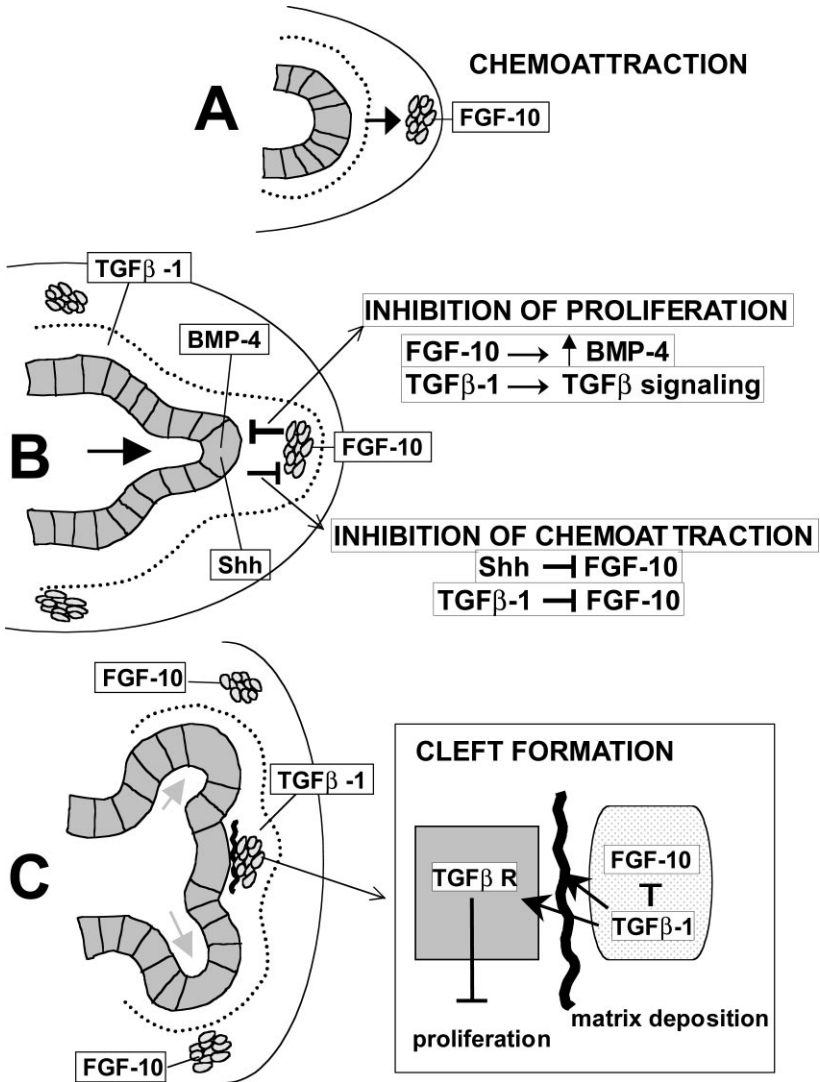


Figure 1 Control of bud formation during branching morphogenesis. Diagram incorporates models proposed by Bellusci and coworkers (6) and Lebeche and coworkers (53). (A) Local expression of FGF-10 in the mesenchyme induces chemo-attraction and epithelial growth. (B) As the bud is induced, FGF-10 is inhibited by Shh expressed at the tips and by TGFβ-1 expressed throughout the subepithelial region. Concomitantly, proliferation is inhibited at the tips by FGF-10-mediated up-regulation of BMP-4. (C) These mechanisms limit bud outgrowth and expansion, resulting in cleft formation. FGF-10-expressing cells appear at other sites to induce a new generation of buds. At the cleft, low levels of FGF-10 are maintained by subepithelial TGFβ-1, which also induces synthesis of extracellular matrix components deposited in the epithelial-mesenchymal interface and prevents local budding (modified from 53).

9–9.5 in the ventral foregut, in the domains of the nascent right and left lung bud (116). Although this suggests a role for BMP-4 in establishing the initial lung field in the foregut mesenchyme, evidence from genetic studies is not currently available.

