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Molecular Mechanisms of Early Lung Specification and Branching Morphogenesis

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ABSTRACT

The "hard wiring" encoded within the genome that determines the emergence of the laryngotracheal groove and subsequently early lung branching morphogenesis is mediated by finely regulated, interactive growth factor signaling mechanisms that determine the automaticity of branching, interbranch length, stereotypy of branching, left-right asymmetry, and finally gas diffusion surface area. The extracellular matrix is an important regulator as well as a target for growth factor signaling in lung branching morphogenesis and alveolarization. Coordination not only of epithelial but also endothelial branching morphogenesis determines bronchial branching and the eventual alveolar-capillary interface. Improved prospects for lung protection, repair, regeneration, and engineering will depend on more detailed understanding of these processes. Herein, we concisely review the functionally integrated morphogenetic signaling network comprising the critical bone morphogenetic protein, fibroblast growth factor, Sonic hedgehog, transforming growth factor-β, vascular endothelial growth factor, and Wnt signaling pathways that specify and drive early embryonic lung morphogenesis. (***Pediatr Res* 57: 26R–37R, 2005**)

Abbreviations
BMP, bone morphogenetic protein
DKK, Dickkopf
EGF (R), epidermal growth factor (receptor)
ERK, extracellular regulated kinase
FGF (R), fibroblast growth factor (receptor)
FN, fibronectin
LRP, lipoprotein receptor-related proteins
MAP, membrane-associated protein
PDGF, platelet-derived growth factor
RAR, retinoic acid receptor
sFRP, secreted Frizzled-related protein
SHH, Sonic hedgehog
Sp-C, surfactant protein C
TGF-α (β), transforming growth factor alpha (beta)
VEGF (R), vascular endothelial growth factor (receptor)

THE 70 M2 ALVEOLAR SURFACE ORIGINATES FROM THE LARYNGOTRACHEAL GROOVE

Lung development begins with the appearance of the laryngotracheal groove, which is a small diverticulum that arises from the floor of the primitive pharynx at E9 in mouse and 4 wk in human. The proximal portion of the laryngotracheal groove gives rise to the larynx and trachea, whereas the distal portion gives rise to the left and right mainstem bronchial buds, which in turn give rise to the left and right lobar branches of the bronchial tree. In mice, four lobes form in the right lung, whereas the left lung forms a single lobe. In humans, the left lung is trilobed, whereas the right lung is bilobed. Lobation of the lungs, as well as the first 16 of 23 airway generations in humans is stereotypic, which implies the presence of a hard-wired, genetic program that controls early embryonic lung branching morphogenesis. The latter seven generations of lower airway branching and onward into the folding and expansion phase of the alveolar surface is nonstereotypic, but nevertheless follows a recognizable, proximal-distal fractal pattern that is repeated automatically at least 50 million times. The lung morphogenetic program thus drives the formation of an alveolar gas diffusion surface 0.1 μm thick by 70 m^2 in surface area that is perfectly matched to the alveolar capillary

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and lymphatic vasculature. This huge gas exchange surface area is packed into the chest in a highly spatially efficient manner (1,2).

SOLUBLE FACTORS ARISING FROM THE MESENCHYME DETERMINE LUNG EPITHELIAL MORPHOGENESIS

Soluble factors arising from the mesenchyme have long been implicated in lung branching morphogenesis distal to the trachea, since it was discovered that transposition of early stage distal lung mesenchyme onto the primitve tracheal epithelium can induce supernumary branching (3–7) as well as distal epithelial marker gene expression (8–10). Null mutation in mice of *Fgf10*, or its cognate receptor *Fgfr2*, as well as misexpression of dominant negative kinase dead FGFR, or of soluble FGFR, all display a spectrum of closely related tracheobronchial truncation phenotypes, in which epithelial, endothelial, and lymphatic branching of the bronchi distal to the trachea is abrogated (11–15). This strongly suggests that the genetic controls of laryngotracheal groove formation are separate from those that induce the peripheral lung. Herein, we review the little that is known about the molecular signals that induce the formation of the laryngotracheal groove. Then, we consider the rather more extensive information on some of the key molecular signaling mechanisms that mediate branching of the airways during early lung development.

THE LARYNX AND TRACHEA ARE GENETICALLY DISTINGUISHABLE FROM THE DISTAL AIRWAYS

Null mutation of *Hnf3β* results in failure of closure of the primitive foregut, thus no laryngotracheal bud can form (16). *Hoxa3* and *5* also play key roles in specifying the larynx and trachea (17–20). Retinoic acid deficiency in mice has long been associated with total agenesis of the lungs, in association with other complex anomalies (21,22). Certain RAR null mutations are also among the few known causes of complete laryngotracheo-pulmonary agenesis in mice (22). The other known null mutations associated with complete absence of the larynx, trachea, and lung being the double *Gli2/3* null mutation (23). Recently, it has been shown that retinoic acid induces *Fgf10* expression within the mesoderm subjacent to the site of origin of the laryngotracheal groove (24). Blockade of RAR signaling in E9 foregut cultures not only blocks the local expression of *Fgf10* at this restricted site but also disrupts expression of *Nkx2.1* and *Sp-C* in the floor of the primitive pharynx. Thus, retinoic acid signaling may connect formation of the larnygotracheal groove with activation of *Fgf10*-dependent bronchial morphogenesis. Further evidence for distinctly separate embryonic cell lineages that form the trachea and proximal bronchi *versus* the peripheral lung was provided by tetracycline driven *Sp-C-Cre* recombination, which activated lineage marker expression within the peripheral lung but not the trachea, upper bronchi, or gastrointestinal tract (25).

LEFTY-1, *LEFTY-2*, AND *NODAL* ARE IMPLICATED IN DETERMINATION OF LEFT-RIGHT LATERALITY OF THE LUNG

Lefty-1 null mutant mice show a variety of left-right isomerisms in visceral organs, but the most common feature is thoracic left isomerism. The lack of *Lefty-1* expression results in abnormal bilateral expression of *Nodal*, *Lefty-2*, and *Pitx2* (a homeobox gene normally expressed on the left side). This suggests that Lefty-1 normally restricts *Lefty-2* and *Nodal* expression to the left side, and that *Lefty-2, Pitx2,* and/or *Nodal* encode a signal for "leftness" in the lung (26,27). We speculate that proximal-distal, anteroposterior, and left-right stereotypy must be superimposed on the automatic morphogenetic branching mechanisms proposed below.

