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# Lung Organogenesis

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## Abstract

Developmental lung biology is a field that has the potential for significant human impact: lung disease at the extremes of age continues to cause major morbidity and mortality worldwide. Understanding how the lung develops holds the promise that investigators can use this knowledge to aid lung repair and regeneration. In the decade since the "molecular embryology" of the lung was first comprehensively reviewed, new challenges have emerged—and it is on these that we focus the current review. Firstly, there is a critical need to understand the progenitor cell biology of the lung in order to exploit the potential of stem cells for the treatment of lung disease. Secondly, the current familiar descriptions of lung morphogenesis governed by growth and transcription factors need to be elaborated upon with the reinclusion and reconsideration of other factors, such as mechanics, in lung growth. Thirdly, efforts to parse the finer detail of lung bud signaling may need to be combined with broader consideration of overarching mechanisms that may be therapeutically easier to target: in this arena, we advance the proposal that looking at the lung in general (and branching in particular) in terms of clocks may yield unexpected benefits.

## 1. Introduction

The concept that lung organogenesis is instructed by coordinated mesenchymal-to-epithelial crosstalk originates in the classical recombination experiments of Alescio and Cassini (1962), in which replacing tracheal mesenchyme with mesenchyme from the lung periphery induced ectopic branching of tracheal epithelium in murine embryonic lung organ culture. This idea was extended in an early review by Warburton and Olver (1997) to include the coordination of genetic, epigenetic, and environmental factors in lung development, injury, and repair. Thereafter, a molecular basis of lung morphogenesis was attempted by Warburton *et al.* (2000). Over the last decade, significant progress has been made in this

field as reviewed by Cardoso and Lu (2006), Maeda *et al.* (2007), and others. Nevertheless, the ultimate goal remains as stated by Warburton and Olver (1997), "to devise new rational and gene therapeutic approaches to ameliorate lung injury and augment lung repair … the ideal agent or agents would therefore mimic the instructive role of lung mesenchyme and would correctly induce the temporospatial pattern of lung-specific gene expression necessary to instruct lung regeneration." To this overall strategy, we can now add (i) the modulation of lung mechanobiology to favor appropriate lung regeneration and (ii) the stimulation of endogenous stem/progenitor cells or supply of exogenous ones for lung regeneration. Therefore, the current review draws together three important strands of information on lung organogenesis as of April 2010: (i) molecular embryology of the lung, (ii) mechanobiology of the developing lung, and (iii) pulmonary stem/progenitor cell biology. Applying advances in these complementary areas of research to lung regeneration and correction of lung diseases remains the therapeutic goal of this field. With the recent human transplanation of a stem/progenitor cell-derived tissue-engineered major airway (Macchiarini *et al.*, 2008), we can clearly see the potential of this field, while recognizing the many problems yet to be solved.

Before concentrating on the molecular biology, mechanobiology, and stem cell biology of the lung, a first step in regenerative strategies is to consider the developmental anatomy of the lung. From this, we can at least see what type of structures we need to generate.

## 2. Developmental Anatomy of the Lung

### 2.1. The bauplan: key steps in lung morphogenesis

A diagrammatic overview of lung morphogenesis is given in Fig. 3.1. Three lobes form on the right side and two lobes on the left side in human lung; in mice four lobes form on the right (cranial, medial, and caudal lobes, plus the accessory lobe) and one on the left. In contrast to humans, in the mouse, there are only 12 airway generations and alveolarization occurs entirely postnatally.

### 2.2. The histological stages of lung development

Histologically, lung development and maturation has been divided into four stages: pseudoglandular, canalicular, terminal saccular, and alveolar (Fig. 3.2).

**The pseudoglandular stage (5–17 weeks of human pregnancy, E9.5–16.6 days in mouse embryo)—**During this, the earliest developmental stage, epithelial tubes lined with cuboidal epithelial cells undergo branching morphogenesis and resemble an exocrine gland (hence the nomenclature). However, this fluid-filled primitive respiratory tree structure is too immature to support efficient gas exchange.

**The canalicular stage (16–25 weeks of human pregnancy, E16.6–17.4 days in mouse embryo)—**The cranial part of the lung develops faster than the caudal part, resulting in partial overlap between this stage and the previous stage. During the canalicular stage, the respiratory tree is further expanded in diameter and length, accompanied by vascularization and angiogenesis along the airway. A massive increase in the number of capillaries occurs. The terminal bronchioles are then divided into respiratory bronchioles and alveolar ducts, and the airway epithelial cells are differentiated into peripheral squamous cells and proximal cuboidal cells.

**The terminal saccular stage (24 weeks to late fetal period in human, E17.4 to postnatal day 5 (P5) in mouse)—**There is substantial thinning of the interstitium during the terminal saccular stage. This results from apoptosis as well as ongoing differentiation of

mesenchymal cells (Hashimoto *et al.*, 2002; Lu *et al.*, 2002). Additionally, at this stage, the alveolar epithelial cells (AECs) are more clearly differentiated into mature squamous type I pneumocytes and secretory rounded type II pneumocytes bearing lamellar bodies that contain surfactant. The capillaries also grow rapidly in the mesenchyme surrounding the saccules to form a complex network. In addition, the lymphatic network in lung tissue becomes well developed during this stage. The thick wall of these saccules, also called primary septae, comprises lining epithelial cells on both sides of a connective tissue core, within which there is a double parallel network of capillaries. Toward the end of this stage, the fetal lung can support air exchange in prematurely born human neonates. Maturation of surfactant synthesis and secretion is a key factor in determining whether the newborn lung can sustain gas exchange without collapsing.

**The alveolar stage (late fetal period to childhood in human, P5–P30 in mouse)—**Alveolarization is the last step of lung development. The majority of the gas exchange surface is formed during this stage.

Genome-wide expression profiling has measured developing human lung transcriptomes in pregnancies terminated between 7 and 22 weeks post conception (Kho *et al.*, 2009). Within the 3,223 gene developing lung-characteristic subtranscriptome, transitions in gene expression correlated with some histological stages, as well as suggesting novel substages exist. For example, induction of surfactant gene expression identifies a “molecular transition” in the pseudoglandular phase.

Hence, the histological account of lung development is complimented by the molecular embryology that we consider in the next main section of the review.

## 2.3. Focus on branching morphogenesis: simplifying the complexities

Branching morphogenesis is a critical part of overall lung development and a crucial phenomenon in the development of several other organs. Understanding this key hurdle in lung regeneration strategies requires us to appreciate that despite the apparent and beautiful complexity of the lung, there are key simplicities that can help us in our task.

Fractal mathematics has revealed that relatively straightforward algorithms, when applied iteratively, could generate patterning of great complexity (Mandelbrot, 1982). Moreover, when the mathematical parameters were chosen appropriately, the approximation to living branched structures was striking. At first sight, intrapulmonary airway branching (distal to the primary bronchi) appears to become increasingly complicated as it proceeds distally and the number of individual branches increases into the millions. But, once the laryngotracheal complex and left–right laterality are established, distal airway branching is now thought to be driven by a relatively simple set of genetically encoded control routines. Echoing the fractal pattern formation achieved in mathematical models, these include the following: (i) a master branch generator routine, with three slave subroutines instructing a periodicity clock which times the appearance of subsequent branches, (ii) a rotational orientation subroutine which determines the orientation of the branches around the axis of the airway, and (iii) a branch tip division subroutine (Metzger *et al.*, 2008; Warburton, 2008). Thus, branching morphogenesis of the bronchi in early mouse embryo lung can be parsed anatomically into three simple geometric forms, termed domain branching, planar bifurcation, and orthogonal bifurcation (Fig. 3.3). These basic forms are repeated iteratively to form different arrangements of branches. The planar or bottlebrush array describes the sequential proximal to distal emergence of secondary branches along the lateral axis of the primary bronchial airway. The bottlebrush mechanism is then reoriented around the branch axis to form a second row of branches at right angles to the first row. The terms *planar array* and *rosette array* describe the patterns formed by sequential bifurcation of the tips of secondary, tertiary,

and subsequent buds at right angles to each other. Repetition of these simple branching modules, together with the hierarchical control and coupling of them, may therefore explain how the genome could possibly encode the highly complex yet stereotypic pattern of early bronchial branch formation, using a relatively simple toolbox of genetic modules. In a further illustration of how the mammalian lung uses simple routines and subroutines to construct itself, substantial homology has been identified between the genetic regulation of lung organogenesis and airway morphogenesis in *Drosophila* (Hacohen *et al.*, 1998; Tefft *et al.*, 1999). Despite the latter's relative simplicity, it is striking to note not only the genetic homology but also the similar epistatic signaling hierarchy into which these regulators are arranged in the fly.

Using real-time microscopic cinematography, individual airway tip branching can be parsed temporally into a branch extension phase, a branch tip arrest phase, and a tip-splitting budding phase, followed once again when the branch budding phase is completed by branch extension until the next round of budding follows once more. A clock mechanism mediated by FGF–FGFR–Sprouty signaling plays a key role in timing the rate of bud extension and hence the inter-branch distance (Unbekandt *et al.*, 2008; Warburton, 2008). Indeed a nested hierarchy of clock routines are likely to be present throughout lung development given the number of oscillating systems intrinsic to the lung (branching, airway peristalsis, calcium oscillations) or visited extrinsically upon it (fetal breathing, circadian rhythms). Branching morphogenesis is accompanied by contractile oscillations (airway peristalsis) that are themselves underpinned by periodic calcium waves (Featherstone *et al.*, 2006; Jesudason *et al.*, 2005). These oscillators appear to be coupled to lung growth, and their precise relation to the timing of branching remains to be determined. However, we postulate that clock routines underlying the linear process of somitogenesis are redeployed three-dimensionally for branching morphogenesis in the lung and other organs (Pourquie, 2003).

### 2.4. The impact of abnormal lung development

The airway is developed sequentially by early epithelial tube branching and later septation of terminal air sacs. Pulmonary vasculature develops within lung mesenchyme in close conjunction with epithelial morphogenesis. Airway and vascular smooth muscle also develop during early morphogenesis. Perturbation of these developmental processes results in abnormal lung structure, deficiency of gas exchange, and neonatal respiratory failure. Clinical examples of such disruption of normal lung growth include cystic adenomatoid malformation of the lung, bronchopulmonary dysplasia (BPD) (a sequel of premature human delivery), and hypoplasia of the lung (seen in congenital diaphragmatic hernia (CDH), a birth defect as common as cystic fibrosis). More subtle lung dysplasias that are not lethal neonatally may emerge later in life with asthmatic wheezing and perhaps predisposition to early onset of chronic obstructive pulmonary disease (COPD). One of the clearest examples of how early development can affect not only lung organogenesis but also long-term health is the ciliary dyskinesia encountered in Kartagener syndrome and primary ciliary dyskinesia (Storm van's Gravesande, 2005). Early in embryogenesis, failure of ciliary function leads to randomization of organ situs and hence a supranormal rate of dextrocardia. This is accompanied by randomization of lung asymmetry. Persisting ciliary dysfunction impairs mucociliary clearance in the sinuses as well as the airways and predisposes to chronic lung disease in later life. Crucially one can note that disruption of lung asymmetry does not itself lead to lung malformation: hence the lung "bauplan" is conserved despite the lungs' left–right asymmetry being the reverse of normal. This observation reiterates to us that the complexities of lung organogenesis may actually be broken down into nested routines and subroutines used to accomplish particular tasks in the overall process.

The implication for lung regeneration is that one need not understand the formation of every last alveolus, but rather that elucidating the iterative routines could suffice to promote pulmonary "self-assembly."

## 3. Molecular Embryology of the Lung

This section of the review serves as a comprehensive reference source. For those with no requirement for such detail, the reader is directed to the summary Fig. 3.4.

We will first use a step-wise "process-driven" description of lung growth followed by a catalogue of the biochemical factors involved: many such factors are involved at multiple stages and do not map neatly on to the "process-driven" account. The biochemical factors are considered as follows: growth and transcription factors in order of first appearance and then other participating factors such as extracellular matrix (ECM) and miRNA.

### 3.1. Process-driven molecular embryology of the lung

**3.1.1. Induction of the early lung anlagen—**Early lung induction is regulated by genes that act cooperatively to define the location of laryngotracheal groove and help specify the spatial axes of the developing organ.

Among the earliest endodermal signals essential for gut morphogenesis and gut tube closure are the GATA (zinc-finger proteins that recognize GATA DNA sequence) and hepatocyte nuclear factor (HNF/Fox) transcription factors. *Foxa2* is required for gut tube closure, while *Gata-6* is required for activation of the lung developmental program within the foregut endoderm. *Hnf-3/Foxa2β* is a survival factor for the endoderm; its expression is induced by Sonic hedgehog (*Shh*). Retinoids and their transcriptional factor receptors also play key roles in induction of early lung branching: retinoic acid (RA) deficiency and compound null mutation of retinoid receptors prevent induction of the laryngotracheal groove. Most recently, Wnt2/2b and β-catenin signaling have been shown to be necessary and sufficient to specify lung progenitors in the foregut (Goss *et al.*, 2009; Harris-Johnson *et al.*, 2009). Embryos lacking Wnt2/2b expression exhibit complete lung agenesis and do not express Nkx2.1, the earliest marker of the lung endoderm. This phenotype is recapitulated by an endoderm-restricted deletion of β-catenin. Conversely, conditional expression of an activated form of β-catenin leads to ectopic expansion of the Nkx2.1 expression domain into esophagus and stomach epithelium. Thus, gain or loss of trachea/lung progenitor identity is accompanied, respectively, by contraction or expansion of esophagus/stomach progenitor identity. Taken together, these findings suggest that Wnt2/2b signaling through the canonical Wnt pathway is required to specify lung endoderm progenitors within the foregut. Furthermore, ectopic lung bud formation can be induced in the esophagus by *Tbx4* misexpression activating *Fgf10* expression (Sakiyama *et al.*, 2003). In addition, left–right asymmetry is controlled by several genes, including *nodal, Lefty-1,2*, and *Pitx-2*. For example, single-lobed lungs are found bilaterally in *Lefty-1*$^{-/-}$ mice, and bilateral isomerism of the lung is found in *Pitx2*-null mutants.