During branching morphogenesis, BMP-4 is dynamically expressed in the distal epithelium of branching airways. The *BMP-4^{lacZ}* reporter gene mouse shows that when buds are forming, signals appear after bud initiation and levels increase at the tips during the period of bud extension (117). Thus BMP-4 appears to be unnecessary for bud induction. Type I and type II serine-threonine kinase receptors and Smad transcription factors transduce BMP-4 signaling (reviewed in 58). Disruption of BMP-4 signaling in the lung of transgenic mice disrupts the proximal-distal pattern of growth and differentiation of the lung. Mice expressing either a dominant-negative type I BMP receptor (*Alk6*) or a secreted BMP-4 inhibitor *Xenopus noggin* (*Xnoggin*) under the control of the *SP-C* promoter do not properly form distal lung. In these mice, proximal cell types, such as ciliated cells, and expression of the proximal genes *HFH-4*, *CC-10* are ectopically found in the periphery of the lung (116). Conversely, over-expression of *BMP-4* in the distal lung results in small lungs with large distal sacs lined by epithelial cells whose morphology is reminiscent of that of alveolar type I cells (4). The concomitant decrease in the number of type II cells (*SP-C* positive) in these mice led the authors to propose that high levels of BMP-4 stimulate distal lung formation but might preferentially induce alveolar type I cell fate. Conversely, cells located far from the tips and exposed to low levels of BMP-4 assume a proximal character (116). Collectively, the data suggest that BMP-4 is part of a distal signaling center that controls proliferation and regulates proximal-distal differentiation.

Levels of *BMP-4* expression at the tips of the growing epithelial buds are influenced by gradients of *FGF-10* in neighboring mesenchymal cells. Recombinant FGF-10 in beads up-regulates *BMP-4* expression in the distal epithelium and induces ectopic expression of *BMP-4* in proximal epithelial explants (53, 117). Moreover, recombinant BMP-4 inhibits epithelial cell proliferation and prevents budding, thus antagonizing the effect of FGF-10 in epithelial explants (117). Presumably, FGF-10 -BMP-4 interaction serves to limit bud outgrowth during budding (Figure 1).

TGF β -1 TGF β -1 is a member of a subfamily of peptides having at least two other members, all expressed in the developing lung. TGF β signaling is mediated by serine-threonine kinase receptors (type I and II) and Smad transcription factors (reviewed in 58). Activation of TGF β signaling is inhibitory for epithelial branching; expression of a dominant-negative TGF β RII in lung organ cultures stimulates branching morphogenesis (127). In the day 11–12 embryonic lung, *TGF β -1* transcripts are uniformly expressed in the subepithelial mesenchyme. TGF β -1 protein accumulates later at sites of cleft formation and along proximal airways. TGF β -1 promotes synthesis of extracellular matrix that, when deposited in the epithelial-mesenchymal interface, is thought to prevent local branching (36). TGF β -1 has

also been identified as a potent negative regulator of epithelial cell proliferation and differentiation *in vitro* and *in vivo*. Recombinant TGF β -1 inhibits branching morphogenesis in cultured lung explants (97). When TGF β -1 is mis-expressed in the distal lung epithelium of transgenic mice, lungs do not develop beyond the late pseudoglandular period and show decreased levels of SP-C expression. Furthermore, blood vessel formation is impaired in these animals, presumably because of decreased levels of VEGF (129). In contrast, lung abnormalities have not been reported in TGF β -1 knockout mice; it is unclear, however, whether this may have resulted from maternal transfer of this peptide rescuing the phenotype (54).

TGF β -1 may control levels of FGF-10 expression in the developing lung mesenchyme. In the day 11–13 embryonic lung, TGF β -1 signals are present in the subepithelial mesenchyme, a site where FGF-10 is normally not expressed. Furthermore, recombinant TGF β -1 markedly inhibits FGF-10 expression, both in lung embryonic mesenchymal cell and in lung organ cultures (53). Therefore, TGF β -1 potentially exerts its effect on lung morphogenesis by at least three mechanisms: limiting epithelial bud proliferation, inhibiting FGF-10-mediated chemoattraction, and synthesizing extracellular matrix components that stabilize clefts (Figure 1).