TRACHEOESOPHAGEAL SEPTATION

Septation of the trachea from the esophagus is normal in the *Fgf10* null mutation, indicating that this process can also be distinguished genetically from peripheral lung branching. However, null mutations of the transcriptional factor *Nkx2.1* and of *Shh* display severe anomalies of larngyo-tracheal-esophageal septation, together with abrogation of distal lung morphogenesis (28–31). Haploinsufficency of *Foxf1*, which is itself a SHH target, causes esophageal atresia and tracheoesophageal fistula (32). SHH also plays a key role in determining correct patterning of the tracheal cartilages, inasmuch as peripheral expression of *Shh* under the control of the *Sp-C* promoter fails to rescue tracheal cartilage morphogenesis on the *Shh* null background (33). SHH also regulates *Gli3* gene expression (34). Laryngeal clefts, tracheal atresias, and esophageal atresias, with or without tracheoesophageal fistula, occur quite frequently in humans. Clinical associations with multiple other anomalies, including the VACTERL association, duodenal atresia and pancreatic malformations, have been noted. In the Pallister-Hall syndrome, among other anomalies, bifid epiglottis and cleft larynx have been described as an effect of Gli3 mutation (34). Defects in the Shh-Gli pathway as well as the FGF pathway have also been noted in the adriamycin-mediated teratogenic model of tracheoesophageal anomalies (35–38). *Hox* family genes are also likely to play a key role in tracheobronchial differentiation, since in *Hoxa-5*$^{-/-}$ mice, tracheal occlusion and respiratory distress are associated with marked decrease in surfactant protein production, together with altered gene expression in the pulmonary epithelium (19). Because *Hoxa-5* expression is restricted to the lung mesenchyme, the null mutant phenotype strongly supports the inference that *Hoxa-5* expression is necessary for induction of epithelial gene expression by the underlying mesenchyme (39,40).

BMP, EGF, FGF, SHH, TGF-β, VEGF, AND WNT SIGNALING PATHWAYS ALL PLAY CRITICAL ROLES TO DRIVE EARLY EMBRYONIC DISTAL LUNG BRANCHING MORPHOGENESIS

EGF, FGF, and PDGF signal through cognate transmembrane tyrosine kinase receptors to exert a positive effect on lung morphogenesis. In contrast, TGF-β and BMP family

peptides signal through transmembrane serine-threonine kinase receptors. In general, TGF-β family peptides exert an overriding inhibitory effect on lung epithelial cell proliferation and hence negatively regulate lung morphogenesis. However, BMP4 appears to exert a complex negative or positive regulatory influence, depending on whether mesenchymal signaling is intact. SHH family peptide signaling represents another key mechanism. The SHH cognate receptor, patched (PTC), exerts a negative effect on SHH signaling both through the release of the transcriptional repressor Smoothened (SMO) and the induction of the *Hedgehog interacting protein* (*Hip*). Recently Wnt signaling has also emerged as exerting a critical influence on matrix fibronectin deposition and hence the specification of branch points. Also, the VEGF pathway has emerged as playing a critical role in early lung morphogenesis. These peptide growth factors and their cognate receptors are expressed in repeating patterns within morphogenetic centers that surround and direct each new branch tip in the early embryonic lung. Important mesenchymally expressed morphogenetic genes include *Fgf10, Sprouty4 (Spry4), patched, smoothened, Wnt,* and *Hox* family members, whereas *Bmp4, Shh, mSpry2, Smads 2, 3,* and *4*, and VEGF are expressed in the adjacent epithelium. The interactions of subsets of these ligand signals, particularly SHH, BMP4, and FGF10, have been extensively reviewed recently and several models have been proposed to explain how they may interact to induce and then regulate epithelial branching morphogenesis (1–3,5,41).

BMP PLAY MULTIPLE ROLES IN LUNG DEVELOPMENT

BMP4 is expressed predominantly in the epithelium and is increased at branch tips. Until recently, BMP4 was postulated to mediate localized suppression of epithelial proliferation, thus providing a negative modulatory influence on FGF signaling to mediate arrest of branch extension and hence to set up branch points. This hypothesis was based upon the hypoplastic phenotype of the epithelium in transgenic misexpression studies of *Bmp4* in the epithelium, as well as upon addition of BMP4 ligand to naked epithelial explants in culture. However, two groups have now shown that BMP4 is actually a potent stimulator of branching in the presence of mesenchyme and at physiologic concentrations in lung explants. Moreover, the effects of BMP4 are in turn negatively modulated by the BMP binding proteins Gremlin and Noggin. Abrogation of Gremlin function results in abnormal lung formation in neonatal Gremlin null mutant mice (42). Therefore, it seems unlikely that BMP4 signaling merely serves to inhibit epithelial proliferation, particularly since BMP4-specific Smads are also expressed in the mesenchyme away from the epithelium at certain developmental stages. BMP have also been reported to control differentiation of the endoderm along the proximal-distal axis (43). Inhibition of BMP signaling at the tip of the lung bud by overexpression in the distal epithelium of Noggin (a secreted inhibitor of BMP) or of a dominant negative form of the Bmp type I receptor, activin receptor-like kinase 6 (Alk6), results in a distal epithelium exhibiting differentiation characteristics, at the molecular and cellular level, of the proximal epithelium. Several additional BMP, including BMP3, 5, and 7, are expressed during embryonic lung development (44–46). The expression of *Bmp5* and *Bmp7* has been detected in the mesenchyme and the endoderm of the developing embryonic lung respectively, whereas *Bmp4* expression is restricted to the distal epithelial cells and the adjacent mesenchyme (44,46). Recently, parallels have been drawn between genetic hard wiring of tracheal morphogenesis in *Drosophila melanogaster* and mammals (1). Dpp, the *Drosophila* BMP4 orthologue, has been reported to be essential for the formation of the dorsal and ventral branches of the tracheal system, controlling tracheal branching and outgrowth possibly through induction of the zinc finger proteins *Kni* and *Knrl* (47,48). Because conventional murine knockouts for BMP4 and BMP-specific *Smads* cause early embryonic lethality, their functions in lung development *in vivo* still need to be further defined. Interestingly, germ line mutations in BMP type II receptors were recently found in familial primary pulmonary hypertension (49).