**3.1.2. Tracheoesophageal septation—**The processes whereby trachea and esophagus form from primitive foregut is of clinical interest due to the common birth defect, tracheoesophageal fistula (TEF) (Fig. 3.5*).*

Usually encountered in conjunction with esophageal atresia (EA), the combined sequence is sometimes found together with other anomalies of heart, vertebrae, anorectum, and limbs. Genetic defects identified in patients with EA-TEF have recently been comprehensively reviewed (Felix *et al.*, 2009). Transgenic murine mutants with deletions in RA receptors or *Gli2*/*Gli3* feature a form of EA-TEF. Moreover, the transcriptomic changes associated with

budding of the lung from the foregut have recently been enumerated. Alongside identifying the known regulators described above, further candidates will need experimental evaluation (Millien *et al.*, 2008). Illustrating that environmental factors may play a role, a EA-TEF phenotype can be generated by exposure of murine embryos to adriamycin (Diez-Pardo *et al.*, 1996). Interestingly, despite the major anomaly of foregut development, lung formation in EA-TEF patients is usually grossly normal. Their respiratory tract morbidity tends to derive from tracheomalacia and, more chronically, reactive airways disease. Whilst the latter is traditionally attributed to gastroesophageal reflux and pulmonary aspiration, it remains possible that some of this pulmonary morbidity stems from subtly abnormal early lung development.

**3.1.3. Tracheal cartilage formation—**Children with EA-TEF may also suffer from tracheal weakness (tracheomalacia) in which inadequate formation of the tracheal cartilages results in potentially life-threatening airway closure during expiration.

Dorsoventral patterning of the trachea during embryonic development is associated with formation of C-shaped cartilage rings ventrally and trachealis muscle dorsally. Ventral mesenchyme segregates into successive cartilaginous and noncartilaginous domains, providing a compromise between flexibility and rigidity.

Tracheomalacia describes weakness of the walls of the trachea and it may result in life-threatening episodes and/or recurrent hospitalizations for lower airway infections (Austin and Ali, 2003; Boogaard *et al.*, 2005; Carden *et al.*, 2005; McNamara and Crabbe, 2004). It can be an isolated idiopathic anomaly or associated with EA-TEF, primary defects of cartilage synthesis (e.g., dyschondroplasia), cartilage degeneration due to trauma (e.g., long-term tracheal intubation), or extrinsic compressive lesions such as vascular rings or tumors (Berdon, 2000). Tracheal stenosis is narrowing of the trachea: it can follow prolonged intubation or accompany a cartilaginous sleeve malformation of the trachea or may again be associated with extrinsic compressive lesions. Tracheal cartilaginous sleeve comprises fusion of the ventral cartilage rings. It is a rare malformation associated with craniosynostosis syndromes like Crouzon syndrome, Pfeiffer syndrome, Goldenhar syndrome or Apert syndrome (Lin *et al.*, 1995).

Tracheal maldevelopment can be modeled: nitrofen administration to pregnant dams results in tracheal malformations as well as CDH in offspring (Diez-Pardo *et al.*, 1996; Xia *et al.*, 1999). Although genetic control of the regulation of tracheal cartilage versus smooth muscle cell (SMC) formation remains unclear, relevant transgenic murine phenotypes have been observed. Miller *et al.* (2004) showed that partial *Shh* inactivation causes tracheobronchial cartilage abnormalities indicative of tracheomalacia. Park *et al.* (2009) demonstrated *Shh* augments *Sox9* expression: Sox9 induces type II collagen (Col2a1) expression and promotes the chondrocyte lineage amongst mesenchymal cells. Bone morphogenic protein 4 (BMP4) also regulates Sox9 to induce chondroprogenitors amongst mesenchymal cells (Hatakeyama *et al.*, 2004). Impaired BMP signaling induces tracheal cartilage defects with EA-TEF (Que *et al.*, 2006). β-Catenin also interacts with Sox9 but to inhibit differentiation of tracheal chondroprogenitor cells (Akiyama *et al.*, 2004).

Recently, FGF18 and FGF10 have also been described to play important roles in tracheal cartilage ring formation (Elluru *et al.*, 2009; Tiozzo *et al.*, 2009; Whitsett *et al.*, 2002). Specific targeted inactivation of *Fgf18*, using the SP-C promoter driving Cre, induced malformation of the cartilage rings (Whitsett *et al.*, 2002). Overexpression of *Fgf18* also resulted in malformation of the cartilage rings, possibly via Sox9 upregulation (Elluru *et al.*, 2009). Tiozzo *et al.* (2009) reported that ectopic fibroblast growth factor receptor (FGFR)2b expression in tracheal mesenchyme renders this hyper-responsive to FGF10, resulting in

cartilaginous sleeve formation reminiscent of the Apert syndrome tracheal phenotype (Fig. 3.6). This abnormal cartilage structure arises secondary to increased proliferation of cartilage progenitor cells within tracheal mesenchyme.

Despite incomplete understanding of such genetics, tissue engineered airway (formed using stem cells and cadaveric scaffold) has been successfully transplanted into adult and a pediatric patients to replace damaged bronchus and trachea, respectively (Macchiarini *et al.*, 2008)

**3.1.4. Branching morphogenesis of airway and vasculature—**A multiplicity of factors are required for normal airway branching morphogenesis. Much of the research in this area is focused on epithelial morphogenesis: this is discussed in detail in subsequent sections dealing with individual signaling pathways. A key insight has been that epithelial morphogenesis proceeds interdependently with vascular development. Indeed tight coupling of endothelial with epithelial development is required for efficient gas transport and fluid clearance at birth. Vascular endothelial growth factor (VEGF) signaling from the epithelium to the developing endothelium is essential for the primitive hemangioblasts to develop into mature capillary networks. Likewise, the endothelium probably signals back to coordinate epithelial morphogenesis. There is a stereotyped anatomical relationship between the developing pulmonary capillaires, arteries, and veins (Fig. 3.7). The arteries run along the superior surface of the developing lobules, while the veins run along the interior surface.

**3.1.5. Alveolar septum formation—**Septation of terminal sacs generates alveoli and involves interacting mesenchymal myofibroblasts, epithelial cells, and endothelial cells. Myofibroblasts are smooth muscle precursors with fibroblast morphology that migrate within nascent septa and deposit elastin (particularly at tips) as the first step of secondary septa development (Bostrom *et al.*, 1996; Lindahl *et al.*, 1997). Alveolar myofibroblast differentiaion requires Lunatic fringe and Notch signaling, and their elastin deposition is PGFa- and FGFR3/4–regulated (Xu *et al.*, 2010). Septal thinning and maturation of the alveolar capillary network are also needed. Interstitial thinning proceeds with expansion of septal epithelial, vascular, and airspace compartments but also myofibroblast apoptosis (Awonusonu *et al.*, 1999; Schittny *et al.*, 1998) that affects lipid-filled interstitial fibroblasts (LFIF) rather than non-LFIF (NLFIF). This apoptosis is associated with downregulation of insulin-like growth factor I receptor (*Igf-IR*) mRNA and cell surface protein expression (Srinivasan *et al.*, 2002).

Finally, the new alveolar septum differentiates into a functional respiratory membrane that consists of type I AECs (AECI), basement membrane, and capillary endothelial cells. The respiratory membrane provides a short distance for diffusion thereby facilitating gas exchange. It is estimated that about 50 million alveoli are present in neonatal lung. However, by age 7–8 years, when alveolarization is largely complete, the number of alveolar units in the lung has increased six-fold to about 300 million. Meanwhile the adut alveolar capillary bed is capable of accommodating the entire adult cardiac output of 5 L/min, rising five-fold to 25 L/min during maximal exercise.

Alveolarization can be adversely affected by premature delivery, hyperoxia, postnatal steroid exposure, and prolonged mechanical ventilation, even with room air (Mokres *et al.*, 2010). Thus, prematurity and hyperoxia plus pressure plus time are well-recognized risk factors for the hypoalveolarization characteristic of BPD in human premature infants. It is postulated that a cascade of events including endotoxin exposure, inflammation, and expression and activation of excessive amounts of transforming growth factor (TGF)-β ligand inhibit aleolarization in BPD and therefore portend an adverse outcome of this disease (reviewed in Shi *et al.*, 2009).

### 3.2. Cataloguing the biochemical regulators of lung development

Having considered the process of building the lung, we next turn to catalogue the factors required for lung growth and maturation. Transgenic mouse technology has allowed us to evaluate roles in organogenesis by, for example, overexpressing or knocking out a specific genes (Costa *et al.*, 2001). Some murine pulmonary phenotypes resulting from a loss or gain of gene function are listed in Table 3.1, modified from Cardoso and Lu (2006).

**3.2.1. Transcription factors—**At least four groups of transcription factors, forkhead box, Nkx homeodomain, RA receptors, and *Gli* family play important roles in lung development.

**Forkhead box transcription factor family:** Members of the forkhead box family transcription factors, such as Foxa1, Foxa2, HFH8, and HFH4, share homology in the winged-helix DNA-binding domain and regulate pulmonary cellular proliferation and differentiation.

HNF-3α (Foxa1) and HNF-3β (Foxa2) share 93% homology in amino acid sequences and were first identified as factors in hepatocyte differentiation (Qian and Costa, 1995). However, *Hnf-3β* is expressed in developing lung, with higher levels in proximal epithelial cells and lower levels in distal type II epithelial cells (Zhou *et al.*, 1996b). Overexpression of *Hnf-3β* under control of epithelial specific *SP-C* promoter inhibits lung branching morphogenesis and vasculogenesis *in vivo* (Zhou *et al.*, 1997). HNF-3α and HNF-3β also regulate expression of CCSP and surfactant proteins in bronchiolar and type II epithelial cells (Bingle *et al.*, 1995; Bohinski *et al.*, 1994; He *et al.*, 2000). *Hnf-3β* is inducible by interferon, and regulates in turn the expression of the *Nkx* homeodomain transcription factor *Nkx2.1* (also termed *Ttf-1* and *Cebpl*), which in turn regulates transcription of the surfactant protein genes in peripheral lung epithelium (Ikeda *et al.*, 1996; Samadani *et al.*, 1995).

HFH8 is restricted to splanchnic mesoderm contacting embryonic gut and presumptive lung at E9.5 suggesting that *Hfh-8* may participate in lung induction. HFH-8 expression continues in lateral mesoderm-derived tissue during development. By E18.5, *Hfh-8* expression is restricted to distal lung mesenchyme and bronchial muscle (Peterson *et al.*, 1997). The level of *Hfh-8* expression is important for normal development: alveolar hemorrhage is observed in *Hfh8*(+/−) mice, while *Hfh8*(−/−) mice die *in utero*. Reduced *Hfh-8* expression in *Hfh-8*$^{+/-}$ mutants is accompanied by decreased expression of VEGF and its receptor 2 (Flk-1), bone morphogenetic protein 4 (BMP-4), and the transcription factors of the Brachyury T-Box family (Tbx2–Tbx5) and Lung Kruppel-like factor (Kalinichenko *et al.*, 2001). HFH8 regulates mesebchymal *Pdgf* receptor (Bostrom *et al.*, 1996; Shinbrot *et al.*, 1994; Souza *et al.*, 1996). HFH8 binding sites are also found in the promoter region of genes, such as *Bmp4, Hgf*, and *Hoxa5*, that are critical regulators of lung morphogenesis (Ohmichi *et al.*, 1998; Weaver *et al.*, 1999).

*Hfh4* (*Foxj1*) regulates ciliated epithelial cell differentiation. It is expressed in E15.5 airway epithelium just before ciliated cells appear (Hackett *et al.*, 1995) and *Hfh4*$^{-/-}$-null mutant mice feature defective ciliogenesis in airway epithelial cells and randomized left–right asymmetry (mimicking human Kartagener syndrome). The latter can result in perinatal lethality, but in low penetrance it gives rise to situs inversus, sinusitis, bronchiectasis, and sterility, all caused by defects in ciliary beat (Brody *et al.*, 2000; Chen *et al.*, 1998). Interestingly, HFH4 and other proximal lung markers such as CCSP are upregulated by BMP antagonist Noggin in mesenchyme-free airway epithelial culture (Hyatt *et al.*, 2002).

*Foxp1, Foxp2*, and *Foxp4* are highly expressed in mouse lung and gut. *Foxp1* and *Foxp4* are expressed in both proximal and distal airway epithelium while Foxp2 is expressed primarily

in distal epithelium. Foxp1 protein expression is also observed in the mesenchyme and vascular endothelial cells of the lung (Lu *et al.*, 2002).

