The molecular genetics of lung morphogenesis and injury repair

David Warburton* and Saverio Bellusci

Developmental Biology Program, Saban Research Institute, Childrens Hospital Los Angeles, Keck School of Medicine at USC, Center for Craniofacial Molecular Biology, USC School of Dentistry, Los Angeles, California, USA

Summary Lung development, as well as epithelial injury repair, is tightly coordinated by a fine balance between stimulatory versus inhibitory genes that appear to co-regulate the function of stem/progenitor cells in the lung. Recently, it has been noted that many of the same genes direct development of the respiratory organs (tracheae) in the fruit fly Drosophila as in mice and men. For example, FGF receptor tyrosine kinase signaling is essential for respiratory organogenesis in both fly and mouse and is negatively regulated by the sprouty genes, a family of inducible FGF pathway inhibitors. Additionally, FGF signaling is required for formation of new alveoli, protection of alveolar epithelial cells from injury, as well as migration and proliferation of putative alveolar stem/progenitor cells during lung repair. Conversely, TGFβ receptor serine-threonine kinase signaling via Smads 2, 3 and 4 inhibits lung morphogenesis and can inhibit postnatal alveolar development, while excessive TGFβ signaling via Smad3 causes interstitial fibrosis. On the other hand, BMP4 stimulates morphogenesis of intact embryonic lung, while inhibiting proliferation of isolated epithelium. We speculate that evolutionary-developmental, functional conservation of the FGF-FGFR-SPROUTY stimulatory pathway as well as of the TGFβ/BMP-SMAD modulatory pathways identifies them as potential therapeutic targets for rational therapy. Novel therapy to activate lung stem/progenitor cells, ameliorate lung injury, augment lung repair and/or induce lung regeneration could be highly beneficial in both children and adults with intractable pulmonary insufficiency.

© 2004 Elsevier Science Ltd.

In humans, emergence of the lung anlage from the anterior foregut, laryngeal and tracheo-esophageal morphogenesis and septation, tracheal bifurcation, patterning of the major bronchi, lobation of the lungs as well as branching morphogenesis of the first 16 generations of airways are highly stereotypic. Branching morphogenesis of the entire 23 generations of airways is completed between 5 and 25 weeks gestation, while alveoli begin to form at 20 weeks and expansion of the alveolar surface to the adult surface area size of 70 m² by 1 micron thick continues for several years postnatally. In contrast to the stereotypy of the upper airways, between 16 and 23 generations as well as at the alveolar level the lungs comprise tightly packed fractal units. The proximal pulmonary vessels develop as angiogenic sprouts from systemic arteries and veins, while pulmonary capillary vasculogenesis depends on transdifferentiation of capillary endothelium from mesenchyme. The pulmonary arteries and veins stereotypically follow the branching pattern of the airways. However, the bronchial arteries originate separately from the systemic vasculature. Intimate juxtaposition of the alveolar epithelium and capillary endothelium is essential for efficient gas exchange. Pulmonary lymphatics and nerves also develop, but their origin is less well understood.

*Correspondence to: David Warburton DSc, MD, FRCP
Tel.: +1-(323)-669-7075; Fax: +1-(323)-671-3613; E-mail: dwarburton@chla.usc.edu

Correspondence address: Developmental Biology Program, Saban Research Institute, Childrens Hospital Los Angeles, 4650 Sunset Boulevard MS35, Los Angeles, CA 90027, USA

© 2004 Elsevier Science Ltd. All rights reserved.
The stereotypy of early lung morphogenesis strongly suggests that pattern formation of the upper airways is genetically “hard-wired”. Mouse molecular genetics and increasingly genomics has become a powerful approach to determine the function of some of these genes and their position in the temporospatial hierarchy that makes a lung. Null mutation has revealed that Hepatocyte nuclear factor-α (Hnfα) is required for closure of the primitive gut tube. Hnfα is normally expressed throughout the gut epithelium and continues to be expressed in the airway epithelium throughout gestation. Hnfα functions as a transcriptional factor regulating airway epithelium gene promoters including Sp-c, Sp-b, Sp-a and Cc10. In Gli 2 and Gli 3 double null mutants the gut tube closes but the laryngotracheal groove fails to emerge from the primitive foregut. Gli 2 null mutation and partial mutation cause lobation abnormalities. Lobation abnormalities are also observed in Fox and RAR family mutants. Null mutation of Fgf10 or misexpression of dominant negative Fgfr2 completely abrogates branching of the airway distal to the trachea. Sonic hedgehog (Shh) and Nkx2.1 null mutation disrupts branching of the airways as well as interfering with formation of the tracheoesophageal septum. Null mutation of Egr and of Tace reduces branching of the distal airways by 50% or more, while Tace null mutation also interferes with peripheral angiogenesis. Null mutation of Tgfβ3 causes a neonatal lethal phenotype by interfering with the final stage of lung epithelial maturation as well as causing pulmonary telangiectasias and failure of medial edge fusion in the palate. For more detailed recent reviews see Cardoso, Perl & Whitsett, Warburton et al.