EGFR NULL MUTATION REDUCES BRANCHING BY 50%

EGF is expressed in the distal epithelium and mesenchyme. Null mutation of *Egfr* results in a 50% reduction in branching and a neonatal lethal failure of lung maturation (50,51). EGF was the first growth factor to be shown to exert an inductive role on chick trachea to induce ectopic branching (52), as well as to accelerate branching through activation of its cognate EGFR tyrosine kinase in mouse embryonic lung in culture (53,54). In addition, null mutation of TNF-α converting enzyme (TACE), a cell surface protein sheddase that regulates release of active from latent forms of TGF-α, amphiregulin, neuregulin-1, HB-EGF, and HerbB4, also causes a hypoplastic phenotype of lung in the perinatal stage and lethality in new born mice (55).

FGF LIGANDS AND RECEPTORS DIRECT EPITHELIAL MIGRATION AND DIFFERENTIATION

FGF10 promotes directed growth of the lung epithelium and induces both proliferation and chemotaxis of isolated endoderm (56,57). The chemotaxis response of the lung endoderm to FGF10 involves the coordinated movement of an entire epithelial tip, containing hundreds of cells, toward an FGF10 source. How this population of cells monitors the FGF gradient and which receptors trigger this effect remains unknown. FGF10 also controls the differentiation of the epithelium by inducing SP-C expression and by up-regulating the expression of BMP4, a known regulator of lung epithelial differentiation (44,57–59). *In vitro* binding assays have shown that FGF10 acts mostly through FGFR1b and FGFR2b (60). Although there is good evidence that FGF10 acts through FGFR2b *in vivo*, there are as yet no conclusive data involving FGFR1b (or any other receptor) *in vivo*. The biologic activities mediated through these two epithelial receptors are likely to be different, as FGF7 (acting mostly through FGFR2b) exhibits a different activity compared with FGF10 (56). This hypothesis is also supported by our recent unpublished findings showing

that *Fgf10*$^{-/-}$ lungs exhibit a more severe phenotype than *Fgfr2b*$^{-/-}$ lungs.

THE MAMMALIAN FGF RECEPTOR FAMILY COMPRISES FOUR GENES (FGFR1 TO FGFR4), WHICH ENCODE AT LEAST SEVEN PROTOTYPE RECEPTORS

Fgfr1, *2*, and *3* encode two receptor isoforms (termed IIIb or IIIc) that are generated by alternative splicing, and each binds a specific repertoire of FGF ligands (60). FGFR2-IIIb (FGFR2b) is found mainly in epithelia and binds four known ligands (FGF1, FGF3, FGF7, and FGF10), which are primarily expressed in mesenchymal cells. Peters *et al.* (61) reported the first evidence of a key role for *Fgfr2* during lung development. They showed that misexpression of a dominant negative form of *Fgfr2* in the embryonic lung under the *SP-C* promoter led to a severe reduction in branching morphogenesis. Further evidence came from *Fgfr2* inactivation in the embryo. Whereas mice null for the *Fgfr2* gene die early during embryogenesis, those that are null for the *Fgfr2b* isoform, but retain *Fgfr2c,* survive to birth (62–65). Mice deficient for *Fgfr2b* show agenesis and dysgenesis of multiple organs, including the lungs, indicating that signaling through this receptor is critical for mesenchymal-epithelial interactions during early organogenesis. This idea is supported by the recent finding that prenatally induced misexpression of a dominant negative FGFR, to abrogate FGF signaling, results in a hypoplastic, emphysematous lung phenotype (66). In contrast, induced abrogation of FGF signaling postnatally did not produce any recognizable phenotype.

THE FGF LIGAND FAMILY IS COMPRISED OF AT LEAST 23 MEMBERS

Many FGF have been implicated in multiple aspects of vertebrate development (for review, see Ref. 67). FGF 1, 2, 7, 9, 10, and 18 play overlapping, yet distinct roles in the lung. In particular, FGF10 has been associated with instructive mesenchymal-epithelial interactions, such as those that occur during branching morphogenesis, whereas FGF9 and 18 appear to play a role in the mesenchyme and FGF1 and 7 appear to play roles in mediating postnatal lung repair (68). In the developing lung, *Fgf10* is expressed in the distal mesenchyme at sites where prospective epithelial buds will appear. Moreover, its dynamic pattern of expression and its ability to induce epithelial expansion and budding in organ cultures have led to the hypothesis that FGF10 governs the directional outgrowth of lung buds during branching morphogenesis (56). Furthermore, FGF10 was shown to induce chemotaxis of the distal lung epithelium (57,69). Consistent with these observations, mice deficient for *Fgf10* show multiple organ defects including lung agenesis (70–73). FGF10 is the main ligand for FGFR2b during the embryonic phase of development as evidenced by the remarkable similarity of phenotypes exhibited by embryos where these genes have been inactivated (64,73,74). Thus, the paradigm proposed so far is that FGF10 expressed by the mesenchyme acts on the epithelium (which expresses FGFR1b and 2b).

CONSERVED FUNCTIONS OF FGF SIGNALING IN *DROSOPHILA* TRACHEA MORPHOGENESIS

In *D. melanogaster*, Branchless (bnl), the *Drosophila* counterpart of FGF10, has been involved in the directional growth of the ectoderm-derived cells from the tracheal placode (75). Bnl expressed by the cells surrounding the placode acts on the ectoderm expressing the *Fgfr2b* ortholog, *breathless* (*btl*). An additional unsuspected function of bnl in the development of the male genital imaginal disc has been recently reported (76). Here, FGF signal expressed by ectoderm-derived cells of the male genital disc induces the FGFR-expressing mesodermal cells to migrate into the male disc. These mesodermal cells also undergo a mesenchymal to epithelial transition. The authors suggest that bnl, the FGF10 ortholog, is likely to be involved in this process. Thus, FGF10 is a multifunctional growth factor and additional roles for FGF10 in lung development likely remain to be identified.