**Nkx and Hox homeodomain transcription factors:** One of the most prominent homeodomain transcription factors in lung development is NKX2.1, also called TTF-1 (thyroid-specific transcription factor) or CEBP-1. *Nkx2.1* is expressed in epithelial cells derived from foregut endoderm in lungs, thyroid, and pituitary, as well as restricted regions of fetal brain (Guazzi *et al.*, 1990; Lazzaro *et al.*, 1991). Hence, human Nkx2.1 mutants may feature benign hereditary chorea, congenital hypothyroidism, and neonatal respiratory distress at term (sometimes retrieved by the transactivating activity of Pax8) (Carre *et al.*, 2009). *Nkx2.1*$^{-/-}$ mice exhibit impaired tracheoesophageal separation and early arrest of lung development featuring two main bronchi but no distal branches (Kimura *et al.*, 1996; Minoo *et al.*, 1999). In developing mouse airway epithelium, *Nkx2.1* is initially expressed proximally and distally becoming restricted at later stages to distal AECs (Zhou *et al.*, 1996b). Overexpression of *Nkx2.1* causes dose-dependent morphological alterations in postnatal lung: modest overexpression raises type II pneumocyte proliferation and SP-B levels; greater overexpression disrupts alveolar septation with emphysema due to alveolar hypoplasia. The highest overexpression of *Nkx2.1* in transgenic mice causes severe pulmonary inflammation, fibrosis, and respiratory failure, associated with eosinophil infiltration and increased eotaxin and IL-6 expression (Wert *et al.*, 2002). Nkx2.1 signaling is critical for surfactant protein, *T1a*, and *CC10* gene expression (Boggaram, 2003; Bruno *et al.*, 1995; Guazzi *et al.*, 1990; Ramirez *et al.*, 1997; Whitsett and Glasser, 1998; Yan *et al.*, 1995; Zhang *et al.*, 1997). *Nkx2.1*-deficient pulmonary epithelial cells fail to express nonciliated marker genes, including differentiated *Sp-B*, *Sp-C*, and *CC10. Bmp4* expression in these cells is also reduced. In addition to modulating expression of other lung-related genes, it is clear that NKX2.1 phosphorylation plays a crucial role in its signaling: mice with point mutation of seven serine phosphorylation sites of NKX2.1 died immediately following birth with malformation of acinar tubules, pulmonary hypoplasia, and reduced expression of surfactant proteins, CC10/secretoglobulin 1A, and *Vegf* (DeFelice *et al.*, 2003). Whilst regulating expression of numerous genes, *Nkx2.1* expression can itself be activated by transcription factors HNF-3β (Ikeda *et al.*, 1996) and GATA-6 (Shaw-White *et al.*, 1999) during lung morphogenesis.

**Hox family transcription factors:** Hox transcription factors are expressed with proximodistal polarity in developing lung: *Hoxa5, Hoxb2*, and *Hoxb5* are restricted to distal lung mesenchyme, whilst *Hoxb3* and *Hoxb4* are expressed in proximal and distal mesenchyme (Aubin *et al.*, 1997; Bogue *et al.*, 1996; Volpe *et al.*, 1997). Illustrating their functional role, *Hoxa5*$^{-/-}$-null mutant mice have tracheal defects and occlusions, impaired lung branching morphogenesis, diminished surfactant protein expression, and alveolar wall thickening (Aubin *et al.*, 1997).

**GLI family zinc-finger transcription factors:** GLI 1, 2, 3 are zinc-finger transcription factors and activated by SHH. All are mesodermally expressed, particularly in the distal lung (Grindley *et al.*, 1997). Combined *Gli2*$^{-/-}$ and *Gli3*$^{-/-}$ mutant mice feature lung agenesis. *Gli3*$^{-/-}$ mice are viable but have small dysmorphic lungs (Grindley *et al.*, 1997). *Gli2* regulates normal lung asymmetry: *Gli2*$^{-/-}$ mice have a fused right and left lung (a small single lobe with defective primary branching in the right lung) and hypoplastic trachea and esophagus that are nevertheless distinct and retain normal proximal–distal differentiation (Motoyama *et al.*, 1998).

**3.2.2. Peptide growth factors—**Embryonic lung mesenchymal and epithelial cells communicate through autocrine and paracrine factors, as demonstrated by effects of added

growth factors on cultured embryonic lung growth (Jaskoll *et al.*, 1988; Warburton *et al.*, 1992).

**FGF family:** FGF family members are found throughout the vertebrates and invertebrates. Their functions in respiratory organogenesis are conserved from *Drosophila* to mammals (Glazer and Shilo, 1991; Sutherland *et al.*, 1996). Based on protein sequence homology, FGFs have been divided into 23 subgroups. Similarly, their cognate transmembrane protein tyrosine kinase receptors (FGFRs) are classified into four types, contributing to the specificity of FGF ligand binding (Ornitz and Itoh, 2001). Heparan sulfate proteoglycan, an ECM glycoprotein, has been reported to be essential for FGF ligand–receptor binding and activation (Izvolsky *et al.*, 2003a,b; Lin *et al.*, 1999). FGFs play critical roles in cell proliferation, migration, and differentiation during development. Early inhibition of murine FGFR signaling shows it is required for early lung branching morphogenesis. Later FGFR inhibition in E14.5 lung decreases prenatal airway tubule formation and is associated with severe emphysema at maturity. At E16.5, FGFR inhibition causes mild focal emphysema. Murine mutants lacking FGFR3 and FGFR4 fail to undergo normal alveolarization, with poorly organized myofibroblasts and excessive amounts of poorly organized elastin. However, inhibition of FGFR signaling after birth did not appear to alter postnatal alveolarization (Hokuto *et al.*, 2003).

FGF10 is one of the most-studied family members during lung development. *Fgf10*-null mice lack distal lung despite formation of larynx and trachea (Min *et al.*, 1998). *Fgf10* is expressed focally in E11–12 mouse peripheral lung mesenchyme and signals through adjacent distal epithelial FGFR2IIIb (whose loss also disrupts lung development) (De Moerlooze *et al.*, 2000). These sites of expression change dynamically, compatible with the idea that FGF10 appears at sites of bud formation (Bellusci *et al.*, 1997b). FGF10 has a chemotactic effect on nearby epithelium in culture: epithelial tips will proliferate and migrate toward FGF10 in mesenchyme or on beads (Park *et al.*, 1998; Weaver *et al.*, 2000). FGF10 controls epithelial differentiation, inducing *Sp-C* expression and downregulating *Bmp4* expression (Hyatt *et al.*, 2002). FGF10 dosage and signal transduction level is critical: mice with 20% of normal FGF10 expression (due to an enhancer trap bearing LacZ inserted 100Kb upstream in the FGF10 promoter) feature lung hypoplasia (Ramasamy *et al.*, 2007); similarly, downstream signaling inhibition by misexpression of Sprouty2 under control of the *Sftpc* promoter induces lung hypoplasia (Mailleux *et al.*, 2001). Several key regulatory molecules such as SHH, BMPs, and TGF-βs crosstalk with FGF10 during embryonic lung morphogenesis: their interactions will be discussed later.

FGF7 (KGF) is found in developing lung mesenchyme at late stages (Post *et al.*, 1996). In early cultured mouse embryonic lung, addition of FGF7 promotes epithelial growth and formation of cyst-like structures with extensive cell proliferation. FGF7 can also contribute to distal airway epithelial cell differentiation (Cardoso *et al.*, 1997; Deterding *et al.*, 1996). *Erm* and *Pea3* are ETS domain transcription factors known to be downstream of FGF signaling. FGF7 can induce *Erm/Pea3* expression more effectively than FGF10. *Erm* is transcribed exclusively in the epithelium, while *Pea3* is expressed in both epithelium and mesenchyme. When examined at E18.5, transgenic expression of a repressor form of Erm specifically in the embryonic lung epithelium shows that the distal epithelium of *Sp-C-Erm* transgenic lungs is composed predominantly of immature type II cells, while no mature type I cells are observed. By contrast, the differentiation of proximal epithelial cells, including ciliated cells and Clara cells, appears to be unaffected (Liu and Hogan, 2002; Liu *et al.*, 2003). FGF7 does not seem to protect against hyperoxic inhibition of normal postnatal alveoli formation and early pulmonary fibrosis, but FGF7 consistently had a significant protective/preventive effect against the development of pulmonary hypertension during

hyperoxia (Frank, 2003). However, *Fgf7*$^{-/-}$ mutant mice have no gross lung abnormalities (Guo *et al.*, 1996), suggesting a FGF7 redundancy during lung development.

FGF9, which signals through FGFR2IIIc, also regulates branching morphogenesis. In E10.5 lung, *Fgf9* is expressed in visceral pleura outlining the lung bud and in bronchial epithelium, while *Fgfr2IIIc* is predominantly expressed in lung mesenchyme. At E12.5 and E14.5, *Fgf9* expression persists in visceral pleura but is no longer detected in epithelium (Colvin *et al.*, 1999). *Fgf9*-null mice exhibit reduced mesenchyme and decreased airway branching but show significant distal airspace formation and pneumocyte differentiation. The reduction in the amount of mesenchyme in *Fgf9*$^{-/-}$ lungs limits expression of mesenchymal *Fgf10* (Colvin *et al.*, 2001). By contrast, addition of recombinant FGF9 protein inhibits the differentiation response of the mesenchyme to N-SHH, but does not affect proliferation (Weaver *et al.*, 2003).

The signaling cascade activated by FGF10 and FGF9 involves FGFR2b and 2c, respectively, as well as Shp2, Raf, MAP ERK kinase (MEK), and extracellular-regulated kinases (ERK) 1 and 2 as signal transducers. MEK inhibition has been shown to reduce lung branching and epithelial cell proliferation, but increase mesenchyme cell apoptosis in fetal lung explants (Papadakis *et al.*, 1997). FGF signaling is regulated at several levels. One of the key inducible negative regulators is the Sprouty family. There are four sprouty (Spry) genes in mouse (*mSpry1–4*) and human (*hSpry1–4*). Murine *Spry2* is expressed in the distal tip of embryonic lung epithelial branches, but is downregulated between the sites of new bud formation. Murine *Spry4* is predominantly expressed in the distal mesenchyme of the embryonic lung (Mailleux *et al.*, 2001), and may play roles in branching morphogenesis. Sprouties (SPRY1, 2, 4) act as suppressors of Ras–MAP kinase signaling (Hacohen *et al.*, 1998; Kramer *et al.*, 1999; Reich *et al.*, 1999). Overexpression of *mSpry2* or *mSpry4* can inhibit lung branching morphogenesis through reducing epithelium cell proliferation (Hadari *et al.*, 1998; Perl *et al.*, 2003; Tefft *et al.*, 2002). SPRED-1 and SPRED-2 are two sprouty related proteins, which contain Enabled/VAsodilator-Stimulated Phosphoprotein (VASP) Homology-1 (EVH-1) domains. *Spreds* are predominantly expressed in mesenchymal cells. Expression of *Spreds* is especially strong in the peripheral mesenchyme and epithelium of new bud formation. After birth, *Spreds* expression decreases, while the expression of *Sprouties* expression remains high. Both *Sprouties* and *spreds* play important roles in mesenchyme– epithelium interaction during lung development (Hashimoto *et al.*, 2002).

**TGF-β/BMP family:** The TGF-β superfamily comprises numerous structurally related polypeptide growth factors including TGF-β, BMP, and activin subfamilies. TGF-β ligands bind to cognate cell surface receptors, and activate Smad proteins, which translocate to the nucleus and modulate target gene expression (Massague, 1998; Shi and Massague, 2003).

**TGF-β subfamily:** The TGF-β ligand subfamily comprises three isoforms, TGF-β1, 2, and 3. TGF-β1 is expressed in early embryonic lung mesenchyme, particularly underlying distal epithelial branch points; TGF-β2 is localized mainly in distal epithelium; TGF-β3 is mainly expressed in proximal mesenchyme and mesothelium (Bragg *et al.*, 2001; Millan *et al.*, 1991; Pelton *et al.*, 1991a,b; Schmidt *et al.*, 1991). Each TGF-β isoform has nonredundant roles revealed by isoform-specific knockouts. Mice lacking TGF-β1 develop apparently normally, but die within 2 months of life from aggressive pulmonary or gut inflammation, as a result of failure to negatively modulate the immune system (McLennan *et al.*, 2000). TGF-β2$^{-/-}$ mutation results in embryonic lethality around E14.5 in mice featuring complex cardiac anomalies and lung dysplasia amongst others (Bartram *et al.*, 2001). TGF-β3$^{-/-}$ mutant mice display cleft palate, retarded lung development, and neonatal lethality with difficulty swallowing and breathing (Kaartinen *et al.*, 1995; Shi *et al.*, 1999). Furthermore, blockade of TGF-β signaling by null mutation of TGF-β activated kinase-1 binding

protein-1 (TAB1) results in lethal cardiovascular and lung dysmorphogenesis (Komatsu *et al.*, 2002).