Sprouty (Spry) *Spry* genes encode a family of cysteine-rich proteins that antagonize FGF signaling. In *Drosophila*, *Spry* is induced by FGF signaling at the tips of branching tracheal tubules and inhibits lateral budding by a mechanism currently not well understood (34). At least three related murine genes, *mSpry 1*, *2*, and *4*, have been identified in specific cell types of different organs at late stages of development. In vertebrates, not all FGFs induce *Spry* expression, perhaps reflecting differences in sensitivity of these genes to individual FGFs or a requirement for additional cooperative factors (73). *Spry 2* and *Spry 4* are expressed in the developing distal lung in the epithelium and mesenchyme, respectively (18, 108). Antisense oligonucleotide inactivation of *Spry 2* has an stimulatory effect on distal branching and differentiation in organ cultures (108).

FGFs Studies in other developing structures such as the limb bud have shown that FGFs regulate expression of other FGF family members (reviewed in 57). The possibility that this may also be true in the lung is suggested by the sequential pattern of induction of individual FGFs during lung development and by data from FGF-treated lung cultures (see below).

FGF-9 and FGF-10 are the first of all FGFs to be detected in the embryonic lung by *in situ* hybridization (6, 17). At day 9.5–10 lung, FGF-9 is expressed in pleural and epithelial cells. At around day 11.5, low levels of FGF-7 and FGF-2 transcripts are detected by RT-PCR; however, transcripts are found in the mesenchyme by *in situ* hybridization only by day 13–14. At this stage, when expression of these FGFs is well established, FGF-1 signals first appear in the lung epithelium and mesenchyme (6, 53, 85). In embryonic lung mesenchymal cell and organ cultures, treatment with recombinant FGF-1 or FGF-2 has been shown to up-regulate FGF-7 gene expression, whereas recombinant FGF-7 up-regulates FGF-1. Based

on these observations, it has been proposed that at early stages, FGF-2 induces *FGF-7*, which in turn induces *FGF-1*; at later stages, FGF-1 may maintain *FGF-7* expression (53). *FGF-10* expression does not seem to be influenced by FGF-1, 2, or 7. Although it remains to be demonstrated, FGF-9 has been suggested as a potential regulator of *FGF-10* expression, via activation of FGFR-1 signaling in the early lung (2).

RETINOIC ACID SIGNALING AND LUNG MORPHOGENESIS

Retinoids are fundamental for normal development and homeostasis of a number of biological systems including the lung. RA (all-*trans*, 9-*cis*, 13-*cis*) is considered to be the active form of the retinoids in most systems. RA results from sequential oxidation of vitamin A from retinol to retinaldehyde and to the acid form (reviewed in 13). Among the several enzymes involved in this pathway, retinaldehyde dehydrogenase-2 (RALDH-2) plays a prominent role in generating RA during organogenesis (79, 80, 115, 128). Expression of *RALDH-2* is developmentally regulated and represents one of the mechanisms that controls RA availability to target cells. RA metabolism represents another way of regulating ligand availability by generating products that are inactive or that differentially affect morphogenesis or regeneration. P450RAI (CYP26) is an RA-inducible, RA-metabolizing enzyme of the cytochrome P450 family, which specifically converts RA into hydroxylated products (26, 121). P450RAI can serve as a mechanism to protect RA-sensitive tissues from high RA levels (42, 76). Recently, another related metabolizing enzyme, P450RAI-2, has been identified in adult human tissues, with highest levels in cerebellum (122).

RA signaling is mediated by nuclear receptors from the steroid hormone superfamily RAR (α , β , γ) and retinoid RXR (α , β , γ), each having multiple isoforms (13). RAR/RXR heterodimers have been shown to transduce RA signaling in vivo (46). These receptors are widely expressed in the embryo, and their genetic inactivation in mice results in abnormalities that resemble those found in vitamin A-deficient animals (65, 123). RA signaling is modulated by a variety of factors that serve as coactivators or corepressors (13). In some systems, nuclear factors such as COUP-TFs (chicken ovalbumin upstream promoter-transcription factors) interfere with RA-mediated transactivation by mechanisms that include sequestration of RXRs (reviewed 113). Retinoid-binding proteins (CRBPs and CRABPs) have been also identified in the embryo, but they do not seem to play a role in morphogenesis, as assessed by genetic studies (29, 51).