SPROUTY FAMILY MEMBERS FUNCTION AS INDUCIBLE NEGATIVE REGULATORS OF FGF SIGNALING IN LUNG DEVELOPMENT

The first example of an FGF-inducible signaling antagonist arose from the discovery of the *sprouty* mutant during *Drosophila* trachea development, in which supernumerary tracheal sprouts arise. In the *Drosophila* tracheae, bnl binds to btl, inducing primary, secondary, and terminal branching. The function of bnl is inhibited by Sprouty (Spry), a downstream effector in the bnl pathway (77). Spry feeds back negatively on bnl, thereby limiting the number of sites at which new secondary tracheal buds form. Spry is not only found downstream in the FGFR pathway, but also appears to be an inhibitor of other tyrosine kinase signaling pathways such as EGF and Torso (78). *mSpry2* is the gene that is most closely related to *Drosophila Spry* and is 97% homologous to *hSpry2*. *mSpry2* is localized to the distal tips of the embryonic lung epithelial branches and is down-regulated at sites of new bud formation (74). On the other hand, *mSpry4* is predominantly expressed throughout the distal mesenchyme of the embryonic lung. Abrogation of *mSpry2* expression stimulates murine lung branching morphogenesis and increased expression of specific lung epithelial maturation/differentiation markers (58). Conversely, overexpression of *mSpry2* under the control of a *SP*-C promoter or by intratracheal microinjection of an adenovirus containing the *mSpry2* cDNA, results in smaller lungs with a particular "moth-eaten" dysplastic appearance along the edges of the lobes, with decreased epithelial cell proliferation (74). Thus, not only is the function of *Spry* conserved during respiratory organogenesis, but also as seen by loss of function and gain of function studies, *Spry* plays a vital role in regulating lung branching morphogenesis. In *Drosophila*, *in vitro* coprecipitation studies show that Spry binds to Gap1 and Drk (a Grb2 orthologue), resulting in inhibition of the Ras-MAPK pathway (77). Upon further investigation of the mechanism by which mSPRY2 negatively regulates FGF10 in mouse lung epithelial cells (MLE15), we recently determined that mSPRY2 differentially binds to FGF downstream effector complexes (78).

FGFR ARE DIFFERENT FROM OTHER TYROSINE KINASE RECEPTORS IN THAT THEY REQUIRE ADAPTER OR DOCKING PROTEINS

These adapter or docking proteins include phospholipase C γ, Shc, FRS2, and various others to recruit the Grb2/Sos complex upon stimulation. Stimulation of FGFR not only results in formation of the FRS2/Grb2/Sos complex, but the binding of a positive tyrosine phosphatase regulator, Shp2, to FRS2, which is required for full potentiation of MAP-kinase activation (79). Complex formation leads to catalysis of GDP to GTP on Ras, which is required for Raf (serine/threonine kinase) activation. Raf causes direct activation of ERK, leading to phosphorylation of cytoplasmic proteins followed by cell growth and differentiation (80). We found in unpublished studies that in the native state mSPRY2 associates with Shp2 and Gap, which is a GTPase-activating protein that hydrolyzes GTP to GDP. It is possible that in this state the binding of Shp2 to mSPRY2 regulates mSPRY2 activity. Upon FGFR activation, mSPRY2 disassociates from Shp2 and Gap and translocates to the plasma membrane, where it binds to both FRS2 and Grb2, thus blocking the formation of the FRS2/Grb2/Sos complex, resulting in a net reduction of MAP-kinase activation. Thus, Sprouty would inhibit the formation of specific signaling complexes downstream tyrosine-kinase receptors resulting in modulation and co-ordination of cell growth and development during organogenesis.

DYNAMIC RELATIONSHIP BETWEEN FGF SIGNALING AND SPRY DURING DEVELOPMENT

It is interesting to note that overexpression of *Spry* in chick limb buds results in a reduction in limb bud outgrowth that is consistent with a decrease in FGF signaling (81). This suggests a possible co-regulatory relationship between FGF signaling and Spry during development. In further support of this model, *Spry4* inhibits branching of endothelial cells as well as sprouting of small vessels in cultured mouse embryos. Endothelial cell proliferation and differentiation in response to FGF and VEGF are also inhibited by *mSpry4*, which acts by repressing ERK activation. Thus, Spry4 may negatively regulate angiogenesis (82).

SPRY2, SPRY4, AND SPRED SHARE COMMON INHIBITORY MECHANISMS

It has been suggested that both Spry2 and Spry4 share a common inhibitory mechanism. Both Sprouty translocate to membrane ruffles upon EGF stimulation. However, only SPRY2 was shown to associate with microtubules (83). The C-terminus of hSPRY2 has been shown to be important for modulation of cellular migration, proliferation, and membrane co-localization (83,84). Interestingly, the C-terminus is the region that is most conserved throughout the Spry family and contains potential regulatory sites that would modulate Spry activity. Spry has also been shown to interact with c-Cbl, resulting in increased EGFR internalization (85). Although Spry is not a specific inhibitor to the FGFR signaling pathway nor to respiratory organogenesis, it appears that Spry plays a vital role in modulating several signaling pathways to limit the effects of excessive growth factor receptor tyrosine kinase signaling. The Sprouty-related *Spred* family of genes are also expressed in the developing lung, however, their function is less clear (86). Nevertheless, SPRED can inhibit cell motility and Rho-mediated actin reorganization as well as growth factor–induced ERK activation (86–89) and therefore may function in a similar fashion to Sprouty to negatively modulate key aspects of lung morphogenesis.

PROTEOGLYCANS

Proteoglycans are deposited within the extracellular matrix during early embryonic lung branching morphogenesis and inhibition of proteoglycan synthesis or treatment with heparitinase severely affects branching (90–92). More recently, it has been shown that both heparan sulfate and chondroitin sulfate proteoglycans are required for lung branching and in fact mediate the inductive effects of FGF10 binding to the epithelium (93–95).