As with the FGFs, the timing and dosage of TGF-β signaling are critical during lung development. Optimal physiological levels of TGF-β-Smad3 signaling appear essential for secondary alveolar septa formation: abrogation of TGF-β type II receptor in lung epithelial cells reduces alveolar septation and allows emergence of AECI (Chen *et al.*, 2008). However, TGF-β1 overexpression in early mouse embryonic lung epithelium inhibits branching morphogenesis (Zhao *et al.*, 1999), whereas misexpression of *Sp-C* promoter-controlled TGF-β1 in embryonic lung epithelium arrests embryonic lung growth and epithelial cell differentiation whilst inhibiting pulmonary vasculogenesis (Zhou *et al.*, 1996a, 2001). Suggesting a crucial role for optimal TGF-β1 levels in human lung maturation, excessive activated TGF-β1 has been reported in tracheal aspirates of human premature infants who develop more severe BPD (Lecart *et al.*, 2000; Toti *et al.*, 1997). Furthermore, misexpression of TGF-β1 in neonatal rat lung using recombinant adenoviral vectors resulted in neonatal alveolar hypoplasia and interstitial fibrosis; this histological picture closely phenocopies human BPD (Gauldie *et al.*, 2003). By contrast, misexpression of TGF-β1 in adult rats results in chronic progressive interstitial pulmonary fibrosis with increased proliferation and matrix secretion by the mesenchyme (Sime *et al.*, 1997; Zhao *et al.*, 2002). In addition, TGF-β1 may be centrally involved in pulmonary fibrotic responses to bleomycin, or endotoxin and infection (Bonniaud *et al.*, 2005). Blockade of TGF-β signaling via Smad3-null mutation strongly attenuates bleomycin-induced pulmonary fibrosis (Zhao *et al.*, 2002).

The activity of TGF-β signaling is multiply regulated: β6-integrin, Latent transforming growth factor-beta binding proteins (LTBPs), and thrombospondin regulate TGF-β release, whilst β-glycan, endoglin, or decorin modulate TGF-β receptor binding affinity. As expected, mutation of the above genes causes some phenotypes similar to those of TGF-β mutants: loss-of-function mutation in human and mouse endoglin (whose protein binds TGF −β and Alk1, its type I receptor) causes hereditary hemorrhagic telangiectasia (Li *et al.*, 1999; Massague, 2000; McAllister *et al.*, 1994; Urness *et al.*, 2000). Null mutation of LTBP-3 or LTBP-4 causes profound defects in elastin fiber structure and lung alveolarization similar to Smad3 knockout mouse lung (Sterner-Kock *et al.*, 2002; Colarossi *et al.*, 2005; Chen *et al.*, 2005).

In addition, TGF-β signaling blockade has distinct impacts on lung branching morphogenesis and alveolarization depending on whether epithelial or mesenchymal cells are targeted. Mesenchymal TGF-β signaling blockade driven by Dermo1 retards branching after mid-gestation; by contrast, epithelial TGF-β signaling abrogation lacks prenatal impact and only disrupts postnatal lung alveolarization (Chen *et al.*, 2008). Meanwhile, human TGF-β pathway mutations in, for example, TGF-β type II receptor (Alk5) or TGF-β binding proteins such as fibrillin underlie dysplastic matrix elastin defects that predispose to aortic dissection or sudden alveolar rupture in Marfan's syndrome patients (Kaartinen and Warburton, 2003). Thus, TGF-β signaling has to be regulated "just right" with both deficiency and excess deleterious to normal alveolarization (a concept we term the Goldilocks hypothesis).

**BMP subfamily:** BMPs, with more than 20 ligand family members, regulate many processes, including lung development (Hogan, 1996). Expression of *Bmp3, 4, 5*, and *7* is detected in embryonic lung (Bellusci *et al.*, 1996; King *et al.*, 1994; Takahashi and Ikeda, 1996). BMP4 plays a central role in normal lung development (Hogan, 1996). Addition of BMP4 to whole embryonic lung explants stimulates branching (Bragg *et al.*, 2001; Shi *et al.*, 2001). However, BMP4 inhibited FGF10-induced growth of isolated E11.5 mouse lung

endoderm cultured in Matrigel (Weaver *et al.*, 2000). Transgenic overexpression of BMP4 in distal fetal lung endoderm, driven by a 3.7 kb human surfactant protein C (SP-C) promoter, causes abnormal morphogenesis with cystic terminal air sacs (Bellusci *et al.*, 1996). Conversely, *Sftp-C* promoter-driven overexpression of BMP antagonists Noggin or Gremlin severely reduces distal epithelial cell phenotype whilst increasing proximal cell types (Lu *et al.*, 2001; Weaver *et al.*, 1999). Interestingly, blockade of endogenous BMP4 in embryonic mouse lung epithelial cells using a conditional gene knockout approach results in abnormal lung development with similar dilated terminal sacs as seen in BMP4 transgenic mouse lung (Eblaghie *et al.*, 2006). This suggests optimal BMP4 levels are essential for normal lung development. As extracellular growth factors, BMPs bind heteromeric complexes of BMP serine/threonine kinase type I and type II receptors to activate intracellular signal pathway (Massague, 1998; Shi and Massague, 2003). Three cognate BMP type I receptors (Alk2, Alk3, and Alk6) have been identified. Among them, Alk3 is expressed predominantly in distal airway epithelial cells during mouse lung development. Alk3 abrogation in mouse lung epithelia either from early lung organogenesis or from late gestation resulted in similar neonatal respiratory distress phenotypes, accompanied with collapsed lungs (Sun *et al.*, 2008). Early induction of Alk3 knockout in lung epithelial cells causes retardation of early lung branching morphogenesis and reduces cell proliferation and differentiation. But late gestation induction of Alk3 knockout also causes significant epithelial apoptosis accompanied by lack of surfactant secretion (Sun *et al.*, 2008). Furthermore, canonical Wnt signaling was perturbed, possibly through reduced WIF-1 expression in Alk3 knockout lungs (Sun *et al.*, 2008). Therefore, deficiency of appropriate BMP signaling in lung epithelial cells results in prenatal lung malformation, neonatal atelectasis, and respiratory failure.

In addition, BMP signaling is also important in lung vasculogenesis and angiogenesis. Mutations of BMP type II receptor (BMPRII) and change in expression of BMP antagonist Gremlin are associated with primary pulmonary hypertension (PPH) (Lane *et al.*, 2000; Costello *et al.*, 2008). Moreover, upregulation of Gremlin is also associated with pulmonary fibrosis and the severity of the fibrotic pathology (Koli *et al.*, 2006; Myllarniemi *et al.*, 2008)

**Sonic hedgehog (Shh) pathway:** Sonic hedgehog is a vertebrate homolog of *Hedgehog* (Hh) that patterns the segment, leg, wing, eye, and brain in *Drosophila*. Hh binds to patched (Ptc), a transmembrane protein, and releases its inhibitory effect on downstream smoothened (Smo), which is a G protein-coupled transmembrane spanning receptor. This leads to the activation of cubitus interruptus (Ci), a 155-kDa transcription factor that is cleaved to form a 75-kDa transcription inhibitor in cytosol. Elements of the *Drosophila* Hh signaling pathway and their general functions in the pathway are highly conserved in vertebrates, albeit with increased levels of complexity. Gli1, 2, and 3 are the three vertebrate Ci gene orthologs (van Tuyl and Post, 2000).

The SHH signal transduction pathway plays important roles in mesenchyme–epithelium interaction. In developing mouse lung, *Shh* is detected in the tracheal diverticulum, the esophagus, and later in the trachea and lung endoderm. *Shh* is expressed at low levels throughout the epithelium, whilst at higher level in the growing distal buds (Bellusci *et al.*, 1997a; Urase *et al.*, 1996). Null mutation of *Shh* produces profound lung hypoplasia and failed trachea–esophageal septation. Mesenchymal *Ptc, Gli1*, and *Gli3* expression are all downregulated in *Shh* knockout lung. Nevertheless, proximodistal differentiation of airway epithelium is preserved (Litingtung *et al.*, 1998; Pepicelli *et al.*, 1998). Also, *Fgf10* expression is dysregulated in *Shh*-null mutant lung compared to the precisely restricted expression seen normally. Lung-specific *Shh* overexpression results in severe alveolar hypoplasia and significant increase in interstitial tissue caused by increased epithelial and

mesenchymal proliferation (Bellusci *et al.*, 1997a). Defective hedgehog signaling may lead to EA and TEF (Spilde *et al.*, 2003).

The membrane-bound Hedgehog interacting protein 1 (HIP1) directly binds mammalian Hedgehog (HH) proteins and attenuates their signaling (Chuang and McMahon, 1999). *Hip1* is transcriptionally activated in response to HH signaling, overlapping the expression domains of *Ptc1* (Chuang and McMahon, 1999; Goodrich *et al.*, 1996). Targeted disruption of *Hip1* results in upregulated Hedgehog signaling and lethal neonatal respiratory failure: left– right asymmetry persists but initial branching from the two primary buds is absent; *Fgf10* expression is slightly downregulated at the tips of the primary buds in $Hip1^{-/-}$ lungs at E10.5 but completely absent from the mesenchyme where secondary branching normally initiates (Goodrich *et al.*, 1996). Attenuated PTC1 activity in a $Hip1^{-/-}$ mutant lungs leads to an accelerated lethality. *Hip1* and *Ptch1* have redundant roles in lung branching control (Goodrich *et al.*, 1996). Both of them can attenuate SHH signal in lung development and pancreas development (Goodrich *et al.*, 1996; Kawahira *et al.*, 2003).

**Wnt/β-catenin pathway:** Wnt signals are transduced via seven transmembrane Wnt receptors encoded by *Frizzled* (*Fzd*) genes to activate the β-catenin T Cell transcription Factor (TCF) pathway, the c-Jun N-terminal kinases (JNK) pathway, or the intracellular $Ca2^{+}$-releasing pathway. The Wnt/β-catenin pathway plays a critical role in many developmental and tumorigenesis processes. Following Wnt binding to the receptor, β-catenin is dephosphorylated and translocates to the nucleus to activate downstream gene expression (Wodarz and Nusse, 1998).

TOPGAL and BATGAL reporter transgenes have been used to analyze patterns of β-catenin stabilization in developing lung. Within the respiratory precursor region, the TOPGAL reporter is expressed in the undivided proximal endodermal tube and then the lung buds as early as E9.5 (Okubo and Hogan, 2004). This pattern is maintained as the trachea and esophagus separate and the lung buds grow out between E10 and E11.5 (Dean *et al.*, 2005; De Langhe *et al.*, 2005; Okubo and Hogan, 2004; Shu *et al.*, 2005). Between E12.5 and E18.5, analysis of TOPGAL and BATGAL transgene activity suggests a dynamic pattern of TCF/β-catenin-dependent gene expression. Reporter gene activity is found in the tracheal epithelium and cartilaginous condensations at E12.5 but is restricted to the bronchial mesenchyme at E13.5 (De Langhe *et al.*, 2005; Shu *et al.*, 2005). The distal lung epithelium expresses both reporters by E9.5. The pattern of TCF/β-catenin-dependent gene activity in the distal lung at later time points is somewhat variable and dependent on the reporter transgene analyzed. In general, transgene activity clears from the central airways between E13.5 and postnatal day 14 (Okubo and Hogan, 2004; Shu *et al.*, 2005). At E14.5, expression in the distal tip epithelium is either extinguished (TOPGAL) (De Langhe *et al.*, 2005) or restricted to a subset of early alveolar type 2 cells (BATGAL) (Shu *et al.*, 2005). In the adult lung, the TOPGAL transgene is highly expressed in the distal trachea and in clusters of airway secretory and ciliated cells but rarely in the alveolar region (De Langhe, unpublished data).

*β-catenin* deletion in proximal airway epithelium during development resulted in no obvious alteration to lung structure (Mucenski *et al.*, 2003). By contrast, embryonic deletion of *β-catenin* in the distal lung epithelium resulted in profound perturbation of normal epithelial, mesenchymal, and vascular development. The latter mice feature proximalization of lung epithelium with decreased expression of alveolar type 2 cell marker Sftpc, vascular endothelial marker PECAM, and α-smooth muscle actin; upper airway epithelial markers (Scgb1a1, FoxJ1, and β-tubulin) were unaltered.

Stabilization of β-catenin in proximal epithelium using the *Catnb*$^{floxedExon3}$ allele raised epithelial β-catenin levels, resulting in squamous, cuboidal, and goblet cell dysplasia in intrapulmonary conducting airways and the appearance of alveolar type 2-like cells in the bronchioles (Mucenski *et al.*, 2005). Epithelial levels of Scgb1a1 immunopositive cells were low whilst SPC expression increased, indicating an increase in Scgb1a1/Sftpc double-positive cells. Similar expansion of Scgb1a1/Sftpc double-positive bronchioalveolar stem cells (BASCs) in response to increased canonical Wnt signaling has been shown in the lung epithelium upon *Gata6* loss (Zhang *et al.*, 2008). These authors also showed that canonical Wnt signaling is activated within the niche containing BASCs during lung epithelial regeneration, while forced Wnt activation greatly increases BASC numbers.

Li *et al.*, (2009) stabilized β-catenin in the entire developing lung epithelium using Nkx2.1-cre and Catnb[+/lox(ex3)] mice: in trachea and main bronchi, polyp-like structures formed featuring intracellular β-catenin accumulation suggesting blocked differentiation of spatially-appropriate airway epithelial cell types, Clara cells, ciliated cells, and basal cells (BCs), while activating UCHL1, a marker for pulmonary neuroendocrine cells.

Alternatively, the method of using a *Spc* promoter-regulated *Lef1-dN89β-catenin* to stabilize β-catenin from about E10.5 was employed by Okubo and Hogan (2004) to generate mice with widened primary bronchial tubes opening directly into saccules (lined with simple cuboidal or columnar epithelium), decreased progenitor differentiation into secretory and ciliated cells, and absence of alveolar type 2 and type 1 cells. Thus, constitutive β-catenin signaling in developing foregut endoderm partially inhibited branching morphogenesis and blocked expression of lung-specific differentiation genes.