RA in the Embryonic Lung

Despite the large number of studies showing teratogenic effects of excess or deficiency of vitamin A during organogenesis, the role of retinoids in lung development

is still not clearly defined. There is evidence that RA signaling is required for lung bud initiation. Acute vitamin A deprivation in pregnant rats at the onset of lung development results in blunt-end tracheae and lung agenesis in some embryos, a phenotype similar to that described in *FGF-10*^{-/-} mice (20, 96). Day 8.0 whole embryos cultured in the presence of a pan-RAR antagonist show disruption of lung bud formation (73a). Furthermore, disruption of RA signaling in *RAR α/β 2* knockout mice leads to agenesis of the left lung and right lung hypoplasia (65).

A recent analysis of the ontogeny of the RA pathway in the embryonic mouse lung shows that, when primary buds are forming (day 9.5), RA signaling is locally active. As suggested by *RALDH-2* expression, RA is synthesized by pleural and tracheal mesenchymal cells (56). Sites where RARs are being activated can be mapped using a reporter mouse carrying a RA-responsive element-driven *lacZ* (*RARE-lacZ*) transgene (95). Ubiquitous distribution of *RARE-lacZ* signals in the lung primordia suggests that RARs are activated in all layers (56).

Interestingly, subsequent branching morphogenesis is characterized by a dramatic down-regulation of RA signaling in the lung. There is evidence that despite continued expression of *RARs* and *RALDH-2*, *RARE-lacZ* signals are not detected in the epithelium and in most of the mesenchyme (21, 56). This appears to result from activation of mechanisms that antagonize RA signaling, such as increased RA degradation in the epithelium via P450RAI-mediated metabolism and inhibition of RA signaling in the mesenchyme presumably by COUPTF-II expression (56) (Figure 2). The efficiency of these mechanisms may be critical for distal lung morphogenesis. Preventing down-regulation of RA signaling by treating embryonic lung explants with high concentrations of RA (10^{-6} – 10^{-5} M) results in dramatic disruption of distal budding and formation of proximal-like immature airways (10, 56). In these cultures, RA inhibits expression and alters distribution of *FGF-10* and *BMP-4*, pattern-related genes that are involved in distal lung morphogenesis. It is also noteworthy that during early stages of branching (day 11–12.5), *RALDH-2* expression is concentrated in trachea (mesenchyme) and proximal lung (mesothelium) at sites of low branching activity. The *RALDH-2* pattern is non-overlapping with that of *FGF-10*, supporting the idea that RA signaling restricts *FGF-10* expression and may have to be inhibited to allow proper distal lung morphogenesis.

The mechanism involved in RA-induced inhibition of *FGF-10* may be mediated by up-regulation of Shh, although there are data suggesting a Shh-independent pathway (6, 11, 56). The RA effect on pattern formation appears to involve signaling by *RAR β* ; the inhibitory effect of exogenous RA on distal bud formation in vitro is reduced in lungs of *RAR β* knockout mice. RA also alters expression of *Hox* genes in the lung (7, 11, 84), but how these changes influence the lung phenotype remains to be determined. Hox proteins regulate anterior-posterior specification of the body axis during development. Several Hox genes are expressed in the lung (reviewed in 9, 45); however, to date, only *Hoxa-5* has shown a lung phenotype (laryngotracheal malformation and lung immaturity) when inactivated in mutant mice (3).