Recently, informative evolutionary-developmental (evo-devo) and functional conservation parallels have been drawn between genetic mechanisms governing branching morphogenesis of the respiratory organs in flies, mice and humans. In the respiratory system of the fly embryo, gas is delivered to individual cells by segmental paired networks of tubes termed tracheae. The terminal tracheal branches deliver oxygen to and remove carbon dioxide from individual cells in a highly stereotypic, branched network. Among the 50 or more genes that have emerged from functional screening studies as directing fly respiratory organogenesis, Sonic hedgehog, patched, smoothened, Gli, Fgf, Fgfr, sprouty, Bmp4, Tgfβ and Smads are functionally conserved in mice and by homology-based inference, also in humans. Among these, FGF signaling is absolutely required for the initiation and maintenance of tracheal branching in the fly as demonstrated by the branchless (Fgf orthologue) and breathless (Fgfr orthologue) null mutant phenotypes. In contrast, the sprouty null phenotype is characterised by exuberant tracheal branching. Hypoxia and the accompanying induction of Hif1α drive FGF-mediated branching in flies.

In mice, FGF signaling is essential for all stages of lung morphogenesis. Not only is FGFindispensable for branching morphogenesis of the bronchi, but also for postnatal modelling of alveoli as well. Null mutation of Fgf10 abrogates bronchial modelling distal to the carina, while double null mutation of Fgfr3 and Fgfr4 abrogates postnatal alveolar morphogenesis. Interestingly, the latter phenotype is associated with abnormal elastin deposition, underlining the intimate functional association between correct modelling of the epithelium with the matrix.

Recent studies on the sprouty family have provided further evidence supporting evolutionary, developmental and functional conservation of key positive and negative regulatory elements in the FGF signaling pathway. Null mutation of sprouty in flies results in exuberant over growth of the distal tracheae, suggesting that SPROUTY functionally antagonises FGF signaling. Loss of function studies in early mouse embryonic lung culture using mSpry2 antisense oligonucleotides also produced an increased branching phenotype, while transgenic misexpression of mSpry2 using the SP-C promoter produced a decreased branching phenotype. Sprouty is an inducible downstream inhibitor of ras activation.

Finely coordinated expression of Fgf10 in murine lung peripheral mesenchyme with mSpry2 in the adjacent epithelium at E11/12 suggests a regulatory paradigm by which these genes may serve to determine inter bud branch length. At the point of initiation of a new lobar branch, Fgf10 is highly expressed in the mesenchyme overlying the new bud. Meanwhile mSpry2 is expressed at a relatively low level in the adjacent bud epithelium, while Fgfr2 is widely expressed throughout the epithelium. As the bud grows out towards the mesenchymal source of FGF10, epithelial mSpry2 expression increases to high levels in the growing tip. Immediately before the next step of bud bifurcation into two segmental bronchi, the site of Fgf10 expression divides laterally into two. Simultaneously, the epithelial mSpry2 expression locus divides into two, adjacent to the two new Fgf10 foci. Bud bifurcation occurs and the cycle of elongation and gene expression is repeated many times until branching is complete. During subsequent stages of murine lung modelling (E14-16), Fgf10 is not only expressed in each individual bud tip, but is also expressed in a band along the edge of each developing lobe, coincident with the pattern of increased expression of SP-C.
The latter Fgf10 spatial distribution resembles the distribution of Fgf8 in the morphogenetic apical ectodermal ridge in the limb bud. Thus, inviting speculation that Fgf10 also plays an inductive role in lobar shape modelling as well as in branching. Somewhat similar counter-regulatory inductive interactions between Fgf10 and Bmp4 have recently been mooted in experiments with isolated embryonic lung epithelium.14 However, addition of either Fgf10 or Bmp4 to intact embryonic lung explants at E11/12 stimulates branching morphogenesis.15-17 This raises a number of interesting questions about growth factor interactions and signaling mechanisms that remain to be addressed.