Using a hypomorphic *Fgf10* allele, Ramasamy *et al.* (2007) showed that FGF10 signaling via FGFR2b controls the proliferation of the pulmonary epithelial progenitors in part by autoregulation of β-catenin signaling in the epithelium. This correlation of a reduction in epithelial FGF signaling and epithelial TOPGAL activity has also been demonstrated in lungs of a mouse Apert disease model (De Langhe *et al.*, 2006). Intriguingly, the regulation of epithelial β-catenin signaling by FGF10 and concomitant upregulation of *Fgfr2b* receptor expression result in potentiating this signaling cascade locally, thus maintaining the distal epithelial progenitor state.

By contrast, the lack of significant activity of well-established Wnt reporters in mesenchyme (including TOPGAL and BATGAL mice) does not support an important role for mesenchymal Wnt signaling during organogenesis. However, expression of several mesenchymal Wnt receptors in the lung has been reported (De Langhe *et al.*, 2005). Furthermore, Wnt5a overexpression either directly or indirectly regulates mesenchymal *Fgf10* expression (Li *et al.*, 2005), while Wnt7b acts on lung vascular SMCs via Frizzled 1 and LRP5 (Wang *et al.*, 2005). Besides Lef1/TCF-mediated β-catenin signaling, β-catenin also acts via PITX family transcription factors (Kioussi *et al.*, 2002), which are abundantly expressed in developing mesenchyme (Kitamura *et al.*, 1999). Using *Dermo1*$^{Cre/+}$-mediated conditional inactivation (CKO) of *β-catenin*, De Langhe *et al.* (2008) showed *Dermo1-cre/β-catenin* CKO embryos have multiple defects reminiscent of double knockout of *Pitx1* and *Pitx2* genes (Marcil *et al.*, 2003). Combining fate analysis and global gene expression studies, mesenchymal β-catenin signaling was shown to have dual, lineage-dependant functions: it regulates formation and amplification but not differentiation of *Fgf10*-expressing parabronchial smooth muscle progenitors (in part via regulation of *Fgfr2c* expression) but is required for normal endothelial cell differentiation (De Langhe *et al.*, 2008). Cohen *et al.* (2009) confirmed the role of Wnt in parabronchial smooth muscle development and showed Wnt pathway upregulation in experimental asthma.

**Epidermal growth factor (EGF) family growth factors:** EGF, TGF-α, and amphiregulin are EGF receptor (EGFR) ligands. Loss- or gain-of-function experiments in mouse, rat, or other animal models prove that EGF ligands positively modulate early mouse embryonic lung branching morphogenesis and cytodifferentiation through EGFR (Schuger *et al.*, 1996a; Seth *et al.*, 1993; Warburton *et al.*, 1992). EGF is expressed in mature AECs and regulates type 2 cell proliferation via autocrine mechanism in culture and *in vivo* (Raaberg *et al.*, 1992). However, epithelial TGF-α overexpression under *Sp-C* promoter control induces postnatal lung fibrosis (Korfhagen *et al.*, 1994). TGF-α overexpression caused severe pulmonary vascular disease mediated via EGFR in distal epithelium, but reductions in VEGF may also contribute (Le Cras *et al.*, 2003).

EGFR is a tyrosine kinase receptor whose deletion (*Egfr*$^{-/-}$) causes abnormal branching, poor alveolarization, and aberrant matrix metalloprotease protein (MMP) expression (Kheradmand *et al.*, 2002). EGFR phosphorylation in response to stretch induces, at least in part, fetal epithelial cell differentiation via ERK pathway activation. Specific EGFR or ERK pathway blockade reduces stretch-inducible *Sp-C* mRNA expression. Thus, EGFR may represent a mechanical signal sensor during lung development (Sanchez-Esteban *et al.*, 2003).

Tumor necrosis factor-α (TNFα)-converting enzyme (TACE) is a transmembrane metalloprotease disintegrin that functions as a membrane sheddase to release the ectodomain portions of many transmembrane proteins, including the precursors of TNFα and several other cytokines, as well as the receptors for TNFα, and neuregulin (ErbB4) (Shi *et al.*, 2003). Neonatal TACE-deficient mice had visible respiratory distress and their lungs failed to form normal saccular structures. Mouse embryonic lung explant cultures show that TGF-α and EGF can rescue the inhibition of TACE activity (Zhao *et al.*, 2001).

**Platelet-derived growth factors (PDGFs):** PDGF-A and PDGF-B form homodimers (AA or BB) or heterodimers (AB). Two PDGF receptor types, α and β, occur in embryonic mouse lung and are differentially regulated in fetal rat lung epithelium and fibroblasts (Buch *et al.*, 1994). PDGF-A regulates DNA synthesis and branching in cultured embryonic murine lung epithelium (Souza *et al.*, 1995b). *Pdgf-a*$^{-/-}$ or *Pdgf-α*$^{-/-}$ mutants die perinatally with loss of alveolar myofibroblasts and SMCs and reduced parenchymal elastin fiber deposition; furthermore, failed alveolar septation is associated with emphysema (Bostrom *et al.*, 1996, 2002). Antisense oligodeoxynucleotide abrogation of PDGF-B reduces the epithelial component of embryonic mouse lung explants but not branch number (Souza *et al.*, 1994). PDGF-B and its receptor are crucial for vascular development during the alveolar phase (Lindahl *et al.*, 1997). PDGF-C and D also dimerize and bind PDGF α or β receptor (LaRochelle *et al.*, 2001; Li *et al.*, 2000). PDGF-C mRNA expression shows a significant increase in bleomycin-induced lung fibrosis (Zhuo *et al.*, 2003).

**Insulin-like growth factors (IGFs):** IGFs and their receptors are expressed in rodent and human fetal lung (Batchelor *et al.*, 1995; Lallemand *et al.*, 1995; Maitre *et al.*, 1995; Retsch-Bogart *et al.*, 1996; Schuller *et al.*, 1995). Null mutant mice for the cognate type 1 IGF receptor (*Igf1r*) gene die at birth with respiratory failure and growth deficiency (45% of normal birth weight). Dwarfism is further exacerbated (70% of size reduction) in either *Igf1* or *Igf2* double null mutants or in *Igf1r* and *Igf2* double null mutants. There does not appear to be a gross defect in branching morphogenesis *per se*; the lungs merely appear hypoplastic (Liu *et al.*, 1993). IGF signaling may play a role in facilitating other peptide growth factor pathways during lung morphogenesis, e.g., IGF1R signaling is required for both mitogenic and transforming activities of EGFR (Coppola *et al.*, 1994). *Igf1*-deficient mice have reduced airspaces, which are exacerbated in mice with additional leukemia inhibitory factor (*Lif*) deletion (featuring abnormal epithelium and decreased Sp3, *Nkx2.1*, and *Sp-B*

expression) (Pichel *et al.*, 2003). IGF1 is also trophic for fetal lung endothelial cells: in human fetal lung explants, IGF-IR inactivation results in endothelial cell loss, attenuates time-dependent increase in budding of distal airway, and increases mesenchymal cell apoptosis (Han *et al.*, 2003).

**Vascular endothelial growth factor (VEGF) isoforms and cognate receptors:** Effective pulmonary gas exchange requires alignment between alveoli and pulmonary capillaries. VEGFs regulate pulmonary vascular development (reviewed extensively in Pauling and Vu, 2004 and in Warburton *et al.*, 2000 and in Warburton *et al.*, 2003; Shi *et al.*, 2007) and signal through cognate receptors Flk-1 (fetal liver kinase-1, VEGFR2) and Flt-1 (fetal liver tyrosinase-1, VEGFR1) (Larrivee and Karsan, 2000). VEGF is regulated by hypoxia-inducible transcription factor-2α (Compernolle *et al.*, 2002) and expression is controlled transcriptionally by hypoxia inducible factor-1 (HIF1) (see Dunwoodie, 2009 for an excellent review). Isoforms VEGF 120, 164, and 188 are expressed in E12.5 murine lung epithelial and mesenchymal cells and regulate endothelial proliferation and microvascular structure (Greenberg *et al.*, 2002; Ng *et al.*, 2001). As epithelial branching progresses, *Vegf-A* expression becomes restricted to distal lung (Healy *et al.*, 2000), which may be partly due to the high affinity of VEGF-A for matrix components concentrated around branching tips (Acosta *et al.*, 2001; Ng *et al.*, 2001). Epithelial and vascular branching morphogenesis of cultured mouse embryonic lung features epithelial, mesenchymal, and endothelial crosstalk mediated in part by VEGF-A signaling via Flk-1 (Del Moral *et al.*, 2006a). Inhibited VEGF signaling disrupts pulmonary endothelial survival and reduces postnatal alveolarization (Kasahara *et al.*, 2000). Neonatal lungs treated with antibodies to Flt-1 are small with simplified alveoli (Gerber *et al.*, 1999). VEGR3$^{-/-}$ mice have lymphatic hypoplasia and lethally delayed removal of lung liquid at birth. Excessive VEGF signaling also disrupts vascular and epithelial lungmorphogenesis: *Vegf* misexpression under SP-C promotercontrol yields decreased acinar tubules and mesenchyme (Zeng *et al.*, 1998).

**ROBO/SLIT:** Roundabout (ROBO) is a receptor involved in repellent signaling and controlling axonal extension and with its ligand SLIT regulates non-neuronal cell migration (Wu *et al.*, 2001). Suggesting interaction during perinatal lung development, *Slit-2* is expressed in saccular mesenchyme whilst *Robo* is expressed in adjacent apical epithelium (Anselmo *et al.*, 2003). A *Robo* knockout mouse loses alvelolar septation with thickened mesenchyme (Xian *et al.*, 2001).

### 3.2.3. Other biochemical factors

**Small noncoding microRNAs (miRNAs) and lung development:** A class of noncoding RNAs called microRNAs (miRNAs) has been recognized for their abundance and conservation between species (Ambros, 2001; Lau *et al.*, 2001; Lagos-Quintana *et al.*, 2001). An intranuclear primary transcript (pri-miRNA) is cleaved by Drosha RNase III endonuclease to liberate the pre-miRNA, a 60–70 nt stem loop intermediate (Lee *et al.*, 2002). Pre-miRNA is transported to the cytoplasm by Exportin-5 (Yi *et al.*, 2003) where Dicer, another RNase III endonuclease, generates the mature miRNA. Mature miRNAs are incorporated as single-stranded RNAs into a ribonucleoprotein complex, the RNA-induced silencing complex (RISC) that downregulates gene expression by mRNA cleavage or translational repression. Mice lacking Dicer die before gastrulation indicating a fundamental developmental role of miRNAs. In lung, conditional epithelial Dicer inactivation arrests branching and disrupts Fgf10 expression (Harris *et al.*, 2006).

Our studies (Carraro *et al.*, 2009) and others' (Lu *et al.*, 2007) highlighted specific miRNAs' importance in lung epithelial cell development. Ubiquitous developing epithelial overexpression of the miR-17–92 cluster augments multipotent Sox9-expressing epithelial

cell number at the expense of delayed epithelial differentiation (Lu *et al.*, 2007). In addition, miR-17 and its paralogs miR-20a and miR-106b target Stat3 and Mapk14 and thereby regulate E-Cadherin expression by modulating FGF10–FGFR2b downstream signaling. Thus, mir17 paralogs control a key pathway modulating the FGF10–FGFR2b–Sprouty-driven bud morphogenesis periodicity clock (Carraro *et al.*, 2009; Warburton *et al.*, 2008).

**Extracellular matrix and lung development:** Differentially expressed protein components of extracellular basement membrane, laminins (LNs), entactin/nidogen, type IV collagen, perlecan, SPARC, and fibromodulin mediate cell–cell and cell–ECM interaction during lung morphogenesis. ECM components provide tissue support, may modulate cell proliferation and differentiation (Lwebuga-Mukasa, 1991), and serve as barrier and reservoir for growth factors. Preventing epithelial interaction with basement membrane disrupts lung development (Hilfer, 1996; Minoo and King, 1994).

LNs are glycoproteins involved in cell adhesion, migration, proliferation, and differentiation during tissue development and remodeling. LNs are composed of three chains, one central (α) and two lateral (β and γ), linked by disulfide bonds to form a cross-shaped molecule (Burgeson *et al.*, 1994). To date five α, three β, and three γ chain isoforms have been identified, which suggests their combination can lead to approximately 30 LN variants (Bernier *et al.*, 1995; Ehrig *et al.*, 1990; Galliano *et al.*, 1995; Iivanainen *et al.*, 1995a,b, 1997a,b, 1999; Koch *et al.*, 1999; Pierce *et al.*, 1998; Vuolteenaho *et al.*, 1994). The α1 chain is principally localized in basement membrane at the epithelial–mesenchymal interface with a predilection for specific zones. LN α1 chain also surrounds some mesenchymal cells. A domain in the cross-region of the α1 chain influences lung epithelial cell proliferation (Schuger *et al.*, 1992). The α4 chain in LN8 and LN9 variants is highly expressed developing murine lung and heart (Frieser *et al.*, 1997; Iivanainen *et al.*, 1995a,b, 1997). LN α4 chain is localized around vessels in fetal lung and may assist organization of lung mesenchyme (Miner *et al.*, 1997; Pierce *et al.*, 1998). The α5 chain in LN10 and LN11 is abundantly expressed during lung morphogenesis (Miner *et al.*, 1995, 1998; Pierce *et al.*, 1998): mutated LN α5 chains impair lobe septation and bronchiolar branching in mutant murine lung.

With roles in cell adhesion, β1 and γ1 are constantly expressed during fetal lung development (Durham and Snyder, 1995): globular domains near their N-termini help regulate cell polarization (Schuger *et al.*, 1995, 1996b). LN β2 chain isoform localizes to the basement membrane of prealveolar ducts, airways, SMCs of airways, and arterial blood vessels, as well as type II pneumocytes.