Reports on the effects of RA on surfactant protein gene expression have been conflicting, possibly because of differences in methodologies or choice of species in the studies. For instance, in one study in cultured rat explants in which all-*trans*

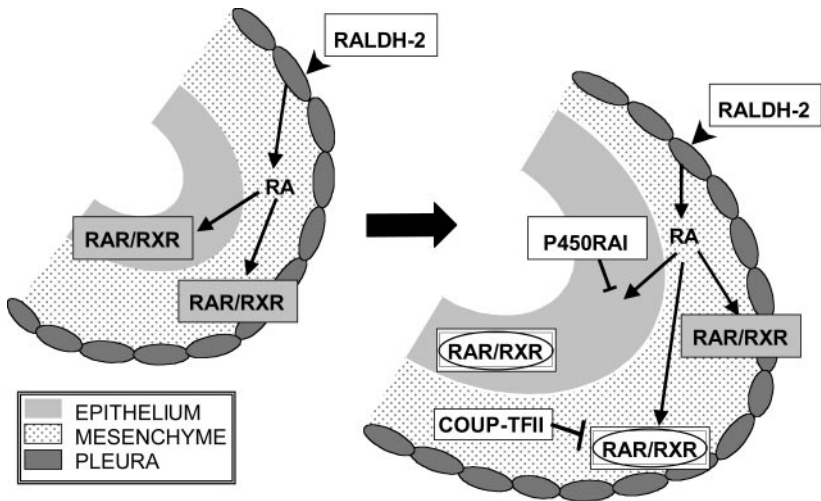


Figure 2 Regulation of RA signaling at the onset of lung development (*left*) and during branching morphogenesis (*right*) based on data from Malpel and coworkers (56). At an initial stage, mesothelial cells expressing RALDH-2 synthesize RA, which diffuses and activates RA signaling ubiquitously (RAR/RXR in gray boxes represents activated RA signaling; RAR/RXR encircled in white boxes represents suppressed RA signaling). During branching, RA signaling is suppressed in the epithelium by P450RAI-mediated RA metabolism and by COUP-TFII inhibition of RAR/RXR activation of target genes (modified from 56).

RA disrupted distal lung formation, *SP-A*, *-B*, and *-C* genes were down-regulated (10). In another study using human fetal lung explants, *SP-A* and *-C* were also down-regulated, but *SP-B* was up-regulated (69).

At late gestation, although *RALDH-2* continues to be expressed in the pleura, data from *RARE-lacZ* mice show little evidence of RA utilization in the lung (56). Whether this implies that RA signaling does not play a role in late developmental events remains to be determined. Paradoxically, data from fetal rat lungs suggest that there is an increase in retinoid availability toward birth. A lipid-rich mesenchymal cell (lipofibroblast or lipid interstitial cell) containing high levels of retinyl esters, the storage form of retinoids, has been identified in embryonic rat lungs (64). Biochemical analysis shows that from late gestation to early postnatal life there is a decline in the retinyl ester content of these cells that coincides with an increase in the levels of retinol and RA (63, 64). Lung lipofibroblasts have been also reported in other species, including humans, hamsters, and mice (44).

RA in Alveolar Formation

The process by which saccules transform into alveolar units involves formation of secondary septae from the pre-existing walls and represents the final major morphogenetic event of the developing lung (126). Some data suggest that RA signaling is involved in alveolization. Low plasma levels of vitamin A have been

reported in premature neonates who develop bronchopulmonary dysplasia, a condition in which alveolization is impaired (100). RA treatment of neonatal rats during the period of alveolization increases the number of alveoli and partially rescues a block in alveolar formation induced by dexamethasone (59, 61). In adult rats, RA has been reported to reverse the anatomical features of elastase-induced emphysema (60). Furthermore, RA treatment also partially rescues the lung defect of the adult *Tight-skin* mouse, a mutant bearing a tandem duplication within the *fibrillin-1* gene with impaired alveolization (103). The mechanisms by which RA exert these effects are still poorly understood.

DEVELOPMENT OF PROXIMAL AIRWAYS

In mice, the primitive trachea emerges once primary lung buds form and the tracheo-esophageal septum separates the digestive tract from the respiratory system (104). The origin and role of this septum in tracheal development is controversial. (125). There is evidence that trachea and lung require different signals to form and that during morphogenesis they can develop independently from each other. This is best exemplified in *FGF-10*^{-/-} mice, in which tracheal morphogenesis and differentiation occur normally, but primary lung buds never form (71, 96).