SHH, PATCHED, AND HIP

The role of SHH signaling in lung morphogenesis has recently been reviewed (96). Hedgehog signaling is essential for lung morphogenesis because *Shh* null produces profound hypoplasia of the lungs and failure of tracheoesophageal septation (30,97). However, proximodistal differentiation of the endoderm is preserved in the *Shh* null mutant, at least in so far as expression of *surfactant protein-C, (SP-C*) and *Clara cell protein 10, (CC10*) genes are concerned. The expression of the SHH receptor, *Patched*, is also decreased in the absence of *Shh* as are the *Gli1* and *Gli3* transcriptional factors. On the other hand, lung-specific misexpression of *Shh* results in severe alveolar hypoplasia and a significant increase in interstitial tissue (98). *Fgf10* expression, which is highly spatially restricted in wild type, is not spatially restricted and is widespread in the mesenchyme in contact with the epithelium of the *Shh* null mutant mouse lung. Conversely, local suppression of SHH signaling by the induction of *Ptc* and *Hip* at branch tips may serve to facilitate FGF signaling locally, where branch outgrowth is stereotypically programmed to take place (99). It is interesting to note that the cecum, which forms as a single bud from the mouse midgut and does not branch, also expresses *Fgf10* throughout its mesenchyme (Burns and Bellusci, unpublished results). Thus, temporospatial restriction of *Fgf10* expression by SHH appears to be essential to initiate and maintain branching of lung.

TGF-B FAMILY PEPTIDE EXPRESSION AND ACTIVATION IS ESSENTIAL FOR NORMAL LUNG DEVELOPMENT

The TGF-β superfamily can be divided into three subfamilies: activin, TGF-β, and BMP (100). There are three TGF-β isoforms in mammals: TGF-β1, 2, and 3. All of them have been detected in murine embryonic lungs (101–104). In early mouse embryonic lungs (E11.5), TGF-β1 is expressed in the mesenchyme, particularly in the mesenchyme underlying distal

epithelial branching points, whereas TGF-β2 is localized in distal epithelium, and TGF-β3 is expressed in proximal mesenchyme and mesothelium (105). Mice lacking *Tgfβ1* develop normally but die within the first month or two of life of aggressive pulmonary inflammation. When raised under pulmonary pathogen–free conditions, these mice live somewhat longer but die of other forms of inflammation (106). Thus, physiologic concentrations of TGF-β1 appear to suppress the pulmonary inflammation that occurs in response to exogenous factors such as infection and endotoxin. On the other hand, *Tgfβ2* null mutants die *in utero* of severe cardiac malformations, whereas *Tgfβ3* mutants die neonatally of lung dysplasia and cleft palate (107,108). Embryonic lung organ and cell cultures reveal that TGF-β2 plays a key role in branching morphogenesis, whereas TGF-β3 plays a key role in regulating alveolar epithelial cell proliferation during the injury repair response (109,110). Thus, finely regulated and correct physiologic concentrations and temporospatial distribution of TGF-β1, 2, and 3 are essential for normal lung morphogenesis and defense against lung inflammation. Overexpression of *Tgfβ1*, driven by the *SP-C* promoter, in lung epithelium of transgenic mice causes hypoplastic phenotypes (111). Similarly, addition of exogenous TGF-β to early embryonic mouse lungs in culture resulted in inhibition of lung branching morphogenesis although each TGF-β isoform has a different IC_{50} (TGF-β2>1>3) (105,111). In contrast, abrogation of TGF-β type II receptor stimulated embryonic lung branching through releasing cell cycle G1 arrest (112). Moreover, overexpression of constitutively active TGF-β1, but not latent TGF-β1, in airway epithelium is sufficient to have significant inhibitory effects on lung branching morphogenesis (109). However, no inhibitory effect on lung branching was observed when TGF-β1 was overexpressed in the pleura and subjacent mesenchymal cells. Furthermore, adenoviral overexpression of a TGF-β inhibitor, *Decorin*, in airway epithelium, completely abrogated exogenous TGF-β1-induced inhibition of embryonic lung growth in culture (113). On the other hand, reduction of decorin expression by DNA antisense oligonucleotides was able to restore TGF-β1-mediated lung growth inhibition (113). Therefore, TGF-β signaling in distal airway epithelium seems to be sufficient for its inhibitory function for embryonic lung growth. Interestingly, TGF-β specific signaling elements, such as Smad2/3/7, are exclusively expressed in distal airway epithelium (114–116). Attenuation of *Smad2/3* expression by a specific antisense oligonucleotide approach blocked the exogenous TGF-β1-induced inhibitory effects on lung growth. Moreover, expression of *Smad7* in airway epithelium, which was induced by TGF-β, had negative regulatory functions for the TGF-β–Smad pathway in cultured cells, specifically blocking exogenous TGF-β–induced inhibitory effects on lung branching morphogenesis as well as on Smad2 phosphorylation in cultured lung explants. Inasmuch as blockade of TGF-β signaling not only stimulates lung morphogenesis in culture *per se*, but also potentiates the stimulatory effects of EGF and PDGF-A, it follows that TGF-β signaling functions downstream of or can override tyrosine kinase receptor signaling.

DEVELOPMENTAL SPECIFICITY OF TGF-B1 OVEREXPRESSION PHENOTYPES

During embryonic and fetal life, epithelial misexpression of TGF-β1 results in hypoplastic branching and decreased epithelial cell proliferation (109). In contrast, neonatal misexpression of TGF-β1 using an adenoviral vector approach phenocopies bronchopulmonary dysplasia (BPD) with alveolar hypoplasia, some interstitial fibrosis, and emphysema (117). Adult misexpression of TGF-β1, on the other hand, results in a chronic, progressive interstitial pulmonary fibrosis, resulting mainly from increased proliferation and matrix secretion by the mesenchyme; a process that depends on transduction through Smad3 (118,119). Thus, the phenotype caused by excessive TGF-β1 production and signaling is always adverse, but the precise effect depends on the developmental stage of the lung: hypoplasia in embryonic, fetal, and neonatal lung; fibrosis in premature and adult lung.