Several key morphogenetic genes are co-expressed in and around peripheral lung buds. This reiterative temporo-spatial pattern is reminiscent of other morphogenetic centres in the body such as the enamel knot in the tooth bud and the apical ectodermal ridge in the limb bud. Mesenchymal morphogenetic genes expressed in the vicinity of peripheral lung bud epithelium include Fgf10, mSpry4, patched, smoothened, Wnt and Hox family members. While Bmp4, Shh, mSpry2, Smads 2, 3 and 4 are co-expressed in the adjacent peripheral epithelium. This invites speculation that the long-anticipated “complete” inductive effects of peripheral lung mesenchyme on airway branching may be mediated through signaling networks that include these genes and their soluble, secreted products and cognate receptors.3 Null mutation of TGFβ1 does not result in a structural lung phenotype. Rather absence of TGFβ1 results in failure to suppress the innate immune response.18 Thus, TGFβ1 null mutant mice die of fulminant pulmonary inflammatory infiltrates at about 1 month of age. In contrast, TGFβ3 null mutant mice are born with cleft palate and a lethal form of late fetal pulmonary epithelial, mesenchymal and vascular dysplasia.19 Over expression of TGFβ1 as well as of Bmp4 during embryonic and fetal life both result in epithelial hypoplasia.20-22 Postnatally, excess TGFβ1 mediates alveolar hypoplasia and interstitial fibrosis. The interstitial fibrosis, caused by TGFβ1 that has been induced by bleomycin is transduced by Smad3. In contrast, excess TGFβ3 does not cause lung fibrosis.

The physiological role of BMP4 remains somewhat controversial.15-17 Some have suggested, based on misexpression studies and culture of dissected embryonic epithelia that BMP4 inhibits lung epithelial cell proliferation and hence branching morphogenesis. However several reports now show that BMP4 positively regulates epithelial branching in intact lung explants. Moreover we found that abrogation of Gremlin, a physiological BMP4 antagonist, enhances branching of embryonic mouse lungs in culture. Misexpression of the BMP4 inhibitor Gremlin results in a phenotype where proximal airways extend all the way to the pleura. Thus, we speculate that BMP4 may play a key role in mediating differentiation of the lung periphery. However, the exact mechanism by which the physiologic BMP4 signal is transduced has not yet been settled. BMP4 ligand is expressed in the epithelium of the terminal lung buds. However, the BMP4 pathway Smads 1, 5 and 8 are expressed in isolated rests of mesenchymal cells. Their target genes and how these mesenchymal cells signal back to the epithelium remains unknown. Interestingly, BMP4 appears to induce senescence in A549 cells and may play a role in retarding the transition from AEC2 to AEC1 phenotype in culture.

Premature delivery places the underdeveloped alveoli of the human fetus suddenly into an environment where tissue pO2 and mean airway pressure are significantly elevated compared to intrauterine values. This can have serious long-term adverse consequences on alveolar modelling, as well as inducing an acute inflammatory reaction, followed by interstitial fibrosis and emphysema, resulting in the clinical syndrome termed bronchopulmonary dysplasia (BPD). Likewise, in adults, exposure to increased levels of inspired oxygen denudes the alveolar epithelium. In human premature babies who get BPD, increased levels of TGFβ1 ligand are found in airway lavage samples early in the course of the disease, and particularly high levels are associated with an adverse prognosis.23 Excessive amounts of TGFβ1 ligand are well known to inhibit embryonic lung morphogenesis in culture, while adenoviral vectors expressing TGFβ1 induce severe, progressive pulmonary fibrosis.24 We have also recently found that the same TGFβ1 adenoviral vectors inhibit alveolar modelling during the neonatal period.25 Thus, dysregulation of the TGFβ1 signaling pathway appears to be pivotal in the abnormal repair response to lung injury. Furthermore, we have recently shown that null mutation of Smad3 in mice renders murine lung refractory to the adverse effects of excess TGF-β1 induced by bleomycin administration.26 Thus, we postulate that the TGF-β1 signaling pathway should yield effective therapeutic targets to ameliorate abnormal lung repair.