Proximal epithelial features of differentiation include those of ciliated, mucous, serous, and Clara cells. At around embryonic day 14.5, expression of *HFH-4* (HNF3/forkhead homologue-4) in areas that do not overlap with *SP-C* domains represents initial evidence of proximal epithelial differentiation (33). By day 16, proximal airways also start expressing *Clara cell 10-kDa protein (CC10)* mRNA, which marks non-mucous and non-serous secretory cells from trachea to terminal bronchioles (91, 102, 120). Development of ciliated cells is regulated by *HFH-4*; in the absence of this transcription factor, cilia are not formed in any cell type, and *HFH-4*^{-/-} mice display random determination of L-R asymmetry of internal organs (15). Conversely, targeted expression of high levels of *HFH-4* to the distal epithelium of transgenic mice results in the ectopic appearance of ciliated cells, *β-tubulin IV* mRNA, and suppression *SP-B* and *SP-C* signals (111). Also, transgenic lungs do not express the proximal marker *CC10*. It has been proposed that *HFH-4* may be part of a mechanism that restricts Clara cell and distal alveolar development and promotes columnar and ciliated cell differentiation.

An important developmental aspect of proximal respiratory tract is the appearance of submucosal glands in the trachea and in bronchi of some species. These structures are formed by mucous, serous, and ciliated cells and produce the secretions that line the airway lumen. In humans, they develop mostly during intrauterine life. Nevertheless, pluripotent progenitor cells have been identified in the respiratory epithelium of adults with the capacity to recapitulate gland development (25). Submucosal glands originate from invagination of the airway epithelium into the interstitium (submucosa), where buds subsequently undergo lateral expansion (110). Invasion of the mesenchyme by the epithelial bud is mediated by epidermal

growth factor (EGF) and $TGF\alpha$ signaling and is inhibited by $TGF\beta$ -1, presumably by stimulation of matrix synthesis and deposition at the epithelial-mesenchymal interface (27, 36). The transcription factor LEF1 (lymphoid enhancer binding factor 1) is found in the epithelium of gland progenitor cells and is essential for gland formation. Although gland development in *LEF-1* knockout mice is impaired, transgenic mice overexpressing *LEF-1* show no significant effect in airways, suggesting that additional factors may be required (23). These observations and the fact that LEF/TCF is a downstream component of the Wnt pathway (39) possibly implicate Wnt signaling in proximal airway development.

CONCLUDING REMARKS

Several important issues such as the role of extracellular matrix components in lung morphogenesis, regulation of epithelial differentiation (surfactant protein and airway secretory product expression), and embryonic lung neovascularization have not been addressed in this review. A dedicated chapter could be written for each of them.

Despite the fact that many of the classical questions about regulation of lung morphogenesis remain unanswered, increasing information from genetic studies and in vitro systems is enlightening. The identification of signaling molecules that regulate pattern formation in species such as *Drosophila* and the evolutionary conservation of their function have helped characterize the molecular mechanisms of the epithelial-mesenchymal interactions in the developing lung. Nothing is known about how territories of the primitive foregut endoderm are demarcated and fated to become lung. The observation that branching patterns are specified before the onset of lung development and that inactivation of a single gene such as *Lefty-1* can modify these programs raises the question about how hierarchies are established within the regulatory network of genes that governs lung pattern formation.

Variable levels of *FGFs*, *Shh*, *TGF β* , *EGF*, retinoid receptors, and other signals that play a role in lung morphogenesis have been reported in the adult lung (8, 24, 28, 87, 94, 124). It is unclear whether and to what extent these regulators are recruited during adult life to mediate cellular activities in processes such as post-injury repair, compensatory lung growth, or asthma. Recent studies implicate EGF and $TGF\beta$ signaling as mediators of epithelial-mesenchymal interactions during airway remodeling in asthma (reviewed in 37). Exploring these areas will open an array of possibilities to understand the role of these signaling molecules in the above processes and to approach new therapeutic strategies in lung disease.

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