VEGF ISOFORMS INDUCE VASCULOGENESIS, ANGIOGENESIS, AND LYMPHOANGIOGENESIS DURING LUNG DEVELOPMENT

Vascularization must perfectly match epithelial morphogenesis to ensure optimal gas exchange. Several VEGF isoforms are expressed in the developing epithelium, whereas their cognate receptors are expressed in and direct the emergence of developing vascular and lymphatic capillary networks within the mesenchyme. It is possible that VEGF signaling may lie downstream of FGF signaling, inasmuch as *in vivo* abrogation of FGF signaling severely affects both epithelial and endothelial morphogenesis. Vasculogenesis is initiated as soon as the lung evaginates from the foregut (121). A critical growth factor during embryonic lung development is VEGF. The loss of even a single allele of *Vegf* leads to embryonic lethality between days E9.5 and E10.5 in the mouse (121). VEGF is diffusely distributed in pulmonary epithelial and mesenchymal cells and is involved in controlling endothelial proliferation and the maintenance of vascular structure. VEGF is localized in the basement membrane of epithelial cells (122).

BOTH HUMANS AND MICE HAVE THREE DIFFERENT VEGF ISOFORMS

VEGF-120, VEGF-164, and VEGF-188 are all expressed in mice during development, but VEGF-164 isoform is the most highly expressed and active during embryogenesis. VEGF signals through the cognate receptors FLK-1 (fetal liver kinase-1, or VEGFR2) and FLT-1 (fetal liver tyrosinase-1, or VEGFR1). VEGF signaling is responsible for the differentiation of embryonic mesenchymal cells into endothelial cells. Interactions between the epithelium and mesenchyme contribute to lung neovascularisation, which is crucial in normal lung formation (123). In fact, epithelial cells of the airways are positive for VEGF, particularly at the budding regions of the distal airway (124). Also, lung mesenchyme cultured alone in the absence of epithelium degenerates significantly and only a few *Flk-1* positives cells are maintained (120).

VEGF KNOCKOUT MICE HAVE A LETHAL PHENOTYPE WITHIN THE EARLY STAGES OF EMBRYONIC DEVELOPMENT (E8.5–E9)

Vegf misexpressing transgenic mice, where the *Vegf* transgene is under the control of the *SP-C* promoter, show gross abnormalities in lung morphogenesis that are associated with a decrease in acinar tubules and mesenchyme (121). VEGF-treated human lung explants show an increase of cellular proliferation in the distal airway epithelial cells with an up-regulation of the mRNA expression of *Surfactant Protein-A* (*Sp-A*) and *C* (*Sp-C*) but not *Sp-B* (124).

VEGF PLAYS A ROLE IN MAINTAINING ALVEOLAR STRUCTURE

VEGF has also been demonstrated to play a role in maintaining alveolar structure (125,126). Lungs from newborn mice treated with antibodies to FLT-1 are reduced in size and display significant immaturity with a less complex alveolar pattern (127). In contrast, the accumulation of VEGF in the alveoli appears to make transgenic VEGF mice more resistant to injury by hyperoxia (128,129). VEGF is a target of hypoxia-inducible transcription factor-2α (HIF-2α). *Hif-2α*–deficient newborn mice die from respiratory distress syndrome (129). In *Hif-2α* null mice the expression of VEGF is dramatically reduced in alveolar epithelial type 2 cells. Additionally, we have recently observed that addition of VEGF to early mouse embryonic lung explants markedly stimulates epithelial as well as vascular morphogenesis (unpublished results). Thus, we speculate that VEGF signaling plays an important role in matching the epithelial-capillary interface during lung morphogenesis.

VEGF-C AND VEGF-D ARE TWO ADDITIONAL MEMBERS OF THE VEGF FAMILY

These factors have a restricted expression pattern, with high levels mainly in lung tissues (130). VEGF-C and -D stimulate lymphoangiogenesis through their cognate receptor VEGFR-3 (131). Signaling *via* VEGFR-3 has been shown to be sufficient for lymphoangiogenesis through null mutation (132). Finally, VEGF-C also interacts with VEGFR-2 and is therefore able to induce angiogenesis *in vivo* (133).

WNT SIGNALING CONTROLS EPITHELIAL AND MESENCHYMAL DIFFERENTIATION AND PLAYS AN IMPORTANT ROLE IN LUNG DEVELOPMENT

The Wnt growth factor family in the mouse is comprised of 19 different secreted ligands that interact with 10 known seven-span transmembrane receptors of the Frizzled (Fz) gene family and either one of two single-span transmembrane proteins, low-density-lipoprotein-receptor-related proteins (LRP-5 and LRP-6) (134–136). Historically, Wnt proteins have been grouped into two classes, canonical and noncanonical. Canonical Wnt bind to frizzled receptors, inhibiting glycogen-synthase kinase-3β (GSK-3β)-mediated phosphorylation of β-catenin. Hypophosphorylated β-catenin accumulates in the cytoplasm, after which it translocates to the nucleus, where it heterodimerizes with members of the TCF/LEF transcription factor family to activate the transcription of TCF/LEF target genes. Noncanonical Wnt activate other Wnt signaling pathways, such as the planar-cell-polarity (PCP)-like pathway that guides cell movements during gastrulation (137). Activation of the PCP pathway can antagonize the canonical pathway intracellularly (138–140). Secreted Wnt antagonists can be divided into two functional classes, the secreted Frizzled-related protein (sFRP) class and the Dickkopf class. Members of the sFRP class bind directly to Wnts, thereby altering their ability to bind to the Wnt receptor complex, whereas members of the Dickkopf class inhibit Wnt signaling by binding to the LRP5/LRP6 component of the Wnt receptor complex.

In addition to LRP5/6, DKK1 interacts with the high affinity DKK1 co-receptors Kremen1 (Krm1) or Kremen2 (Krm2), which functionally cooperate with Dkk1 to block Wnt/beta-catenin signaling (141,142). DKK1, DKK3, and DKK4 act as inhibitors of canonical Wnt signaling, whereas DKK2 can function both as an inhibitor in the presence of Kremen2 or as an activator in its absence (143,144).