Nidogen (150 kDa) in basement membrane binds γ1 and γ3 chains helping link LN to collagen IV (Dziadek, 1995; Koch *et al.*, 1999; Reinhardt *et al.*, 1993). Nidogen is mesenchymally synthesized and appears to help basement membrane organization during lung morphogenesis (Senior *et al.*, 1996). Blocking Nidogen's interaction with LN disrupts lung development (Dziadek, 1995; Ekblom *et al.*, 1994; Senior *et al.*, 1996). Nidogen to degradation by matrix metalloproteinases (MMPs) may facilitate remodeling via basement membrane degradation (Mayer *et al.*, 1993).

Proteoglycans comprise a protein core with sulfated carbohydrate side chains. They form flexible structures and function as a reservoir for growth factors, water, and ions. Inhibiting proteoglycan sulfation disrupts branching of E13 mouse lung explants and inhibits epithelial migration toward lung mesenchyme or FGF10-soaked beads (Shannon *et al.*, 2003). Perlecan is a predominant basement membrane proteoglycan, composed of an approximately 450 kDa core protein with three heparan sulfate chains. Pulmonary perlecan synthesis rises sharply with increased fetal SMC proliferation (Belknap *et al.*, 1999).

Fibronectin (FN) is essential for clefting during initial epithelial branching in salivary gland and is accumulated at sites of epithelial constriction and indentation in lung and kidney (Sakai *et al.*, 2003), supporting roles for FN in lung branching morphogenesis (Roman, 1997). Indeed, FN is a Wnt target required in the control mechanism of lung bud tip splitting (De Langhe *et al.*, 2005). Treating lung rudiments with anti-FN antibody or siRNA inhibited branching morphogenesis, while FN supplementation promoted branching (Sakai *et al.*, 2003). Located in epithelium and mesenchyme, the EIIIA segment is one of the alternatively spliced FN segments, modulating FN's cell proliferative effect: expression decreases from pseudoglandular to saccular stages, increases into the alveolar stage, and parallels changes in Proliferating Cell Nuclear Antigen (PCNA)-positive distal pulmonary cell number (Kikuchi *et al.*, 2003).

MMPs are ECM-degrading enzymes that are inhibited by tissue inhibitors of metalloproteinases (TIMPs). MMPs may alter cell fate and behavior by ECM modulation and modulate signaling of bioactive molecules by their cleavage, by their release from bound stores, or by altering activity of their inhibitors (Vu and Werb, 2000). *Timp3*-null mutant mice have attenuated airway branching and alveologenesis (Gill *et al.*, 2003) and develop progressive emphysema-like changes (Leco *et al.*, 2001). MMP2, MMP9, and their tissue inhibitors (TIMP1 and TIMP2) are influenced by early postnatal dexamethasone exposure: *Timp2* expression falls and *Mmp9* expression increases. Such changes may contribute to steroids' effects on neonatal lung structure (Valencia *et al.*, 2003). MT1-MMP, a potent MMP2 activator, is a major downstream EGFR target. $Egfr^{-/-}$ mice had low *MT1-Mmp* expression with 10-fold reduction in active MMP2. Abnormal lung alveolarization in $Mmp2^{-/-}$ mice is similar to but less severe than that in neonatal $Egfr^{-/-}$ mice (Kheradmand *et al.*, 2002).

**Retinoic acid signaling:** Retinoids (all-trans, 9-cis, and 13-cis) are fundamental for development and homeostasis of numerous systems including lung, and there is a well-described mammalian RA synthesis and degradation system (Chambon, 1996). Retinaldehyde dehydrogenase-2 (RALDH-2) plays a prominent role in generating RA during organogenesis (Niederreither *et al.*, 1997, 1999; Ulven *et al.*, 2000). RA signaling is mediated by its nuclear receptors of the steroid hormone receptor superfamily: RAR (α, β, γ) and retinoid RXR (α, β, γ) (Chambon, 1996). RAR/RXR heterodimers also transduce RA signaling *in vivo* (Kastner *et al.*, 1997). Within E13.5 lung, *Rar-β* isoform transcripts are localized to proximal airway epithelium and adjacent mesenchyme, whereas *Rarα1, Rarα2*, and *Rarγ2* isoforms are ubiquitously expressed (Chazaud *et al.*, 2003).

RA signaling is required for lung bud initiation. Acute vitamin A deprivation in pregnant rats at the onset of lung development results in blind-ending tracheae and lung agenesis in some embryos, which is similar to the case of $Fgf10^{-/-}$ mutant mice (Dickman *et al.*, 1997; Sekine *et al.*, 1999). Disruption of RA signaling in *Rarα/β2* knockout mice causes left lung agenesis and right lung hypoplasia (Mendelsohn *et al.*, 1994). Lung branching morphogenesis is characterized by dramatic downregulation of RA signaling. Preventing this by treating embryonic lung explants with high RA concentrations ($10^{-6}$–$10^{-5}$ M) disrupts distal budding with formation of proximal-like immature airways (Cardoso *et al.*, 1995; Malpel *et al.*, 2000). Continued RA activation by overexpression of constitutively activated *Rara* chimeric receptors meant lungs did not form saccules or identifiable type I cells: raised epithelial *Sp-C, Nkx2.1*, and *Gata6* levels (but not *Sp-A* or *Sp-B*) at birth suggested differentiation was arrested early in these lungs. Downregulation of RA signaling is required to allow differentiation to form mature type I and II cells (Wongtrakool *et al.*, 2003). RA inhibits expression and alters *Fgf10* and *Bmp4* distribution (Cardoso *et al.*, 1995; Malpel *et al.*, 2000). Pan-RAR antagonism alters *Tgf-β3, Hnf-3β*, and *Cftr* expression in

proximal tubules and *Bmp4, Fgf10*, and *Shh* expression in distal buds (Chazaud *et al.*, 2003).

In early E11–12.5 murine lung, *Raldh-2* is concentrated in trachea (mesenchyme) and proximal lung (mesothelium) at sites of limited branching: this lack of overlap with *Fgf10* suggests RA may restrict *Fgf10* expression thereby defining the proximal–distal lung axis. However, during postnatal lung development, RA increases alveolar number, partially rescuing dexamethasone's effects. In adult rats, RA reverses features of elastase-induced emphysema (Maden and Hind, 2003; Massaro and Massaro, 1996, 2000). *RAR-γ*-null mice have defective alveolar septation consistent with abnormal elastin deposition (Chytil, 1996). Additional deletion of a retinoid X receptor (*RXRα*) allele decreases alveolar surface area and number (McGowan *et al.*, 2000). RA is a vitamin A metabolite. Vitamin A deficiency injures lung and impairs rat type II pneumocyte function (McGowan *et al.*, 2000). This combined evidence suggests RA may have a complex role in alveolar development.

## 4. Mechanobiology of the Developing Lung

Mechanical stimuli to lung development have been long appreciated. Recent advances in molecular and stem cell biology allow these fields to be integrated with modern mechanobiology (Ingber, 2003). For example, ECM polymers, in addition to binding and presenting growth factors, provide resistance to deformation, fluid flow, and diffusion, and transmit force over surprisingly long distances. Adhesion molecules regulate cell motility and tissue structure, and these in turn interact through forces and deformations. Cytoskeletal components generate and transmit forces and conversely provide resistance to deformation. If we do not understand the relevant physics, we miss major factors in development, physiology, and regulation.

In an inflated rubber glove, raising internal pressure *decreases* curvature with *simplification* of form. By contrast, in lung increased internal pressure is associated with *increased* curvature and more complex morphology. Why this difference? Part of the answer is that the rubber glove is a thin elastic shell whilst embryonic tissue better resembles a viscoelastic fluid (Forgacs *et al.*, 1998; Jakab *et al.*, 2008). Over the timescale of growth, the elastic component of tissue viscoelasticity may be neglected; the tissue therefore behaves mechanically as a fluid, such that a simple mechanobiological model (Lubkin and Murray, 1995) predicts pulmonary pressure–morphology relationships (Unbekandt *et al.*, 2008).

Mechanics also influence differentiation: *in vitro* mesenchymal stem cells differentiate toward neurons at 1 kPa, muscle at 10 kPa, and cartilage at 30 kPa (Engler *et al.*, 2006). Hypothesizing that lung seeks to equilibrate tangential epithelial stress, a mechanobiological model of pseudoglandular lung (Lubkin and Murray, 1995) treated the epithelium as a viscous fluid with surface tension (Foty *et al.*, 1994) to predict that branch size will be inversely related to pressure difference between external medium (and native mesenchyme) and lumen. Indeed embryonic lung epithelium appears to regulate tangential stress by modulating cytoskeletal tension via the Rho– ROCK system (Moore *et al.*, 2005).

### 4.1. Lessons on mechanobiology from human and *in vivo* studies

Human birth defects and *in utero* experiments have demonstrated lung development is subject to mechanics. For example, CDH (Smith *et al.*, 2005) comprises a diaphragmatic defect, intrathoracic herniation of abdominal viscera and lung hypoplasia: affected newborns retain a high mortality rate due to inadequate lung growth. Traditionally, lung hypoplasia was attributed to lung compression by herniated abdominal viscera. Indeed lung growth is impaired when fetal CDH is created surgically (Starrett and de Lorimier, 1975). Similarly, human fetuses with renal agenesis or profound renal failure exhibit Potter's syndrome, in

which an underfilled amniotic cavity is thought to cause lung hypoplasia due to excessive lung fluid loss and/or fetal thorax compression. Certainly, bilateral fetal nephrectomy impairs ovine lung growth (Wilson *et al.*, 1993). Alternatively, lung hypoplasia may result from developmental insults to the lung that precede or coincide with the origins of CDH and renal agenesis, respectively. For example, in the nitrofen-induced CDH model, early lung malformation precedes CDH (Jesudason *et al.*, 2000). Similarly, lung hypoplasia emerges before fetal urine output normally contributes to amniotic fluid in a transgenic murine model of renal dysgenesis (Smith *et al.*, 2006). Synthesizing these positions argues for an early developmental insult to the lung that is then compounded by unfavorable mechanical influences (Keijzer *et al.*, 2000). In addition to extrinsic forces acting on fetal lung, a distending pressure is generated by lung liquid production. Draining this fluid by fetal tracheostomy is associated with lung hypoplasia (Fewell *et al.*, 1983). Likewise, retention of this fluid in congenital laryngeal atresia is associated with lung overgrowth and distension (Harding and Hooper, 1996). This led to development of fetal tracheal occlusion to rescue hypoplastic lung growth in human CDH (Harrison *et al.*, 2003; Hedrick *et al.*, 1994). The normal fetal larynx appears to open only during diaphragmatic contraction (fetal breathing movements: FBMs), which restricts lung liquid efflux (Fewell and Johnson, 1983). Hence, failure of FBM in CDH may also contribute to lung hypoplasia. Experimental FBM abolition by phrenic nerve section is associated with lung hypoplasia (Miller *et al.*, 1993). Loss of skeletal muscle formation also causes lung hypoplasia: thinned diaphragms in MyoD$^{-/-}$ mice cannot support FBM and the lungs are hypoplastic with reduced cell proliferation at E18.5 (Inanlou and Kablar, 2003). Neonatally, mechanical ventilation conspires with factors such as inflammation to generate BPD in premature newborns (Warburton *et al.*, 2001). Mechanical factors appear influential beyond this period: compensatory lung growth follows lung resection (Thurlbeck, 1983) comprising lung distension and parenchymal growth. This postpneumonectomy effect suggests the lung responds to altered mechanics and that the organism to reduced alveolar surface area. At a smaller scale, airway smooth muscle (ASM) hypertrophy and hyperreactivity in asthma are associated with air trapping and acute lung distension; however, with time, this is associated with airway remodeling and chronic lung hyperexpansion. ASM-led airway occlusions in asthma may therefore have analogous effects to fetal tracheal occlusion (which distends and remodels prenatal lung) (Jesudason, 2007). Moreover, transient endogenous ASM-led airway occlusions occur in fetal lung (known as airway peristalsis), and this contractility may be an important regulator of lung growth (discussed below) (Jesudason, 2006a). With this in mind, we next focus on three areas of interest in lung mechanobiology: (i) lung liquid, (ii) airway contractility, and (iii) calcium signaling in this secretory, contractile environment.

### 4.2. The impact of hydraulic pressure on lung organogenesis

Prenatal lung liquid is neither plasma ultrafiltrate nor “inhaled” amniotic fluid (Adamson *et al.*, 1969). Lung liquid is produced throughout prenatal lung development by incompletely understood mechanisms that involve active $Cl^-$ transport from blood/interstitium into lumen (Olver and Strang, 1974). Intracellular $Cl^-$ accumulation is energized by the basolateral $Na^+/K^+$-ATPase (Bland and Boyd, 1986) and accomplished via $Na^+$-linked cellular $Cl^-$ uptake through the $Na^+/K^+/2Cl^-$ co-transporter (Thom and Perks, 1990); indeed $Cl^-$ secretion rate depends on NKCC1 expression (Gillie *et al.*, 2001). Movement of accumulated $Cl^-$ down its concentration gradient via apical $Cl^-$ channels results in accompanying $Na^+$and water flux to generate fetal lung fluid (see Olver *et al.*, 2004 for comprehensive review). Whilst active $Cl^-$ and fluid secretion are crucial to lung growth (Alcorn *et al.*, 1977), they may not contribute to branching per se (Souza *et al.*, 1995a).