The alveolar epithelial type 2 cell (AEC2) has many functions in addition to the production and metabolism of pulmonary surfactant phospholipids and proteins. In fact, the AEC2 can be considered as a remodelling toolbox.27 Two key features of the alveolar remodelling process during recovery from injury are AEC2 migration and proliferation, which rapidly recover and reseal the denuded alveolar surface. AEC isolated from fetal rats
migrate rapidly and aggressively immediately upon isolation: in fact they migrate as fast as A549 lung epithelial adenocarcinoma cells. This migratory capacity of fetal AEC is compatible with their role in alveolar modelling. In contrast, adult rat AEC2 migrate sluggishly and only after 48 hr in culture. However, during the recovery phase from acute hyperoxia, adult rat AEC2 regain the capability to migrate rapidly, suggesting that this is an important function for alveolar repair. AEC2 migration is further optimised when cells are cultured upon fibronectin and exposed to EGF. Fibronectin is also secreted by AEC following injury, while EGF is actively synthesised by AEC2 in adult rats and is released from them following injury.28 Both fetal AEC and adult AEC2 following injury secrete and activate MMP2 and MMP9. The activity of MMP9 is required for AEC migration as well as for KGF to exert its protective effects against hyperoxia induced apoptosis in injured AEC2.

The question therefore arises as to whether the general population of AEC2 can respond to injury by proliferating or whether it is a small sub-population of AEC stem or progenitor cells that do the job. AEC2 are normally quiescent in G0 of the cell cycle, expressing little or no cell cycle proteins.29 Fetal AEC on the other hand do proliferate and express several key cyclins and cdks. Following acute hyperoxic injury, AEC2 transiently regain the capacity to proliferate and express numerous cell cycle related genes.30 We have recently determined that a small (<5%) population of putative stem cells does indeed exist within the AEC population.31 These putative stem cells express telomerase and are relatively resistant to hyperoxic apoptosis, suggesting that they may enjoy a competitive advantage over the general population in terms of injury resistance. We are currently pursuing their further characterisation.

From the foregoing it seems clear that respiratory morphogenesis, injury and repair are under tight genetic control and that many genes are functionally conserved between these processes. Branching morphogenesis is, however, far from unique to the respiratory system of mammals or flies. Branching is a common morphogenetic paradigm in organs where a large surface area is required to maintain a gas or liquid interface within a small volumetric space such as the kidney, salivary gland and lactating breast. Many of the same morphogenetic genes are involved in organogenesis of these structures as in lung. Temporo-spatial specificity of mesenchymal–epithelial reciprocal signaling is clearly a major factor, based on classical mesenchyme recombination studies. However, the question of whence comes specificity in these systems remains incompletely answered.

Branching morphogenesis is also a common physical process in such natural systems as rivers and watersheds, electrolysis and viscous fingering in which nary a gene is present. We therefore speculate that branching of the primitive respiratory tubules and the accompanying vasculature may be initiated by physical force, as proposed by Fleury at the conference. However, we propose that temporospatial gene expression superimposes order: stereotypic order early on and fractal order at later stages of lung development. Fleury proposed that, as in the physical model of viscous fingering, force exerted across a mesenchymal–epithelial interface of differing viscosity will result not only in tip splitting events (bipolar branching), but also monopodial branching further back, strongly reminiscent of early branching in the respiratory tree.

What may be the source of these physical forces? Olver showed at the conference that the fetal lung epithelium actively transports chloride into the lumen, carrying water with it. Tracheal effluent volume and pressure have long been known to be critical for fetal stage lung morphogenesis. There is now a considerable surgical literature on strategies to manipulate intrapulmonary pressure in utero in the hope of augmenting lung growth in the face of lung hypoplasia.

We therefore propose that genes, ions and physical forces should be considered together as key morphogenetic elements in the lung. Disruption of these finely balanced elements would therefore be expected to result in abnormal morphogenesis. This is exactly what happens in children whose lungs are exposed to endotoxin, inflammation, barotrauma, hyperoxia, and/or corticosteroids during critical periods of lung morphogenesis.

We are currently using insights from the molecular genetics of lung morphogenesis to search for therapeutic targets to re-entrain morphogenesis in damaged or hypoplastic lungs, by reactivating pulmonary or systemic stem cells. Some of our recent results showing that FGF10 rescue of nitrofen-induced embryonic lung hypoplasia culture provide an encouraging early proof of this principle.32

ACKNOWLEDGEMENTS

Funded by NHLBI. Due to the brief nature of this minireview we apologise to those colleagues whose valuable work we have failed to mention.
REFERENCES