Between E10.5 and 17.5, β-catenin is localized in the cytoplasm and often also in the nucleus of the pulmonary epithelium and adjacent mesenchyme (146). Wnt ligands, Fz receptors, and the Tcf/Lef1 transcription factors are expressed during early lung development. In the early mouse lung (at E12.5), *Wnt7b* is expressed in the lung epithelium with a more intense expression distally, *Wnt2a* is highly expressed in the distal mesenchyme, and *Wnt5a* shows a low expression in the mesenchyme and epithelium and is highest around the trachea and pharynx. *Tcf1 (T cell factor1), Lef1 (Lymphoid enhancer factor 1), sFrp1 (secreted Frizzled-related protein 1)*, and *sFRP2* are highly expressed in the mesenchyme adjacent to the pulmonary epithelium. *Tcf3* is highly expressed at the apical side of the pulmonary epithelium whereas *Tcf4* and *sFrp4* are detected in both the epithelium and adjacent mesenchyme (145). *Fz8* is highly expressed throughout the epithelium whereas *Fz2, 3, 6* and *7* are expressed both in the epithelium and mesenchyme (145). These expression patterns suggest that Wnt signaling can originate from the epithelium and mesenchyme and can target both tissues in an autocrine and/or paracrine fashion. TOPGAL or BATGAL mice, which harbor a *β-galactosidase* gene under the control of a LEF/TCF and β-catenin inducible promoter (146), reveal that from E10.5 until E12.5, canonical Wnt signaling occurs throughout the epithelium and in the mesenchyme adjacent to the proximal airways where the bronchial smooth muscle cells (SMC) arise (147–149). From E13.5, TOPGAL activity is no longer present in the mesenchyme and the activity in the epithelium is reduced distally concomitant with the onset of expression of *Dkk1* in the distal epithelium (149).

CONDITIONAL INACTIVATION OF THE B-CATENIN GENE IN THE EPITHELIUM OF THE DEVELOPING MOUSE LUNG LEADS TO NEONATAL DEATH RESULTING FROM SEVERE LUNG DEFECTS

In this phenotype, branching of secondary bronchi is altered and the number of small peripheral alveolar ducts and terminal saccules is markedly reduced (150). In addition, the epithelium

fails to undergo proper distal differentiation, lacking the expression of pro-*SP-C* protein and *Vascular endothelial growth factor a*, the latter correlating with a reduction in alveolar capillaries. So far, inactivation of only two WNT ligands have resulted in lung defects. *Wnt7b* is normally expressed in epithelial cells of the lung periphery. *Wnt7b*$^{-/-}$ mice exhibit perinatal death due to respiratory failure. Defects were observed in proliferation of the lung mesenchyme resulting in lung hypoplasia. In addition, *Wnt7b*$^{-/-}$ embryos and newborn mice exhibit severe defects in the smooth muscle component of the major pulmonary vessels, with increased apoptosis of the vascular smooth muscle cells (VSMC), resulting in rupture of the major blood vessels and hemorrhages in the lungs after birth. *Wnt5a* is expressed at high levels in the distal lung mesenchyme. *Wnt5a*$^{-/-}$ mice die perinatally from lung defects including truncation of the trachea, overexpansion of the peripheral airways and delayed lung maturation. Absence of WNT5a activity in the mutant lungs leads to increased cell proliferation and up-regulation of the expression of *Fgf10*, *Bmp4*, *Shh*, and *Ptc*. On the other hand, hyperactive Wnt signaling changes the developmental potential of embryonic lung endoderm to express markers of gut endoderm differentiation (151).

DICKKOPF REVEALS FIBRONECTIN AS AN IMPORTANT MATRIX TARGET OF WNT SIGNALING IN LUNG MORPHOGENESIS

Our recent experiments show that early embryonic mouse lung organ cultures treated with Dickkopf (DKK1), a potent and specific diffusible inhibitor of Wnt action that is also endogenously secreted by the distal lung epithelium, display impaired branching, characterized by failed cleft formation and enlarged terminal buds. The DKK1-treated lung explants show reduced α-smooth muscle actin (α-SMA) expression and defects in the formation of the pulmonary vascular network. These defects coincide with a pattern of decreased Fibronectin (FN) deposition and *Platelet derived growth factor-A* (*Pdgf-a*) expression. All of the DKK1-induced morphogenetic defects can be recapitulated by inhibition of FN with an antifibronectin antibody and conversely can be rescued by addition of exogenous FN (149). This points out the importance of correct orientation of the extracellular matrix in response to growth factor signaling. It also suggests that Fibronectin is a downstream target of Wnt signaling.

FUNCTIONAL INTEGRATION OF KEY GROWTH FACTOR SIGNALING PATHWAYS IN LUNG BUD OUTGROWTH, BUD ARREST, AND BUD BRANCHING

Our current concepts are diagrammed in Figure 1. We postulate that the dynamically changing relative activity of SHH, FGF10, and mSPRY2 may impart automaticity to the branching process. SHH is high and FGF10 is correspondingly low where branching is not supposed to take place. In contrast, SHH is suppressed locally by PTC and HIP, so that FGF10 is therefore high where a branch is supposed to occur. FGF10 in turn dynamically induces its inhibitor *mSpry2* as branches