The identity of the apical $Cl^-$ channel remains unclear. Numerous channels are demonstrated in fetal alveolar type II cells, including a G protein-regulated maxiCl channel (Kemp *et al.*, 1994), cystic fibrosis transmembrane conductance regulator (CFTR) (McCray *et al.*, 1993), at least one member of the Chloride Channel (CLC) channel family (Blaisdell *et al.*, 2004; Murray *et al.*, 1995), and a $Ca^{2+}$-activated $Cl^-$ channel, TMEM16a (Rock *et al.*, 2008). $CFTR^{-/-}$ mice have normal prenatal lungs (Wallace *et al.*, 2008), suggesting CFTR plays no role in producing lung liquid or there is functional redundancy. Although a definitive link between CLC channels and lung liquid production remains to be established *in vivo*, there is evidence that CLC-2 contributes to fluid secretion and cyst expansion *in vitro* (Blaisdell *et al.*, 2004). Interestingly, murine $TMEM16a^{-/-}$ mutants die of respiratory failure at an interval after birth with characteristic tracheomegaly and disruption of trachealis formation (Rock *et al.*, 2008).

The rate of liquid production and the laryngeal valve function help determine hydraulic pressure in the lung. Obstructing the prenatal trachea increases intraluminal pressure two- to three-fold and airway branching three-fold; the rate of bud extension increases about two-fold whilst inter-bud distance is halved. These effects depend on FGF10–FGFR2b–Sprouty signaling (Unbekandt *et al.*, 2008). Several studies have used tracheal obstruction to try to improve lung growth in human CDH (Harrison *et al.*, 2003; Jani *et al.*, 2005). However, clinical evidence of benefit of this potentially hazardous intervention remains limited. An alternative being explored is to exploit spontaneous airway occlusions that may be important for lung growth and perhaps avoid invasive fetal interventions (Jesudason, 2009).

### 4.3. The impact of embryonic airway peristalsis in lung organogenesis

Early mammalian airway exhibits spontaneous transient airway occlusions due to airway peristalsis. This is mediated by spontaneous ASM contractions that occur in birds and humans and which increase in frequency from embryonic stages to birth (Schittny *et al.*, 2000). Peristaltic contractions and airway occlusions direct waves of fluid toward the lung's tips. This results in rhythmic stretch and relaxation of growing buds (Fig. 3.8). Hence airway peristalsis and occlusions are well placed to regulate both pressure and stretch in the tips of developing lung (Jesudason, 2009). These ASM waves emanate from pacemaker areas in proximal airway before transmission distally (Jesudason *et al.*, 2005). This pacemaker-driven airway contractility may even be important postnatally in asthma (Jesudason *et al.*, 2006b). Thus, putative pulmonary pacemakers might be targeted for ablation by bronchial thermoplasty for asthma (Jesudason, 2009). Studying frequency of peristalsis in embryonic lung culture revealed that it is amenable to acceleration by cholingergic agents as well as growth factors (FGF10). These accelerated rates accompany enhanced *in vitro* lung growth. Similarly, *in vitro* inhibition of peristalsis is associated with reduced lung growth (Jesudason *et al.*, 2005). This apparent coupling raised interest in mechanisms linking morphogenesis and peristalsis-led airway occlusions. In particular, $Ca2^+$-imaging studies revealed that prenatal lung features spontaneous regenerative intercellular ASM calcium waves that propagate along main airways immediately prior to the wave of peristaltic contractility (Featherstone *et al.*, 2005). Using pharmacological inhibitors, we showed that ASM calcium waves depend on extra- and intracellular calcium as well as gap junction integrity. Moreover, these calcium waves are abnormal in experimental lung hypoplasia (Featherstone *et al.*, 2006). Thus, if peristaltic airway contractions do regulate lung growth, it means that underlying calcium oscillations govern lung development.

### 4.4. Lung stretch transduction and parathyroid hormone-related protein (PTHrP)

Airway peristalsis is coupled to lung growth, responsible for phasic lung stretch and underpinned by calcium oscillations. Transduction of such mechanical activity involves key modulators and sensors of serum $Ca2^+$. For example, stretching alveolar type II cells

produces parathyroid hormone-related protein (PTHrP) (Torday *et al.*, 2002), which binds in paracrine fashion to its cognate receptor on adjacent adepithelial fibroblasts (Torday and Rehan, 2002); the latter are induced to differentiate into a lipofibroblast lineage (Schultz *et al.*, 2002). The lipofibroblasts, discovered by Vaccaro and Brody (1978), protect against oxidant injury (Torday *et al.*, 2001) and produce leptin, which stimulates synthesis of surfactant phospholipids and proteins, by ligating its receptor on alveolar type II cells (Torday *et al.*, 2002a,b). Alveolar stretch releases PTHrP, which dilates alveolar capillaries (Gao and Raj, 2005) and coordinates surfactant production with alveolar perfusion. The Torday group showed that fluid distension permits cultured lung to differentiate like lung *in vivo*, agreeing with the concept discussed elsewhere that rhythmic mechanical distension serves as a clock or "zeitgeber" mechanism for organogenesis (Gross *et al.*, 1978). Conversely, inhibiting lung fluid in explants inhibits the putative "zeitgeber," while PTHrP treatment rescues the temporal molecular development of the lung. Fluid distension also stimulates endodermal Shh expression, which in turn stimulates the mesodermal Wnt pathway. Wnt increases endodermal PTHrP expression, which feeds back negatively on the Wnt pathway by stimulating protein kinase A. Wnt/β-catenin signaling has recently been linked to both PTHrP and the CFTR (Cohen *et al.*, 2008).

### 4.5. Lung development and the $Ca2^{+}$-sensing receptor (CaSR)

Stretch-induced PTHrP regulates $Ca2^{+}$ and lung growth, and stretch-inducing airway peristalsis is underpinned by $Ca2^{+}$ signaling. However, the developing fetus is actually hypercalcemic compared to the adult, with free extracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_o$) of around 1.7 mM, while maternal $[Ca^{2+}]_o$ is 1.1.–1.3 mM. Studies in mouse lung explant culture demonstrated fetal $[Ca^{2+}]_o$ suppresses branching and cellular proliferation while enhancing $Cl^{-}$-dependent fluid secretion (Finney *et al.*, 2008). Since effects of fetal $[Ca^{2+}]_o$ are mimicked by pharmacological activation of $Ca^{2+}$-sensing receptor (CaSR), the G protein-coupled extracellular CaSR and fetal $Ca^{2+}$ via CaSR may balance branching morphogenesis and lumen distension. CaSR expression has been documented in mouse (Finney *et al.*, 2008) and humans (Finney, Wilkinson, Kemp, and Riccardi, unpublished observations), throughout the pseudoglandular stage. At the start of this phase, CaSR is restricted to the epithelium where its expression is maintained for at least 48 h in the organ culture. From E14.5 in mouse (and week 9 in humans) mesenchymal CaSR expression appears. CaSR expression wanes during the canalicular phase and is absent from adult mouse, rat, and bovine lung (Brown *et al.*, 1993; Finney *et al.*, 2008; Riccardi *et al.*, 1995). CaSR-dependent regulation of branching and fluid secretion occurs via phospholipase C-dependent rises in intracellular $Ca^{2+}$ concentration and phosphoinositide 3 ($PI_3$) kinase activation (Fig. 3.9). Whether CaSR-dependent rises in $Ca^{2+}$ affects lung development through peristaltic waves (discussed above) and/or controls growth by altering epithelial integrity via activation of $PI_3$ kinase-dependent β-catenin signaling remains unclear. CaSR activation could also affect clock mechanisms of airway branching by interfering with FGF–FGFR–Sprouty signaling events: lungs cultured in 1.7 mM $Ca^{2+}$ delay both bud extension and tip-splitting, when compared to lungs cultured in lower, adult levels of $[Ca^{2+}]_o$ (1.2 mM) (Finney *et al.*, 2008). Finally, patients with inactivating CaSR mutations have increased risk of interstitial lung disease (Auwerx *et al.*, 1985a,b). Given that CaSR expression appears confined to the pseudoglandular phase, we need to test if this phenotype originates prenatally.

## 5. Stem/Progenitor Cell Biology of the Lung

A stem cell describes a self-renewing, primitive, undifferentiated, multipotent source of multiple cell lineages. Such cells are critical for development and growth; pools of adult stem cells are hypothetical sources for tissue regeneration and repair as well as cancers. In

contrast to embryonic stem cells and tumor cells, adult stem cells reduce telomere length with age (Warburton *et al.*, 2008).

In the lung, there is limited knowledge about existence of self-renewing cells, whether such cells conform to classic or nonclassic stem cell hierarchies and whether a single stem/progenitor cell suffices to generate the more than 40 distinct cell types required in mature lung. At least five putative epithelial stem/progenitor cell niches are reported in adult mouse airway (Liu and Engelhardt, 2008), as well as endothelial stem cells in the pulmonary vasculature and ASM stem cells. Additionally, circulating stem/progenitors may lodge in the lung. Unlike skin and gastrointestinal tract, postnatal lung turns over slowly, which hampers putative progenitor identification. Specific markers and clonality assays also limit attempts at isolation. Herein we review new information regarding the development and function of lung stem/progenitor cells in organogenesis.

### 5.1. Endogenous epithelial progenitor cells

Failing regeneration and repair with age has been suggested to be due to stem cell failure. In pseudoglandular lung, tips of the branching tubules appear to contain undifferentiated multipotent epithelial progenitors. In adult lung, putative endogenous epithelial progenitors have been located in the basal layer of the upper airways, within or near pulmonary neuroendocrine cell rests as well as at the bronchoalveolar junction and in the alveolar epithelium (Engelhardt, 2001; Giangreco *et al.*, 2002, 2004; Kim *et al.*, 2005; Rawlins and Hogan 2006; Reddy *et al.*, 2004; Reynolds *et al.*, 2000, 2004). The distal-most epithelial cells were shown to be a multi-potent progenitor cell population during branching morphogenesis of the lung (Rawlins *et al.*, 2009a). A recent model suggests, as the lung branches, descendents of distal tip progenitors are left to differentiate in the stalks, whereas self-renewing progenitors remain in the epithelial tips: distal epithelial cells have a unique gene expressionpattern, including high levels of *Sox 9, Id2* (inhibitor of differentiation 2), *N-myc*, and *Etv5/ERM* (ets variant gene 5). In addition, they are exposed to high levels of FGF, Wnt, Shh, and BMP signaling (Bellusci *et al.*, 1996; Liu *et al.*, 2002; Shu *et al.*, 2005). Many of these pathways including *sox9, etv5*, and FGFR signaling are associated with stem/progenitor cells in other endodermally derived organs (Seymour *et al.*, 2007; Zhou *et al.*, 2007). Distal epithelial cells also have different cell cycle kinetics compared with the rest of the epithelium; a higher proportion of them incorporate the thymidine analog bromodeoxyuridine (BrdU) during a short pulse (Okubo *et al.*, 2005).

**5.1.1. Tracheal and bronchial epithelial progenitors—**Candidate endogenous stem or progenitor cells have been identified in trachea and lung, using lung injury/repair models. For example, CC10/*Scgb1a1*$^{+}$ Clara cells self-renew and proliferate after tracheal injury, but seem not the main source for tracheal epithelial regeneration (Rawlins *et al.*, 2009b). However, subsets of Keratin-14 (K-14)-expressing BCs in the trachea (Hong *et al.*, 2004a) can restore differentiated epithelium after injury and are distinct from bronchial BCs (Hong *et al.*, 2004b). Lineage tracing in the adult mouse lung and trachea showed K-14-positive cells act as progenitors and ciliated cells cannot (Hong, *et al.*, 2004a; Rawlins, *et al.*, 2007). Human *SpC* and rat *CC10* promoters have also been used for lineage tracing in lung (Perl *et al.*, 2002, 2005a).

Since pseudostratified epithelium of mouse trachea and human airways contains a BC population expressing cytokeratin 5 (*Krt5*), a recent study using *Krt5-CreER*$^{T2}$ transgenic mouse line for lineage tracing showed BCs of mouse trachea function as progenitors during postnatal growth, in adult homeostasis and also in epithelial repair of experimentally-induced $SO_2$ damage (Rock *et al.*, 2009). A clonality assay also found BCs of mouse and

human airways self-renew and differentiate into mucus and ciliated lineages in the absence of stroma or columnar epithelial cells.

There is also a rare mixed population of pluripotent cells in lower respiratory tract characterized as a Hoechst dye effluxing side population (SP) cells. They express molecular markers of airway and mesenchymal origin (Giangreco *et al.*, 2004). $CD45^-$ SP cells isolated from human tracheobronchial epithelium have proliferative potential. Increased numbers of these cells in asthmatic airways suggest that dysregulation of pluripotent cells may play a role in this chronic disorder (Hackett *et al.*, 2008). In developing lung, some SP cells, which are $CD45^+$ and $CD45^-$ have endothelial progenitor cell (EPC) potential in response to hyperoxia (Irwin *et al.*, 2007).

The submucosal gland ducts in proximal airway are likewise suspected to contain stem cells (Liu and Engelhardt, 2008). However, relatively little is known about glandular stem/progenitor cells and their niche(s). Studies have suggested regenerating tracheal epithelium after naphthalene injury arises from cells migrating from gland ducts (Borthwick *et al.*, 2001).