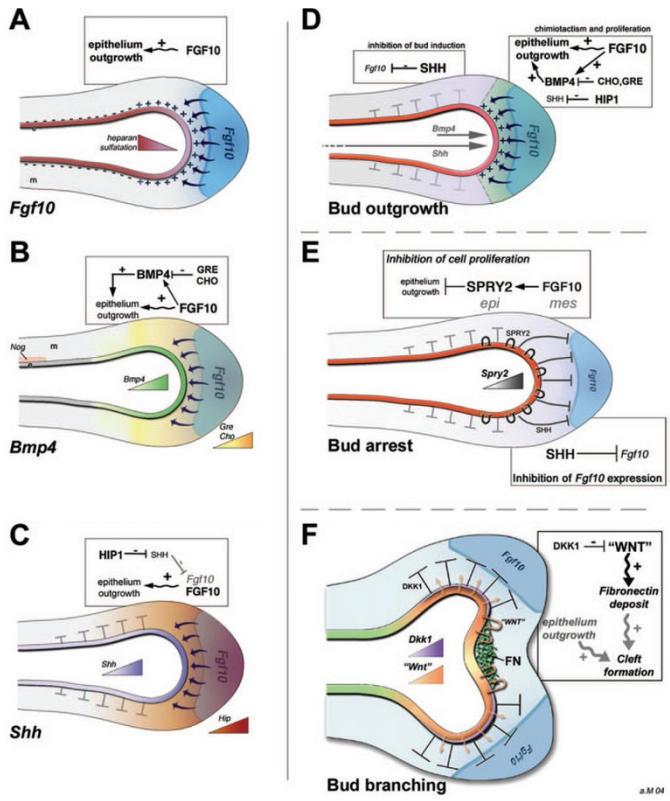

Figure 1. The figure models the functional integration of key growth factor signaling pathways in lung bud outgrowth, bud arrest, and bud branching. *Panel A* depicts the function of FGF10 to stimulate bud outgrowth. *Fgf10* is expressed in the distal mesenchyme so that a decreasing gradient of FGF10 acts to stimulate chemotaxis of the bud tip toward the subpleural source of FGF10. Heparan sulfation is also important for FGF function. *Panel B* depicts the function of BMP4 to stimulate lung branch tip outgrowth together with FGF10. FGF10 is shown stimulating BMP4 expression, whereas the ligand binding proteins Gremlin (GRE) and Chordin (CHO) exert negative modulation on BMP4. *Panel C* depicts the functional interaction of SHH and Hip with FGF10. SHH inhibits *Fgf10* expression away from the branch tip. However at the branch tip, Hip inhibits SHH, releasing the SHH mediated inhibition of *Fgf10* expression. *Panel D* superimposes the functional integration of *Fgf10, Bmp4,* and *Shh* to mediate the delicate balance between chemotaxis and proliferation leading to bud induction *versus* inhibition of bud outgrowth. *Panel E* depicts the events that may determine interbranch length by leading to arrest of bud outgrowth. FGF10 induces SPRY2, which in turn inhibits epithelial outgrowth. Meanwhile, in more proximal regions suppression of branching is mediated by SHH, which inhibits *Fgf10* expression outside the peripheral mesenchyme. *Panel F* depicts a potential mechanism for bud tip splitting in which WNT signaling drives Fibronectin (FN) depostion between the branch tips, leading to epithelial cleft formation. Meanwhile, Dickkopf (DKK1) inhibits Wnt signaling away from the cleft, leading to lower levels of FN deposition where clefting does not occur.

lengthen. Thus, the net relative activities between SHH, FGF10, and mSPRY2 may determine FGF signal strength in the epithelium and hence the relative rate of bud outgrowth rate at a given point and hence interbranch length. As a bud begins to elongate toward a mesenchymal source of FGF10, *mSpry2* begins to be expressed in the distal tip. During subsequent elongation, *Fgf10* continues to be expressed in the distal mesenchyme and the level of *mSpry2* gradually increases as the bud lengthens. When the bud finally approaches the pleura, the *Fgf10* expression domain adjacent to the distal tip appears to thin out and some of it appears to be pushed laterally to lie

between adjacent branch tips. At the time, *mSpry2* expression in the distal tip is at its highest level, perhaps mediating bud outgrowth arrest. A tip-splitting event then occurs. Of note is that *mSpry2* expression is extinguished between the daughter bud tips, but continues to be expressed within the tips of the daughter bud epithelia. This cycle of interaction is then repeated during subsequent branching events. BMP4 and VEGF signaling, as discussed above, appear to play necessary but complementary roles to accelerate this overall process.

MOLECULAR ORIGIN OF THE EPITHELIAL TIP-SPLITTING EVENT

Because tip-splitting events occur widely in nature where genes are not present, in such processes as viscous fingering, electrolysis of metals, river deltas, and oil field deposits, it is not clear whether physical forces are primary or secondary to growth factors in lung development (152). Certainly, physical force can modulate the rate of branching, inasmuch as tracheal ligation, which doubles the intraluminal pressure in embryonic lung, significantly accelerates branching (153). However, the numerous null mutants now available show that growth factors such as FGF10 and SHH are essential and these null mutations can abrogate the influence of increased force. FGF10 clearly is capable of inducing a strong chemotactic response of denuded lung epithelial bud tips toward and indeed to engulf an FGF10 soaked bead. Moreover, terminal buds appear to migrate into and toward the FGF10 epitopes within the embryonic lung mesenchyme. However, one of many puzzles has been why *Spry2* expression, which is controlled by FGF10, is focally extinguished in a small stretch of epithelium right in the cleft between two new daughter buds. Another plausible explanation for tip splitting is the "rock in the stream" hypothesis, wherein a solid bar of something in the mesenchyme acts like a rock to divide the flowing stream of epithelium into two as it chemotaxes toward FGF10. Fibronectin and myofibroblasts have been suggested as candidates to play the role of the rock. Certainly our new data on Wnt signaling, using DKK as a means to abrogate the canonical Wnt pathway, suggest that Fibronectin deposition is indeed a good candidate to function as the rock in the stream, whereas Wnt signaling appears to control its deposition. Netrins, deposited within the extracellular matrix, may also play a role in regulating epithelial behavior in response to FGF signaling during branching morphogenesis in the embryonic lung (154).

POSTNATAL LUNG DEVELOPMENT

Many of the same genes that are responsible for lung organogenesis also play key roles in postnatal lung development, although many are also silent by that time. Null mutation studies have revealed essential roles for PDGF-A chain and for FGFR3 and FGFR4 as well as elastin in the induction of alveolar ridges and the correct orientation of elastic fibers in the postnatal lung. This is interesting because elastin expression by myofibroblasts, in response to PDGF and FGF signaling, has been adduced as a critical event during the alveolar phase of lung development. Because VEGF signaling by the epithelium to the endothelium is also critical for normal alveolarization, it is interesting to speculate that hydraulic force within the capillary vasculature may also be important. Certainly, abrogation of VEGF signaling by inducible misexpression of a dominant negative VEGF receptor under the control of the *Sp-C* promoter abrogates alveolarization as well as peripheral capillary morphogenesis, underscoring that epithelial to endothelial crosstalk is also an important mechanism in lung alveolar morphogenesis. After delivery, particularly premature delivery, exposure to endotoxin, oxygen, and/or barotrauma, with the resulting induction of cytokines including excessive amounts of TGF-β activity, adversely affects alveolarization and hence may induce the human pathobiological condition termed BPD or infantile chronic lung disease.

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