Rawlins *et al.* (2009b) used lineage tracing to circumvent obstacles that hampered earlier studies of lung stem/progenitor cells. Using the restricted expression of *CC10*/*Scgb1a1*, they generated a "knocking" transgenic mouse with a tamoxifen (TM) inducible *Cre-recombinase* (*ScgB1a1-CreER*$^{TM}$) that lineage-tags Clara cells of the airway. By varying dose and timing of TM administration, they discovered that epithelial reconstitution in the bronchioles involves Clara cell self-renewal and differentiation into ciliated cells. These data argue that bronchiolar *ScgB1a1*-expressing cells, largely mature Clara cells, are a self-renewing progenitor pool. The observation that lineage tags are chased into ciliated cells over time is consistent with early findings of Evans and colleagues (1978) that Clara cells are progenitors for ciliated cell renewal. On the other hand, Rawlins *et al.* (2009b) showed that lineage tags introduced into *ScgB1a1*-expressing cells of tracheobronchial airways were depleted within the *ScgB1a1*-expressing population over time. Collectively, these data suggest that *ScgB1a1*-expressing cells of proximal airways behave like transit amplifying (TA) cells, like those of intestinal epithelium, whereas *ScgB1a1* cells of bronchiolar airways behave like self-renewing progenitors present in the interfollicular epidermis (reviewed by Chen *et al.*, 2009).

A different approach by Giangreco and colleagues (2009) to investigate long-term behavior of airway progenitors in normal and injured airways showed in concordance with Rawlins *et al.* (2009b) that during homeostasis an abundant progenitor cell pool maintains the airway epithelium (rather than rare tissue stem cells). However, clonal patches of labeled cells emanate from tissue-specific stem cells located at airway branch points or bronchioalveolar duct junctions, after Clara cell depletion resulting from naphthalene exposure. In this naphthalene injury case, repairing bronchiolar airways more closely resemble the renewing epidermis after wounding, wherein stem cells are recruited from the hair follicle bulge to replace the depleted BC pool of the interfollicular epidermis (Zemke *et al.*, 2009).

Varying dose and timing of TM administration, Rawlins *et al.* (2009a) discovered that reconstitution of bronchiolar epithelium involves Clara cell self-renewal and differentiation into ciliated cells and that Clara cells contribute to tracheal repair. Using lineage tracing, this study showed that a special population of BASCs which coexpress *CC10* and *SP-C*, which have been proposed to contribute to both bronchioles and alveoli, has no apparent function during postnatal growth, adult homeostasis, or alveolar repair. Thus, they propose that trachea, bronchioles, and alveoli are maintained by distinct progenitor populations (Rawlins *et al.*, 2009a). Currently, the significance that some *Scgb1a1*$^+$ bronchiolar Clara cells

express *SftpC* and some alveolar type 2 cells express *Scgb1a1* is not understood. There is accumulating evidence the (*Scgb1a1*$^{+}$, *SftpC*$^{+}$) coexpressing cell population increases in number in murine lung cancer models (Ventura *et al.*, 2007; Yang *et al.*, 2008). However, it is unclear if this is due to preferential proliferation of preexisting (*Scgb1a1*$^{+}$, *SftpC*$^{+}$) cells or oncogenic upregulation of *SftpC* or *Scgb1a1*.

In a recent study (Tompkins *et al.*, 2009), selective *Sox2* deletion in Clara cells with *Scgb1a1-Cre* showed that Clara cell Sox2 is required for differentiation and/or maintenance of ciliated, Clara, and goblet cells in bronchiolar epithelium after birth and caused progressive loss of ciliated, Clara, and goblet cells and an inability to produce goblet cells in response to allergen. The findings indicate Clara cells can serve as common progenitors of ciliated, Clara, and goblet cells in a process requiring *Sox2*.

**5.1.2. Alveolar epithelial progenitors—**Epithelial progenitors of the alveoli have yet to be identified. An interesting model is that the alveolar progenitors are located in distal epithelial tips during the canalicular stage. However, there is no published evidence to support, or refute, this hypothesis. Presumably, because of its vast area a large number of AECs must function as a “ready reserve” to repair damaged alveolar surface. For instance, the expression of telomerase, a stem/progenitor cell marker, after acute oxygen injury is widely upregulated in AECs during recovery (Driscoll *et al.*, 2000). This suggests that either AECs contain a progenitor cell subpopulation or that the majority of AECs undergo reactivation progenitor-like states after injury (Driscoll *et al.*, 2000). In addition, without telomerase, resistance to injury and repopulation of damaged alveoli are compromised, indicating this pathway is likely critical for alveolar progenitor cell activity (Driscoll *et al.*, personal communication). Moreover, Kim and colleagues (2005) described BASCs, which possess stem cell characteristics, are resistant to naphthaline injury and proliferate after airway or alveolar injury. Such BASCs reside near bronchioalveolar junctions and coexpress both alveolar (SP-C) and airway (CC10) epithelial cell markers, as well as coexpressing Sca-1. They are capable of self-renewal and differentiation into Clara cells and alveolar cells, and are also multipotent in clonal assays. Moreover, studies by Hong and coworkers (2001) identified variant Clara cells as endogenous lung stem cells, which infrequently proliferate during steady state but are held responsible for repopulating distal airway epithelium after injury. Variant Clara cells express Clara cell secretory protein, but survive naphthalene injury. As the lung continues to grow postnatally, Clara cells both self-renew and act as progenitors for ciliated cells, based on kinetics of cell labeling after a pulse of tritiated [3H]-thymidine (McDowell *et al.*, 1985; Plopper, *et al.*, 1992). This is supported by recent lineage labeling (Perl *et al.*, 2005a). Whether all Clara cells have this capacity requires investigation. Moreover, type II cells proliferate and give rise to type I cells after adult alveolar injury, and this probably also occurs during postnatal growth (Evans *et al.*, 1975). Several putative endogenous alveolar stem cell populations thus provide targets for directed regenerative therapies. Taking acute oxygen injury as an example, AECs undergo DNA and other forms of damage such as mitochondrial failure, glutathione depletion, and apoptosis (Buckley *et al.*, 1998; Lee *et al.*, 2006; Roper *et al.*, 2004).

### 5.2. Endogenous mesenchymal progenitors

Several studies have shown that signals from lung mesenchyme are essential for branching morphogenesis. Mesothelium-derived FGF9 activates and controls FGF10 signaling from peripheral mesenchyme via FGFR2b, SHP2, Grb2, Sos, Ras, and Sprouty in epithelium (Del Moral *et al.*, 2006b; Bellusci *et al.*, 1997b; Tefft *et al.*, 2002, 2005).

**5.2.1. Smooth muscle progenitors—**Distal *Fgf10*-expressing mesenchymal cells serve as progenitors for peripheral ASM (De Langhe *et al.*, 2006; Mailleux *et al.*, 2005;

Ramasamy *et al.*, 2007). *Fgf10-lacz* lineage tracing reveals ASM progenitors begin as *Fgf10*-expressing cells that, as the airway elongates, become distributed along peripheral airway. Transdifferentiation to express alpha–smooth muscle actin occurs under the control of SHH and BMP4, which are expressed proximal to the airway tip. Thus, increase in population size and localization of peripheral ASM progenitors occur early in development. Another population of ASM progenitors arise in proximal mesenchyme and advance peripherally (Shan *et al.*, 2008).

**5.2.2. Vascular progenitors—**Lung microcirculation is rich in progenitors, but our understanding of these is limited. Mesothelium overlying the lung contains progenitors that give rise to pulmonary vascular (but not airway) SMCs during embryonic development (Que *et al.* 2008). Endothelial progenitors arise from endogenous vascular wall or from circulating progenitors. Similar to lung epithelial cells, heterogenous pulmonary endothelial cells may require a site-specific niche (Clark *et al.*, 2008); alternatively, putative resident endothelial progenitors may constitute a universal pool of progenitors that lack segmental specification (Blaisdell *et al.*, 2009).

Distal airspace and vascular growth are coordinated so injury can affect both (Jakkula *et al.*, 2000). Balasubramaniam *et al.* (2007) examined endothelial progenitors in BPD to show that hyperoxia disrupts alveolar and vascular growth, limiting surface area for gas exchange. In the lung, nitric oxide, VEGF, and erythropoietin contribute to mobilization and homing of EPCs. Several related developmental changes occur after hyperoxia in neonatal mice: expression of endothelial nitric oxide synthase, VEGF, and erythropoietin receptor and the number of EPCs in the blood, bone marrow, and lung were all reduced (Balasubramaniam *et al.*, 2007).

Primitive capillaries surround the laryngotracheal groove as the lung buds from foregut and can be visualized by β-galactosidase expression under control of Flk1 promoter. This promoter is active and the earliest known marker of hemangioblasts. Under stimulation of epithelial VEGF, these hemangioblasts differentiate into a capillary network that surrounds bronchial, lobar, and segmental airways (Del Moral *et al.*, 2006a; Ramasamy *et al.*, 2007). Organization of this plexus appears essential for correct branching and perfusion. Thus, mesothelial–mesenchymal–epithelial–endothelial crosstalk matches epithelial and vascular progenitor function and will likely be essential for lung regeneration to succeed. Further studies are needed to define phenotypes of the pulmonary endothelial cell but also SMCs within the vasculature (Stevens *et al.*, 2008).

### 5.3. Control of lung progenitor cell proliferation

Embryonic progenitors undergo symmetric and asymmetric divisions. To distinguish these, one can look at differences in spindle orientation or differential inheritance of cytoplasmic or membrane-bound proteins such as cell fate determinant Numb and atypical protein kinase C (PKC) (Huttner and Kosodo, 2005; Morrison and Kimble, 2006; Wang *et al.*, 2009; El-Hashash and Warburton, unpublished data). Cells divide asymmetrically in response to extrinsic or intrinsic fate determinants: extrinsically, daughter cells placed in different microenvironments adopt different fates; intrinsically, cytoplasmic cell fate determinants (e.g., Numb) are asymmetrically localized within a cell and segregate differentially into daughters that adopt different fates (reviewed by Yamashita, 2009). Comparing progenitor numbers in mutant and sibling control lungs, we infer that certain molecules promote progenitor self-renewal or differentiation (Rawlins, 2008).

Several transcription factors and signaling molecules control lung growth and therefore probably affect progenitor cell proliferation. Thyroid transcription factor 1 (Ttf-1/Nkx2.1) expression marks lung lineage commitment in the early embryo and is critical for distal lung

progenitor development (Kimura *et al.*, 1999). *Ttf1*$^{-/-}$-null mice have insufficiently differentiated lungs for survival (Kimura, *et al.*, 1996). HMG box transcription factor, *Sox9* is intensively expressed in distal epithelial progenitors from E11.5 to E16.5 (Liu and Hogan, 2002). However, lung-specific conditional deletion has no effect on progenitor cell behavior (Perl *et al.*, 2005b). *Sox9* may therefore act redundantly with other, as yet unknown, regulators: N-myc is also essential for maintaining a distal population of undifferentiated, proliferating progenitor cells, and may promote their self-renewal (Okubo *et al.*, 2005).

In addition, several forkhead/winged helix (fox) family transcription factors have mutant knockout phenotypes and may promote lung epithelial progenitor proliferation. For example, conditional deletion of both *foxa1* and f*oxa2* genes in lung results in small lungs with decreased cell division rates (Wan *et al.*, 2005). A similar phenotype was reported after conditional deletion of both *foxp1* and *foxp2*, which are enriched in the distal epithelial progenitors. In *foxp22/2; foxp11/2* double mutants, the lungs are smaller than normal, with inhibited proliferation, but normal proximal–distal patterning (Shu *et al.*, 2007). This suggests an essential role of fox transcription factors in the maintenance of the progenitor cell population and their self-renewing divisions.

Similarly, five key signaling molecules regulate many processes in embryonic development: Wnt, Notch, Hedgehog, FGF, and TGF-β family. Embryos lacking Wnt2/2b exhibit lung agenesis and do not express Nkx2.1, the earliest marker of lung endoderm. Endoderm-restricted deletion of β-catenin replicates this, suggesting canonical Wnt2/2b signaling is required to specify lung endoderm progenitors in the foregut (Goss *et al.*, 2009, Harris-Johnson, 2009). FGF signaling plays an essential role in specification of distal lung lineages (De Langhe *et al.*, 2008; Ramasamy *et al.*, 2007) and others (Serls *et al.*, 2005). FGF10 is expressed by lung mesenchyme and is a chemotaxin during morphogenesis. FGF10 overexpression maintains epithelial progenitor cell proliferation and leads to goblet cell metaplasia (Nyeng *et al.*, 2008). In addition, FGF10 coordinates alveolar SMC formation and vascular development (Ramasamy *et al.*, 2007). RA signaling is also essential for expansion of lung progenitors and formation of primary lung buds, by affecting *Fgf10* expression through TGF-β signaling (Chen *et al.*, 2007). Similarly, Shh in distal epithelium controls proliferation and branching and is believed to promote progenitor proliferation (Pepicelli *et al.*, 1998). Autocrine Bmp signaling is likewise important for proliferation of the distal epithelial progenitor cell compartment. Wnt5a is also highly expressed around distal epithelial tips. *Wnt5a*$^{-/-}$ lungs have increased cell proliferation and an additional airway branch (Li *et al.*, 2002), but it is unknown if this phenotype relates to defective progenitors. The details of how these signaling pathways regulate distal epithelial progenitor cells remain to be determined.

### 5.4. Embryonic lung progenitors and proximal–distal patterning

Recent studies suggest that Wnt and Bmp signaling controls proximal–distal lung patterning, but there is currently no evidence to confirm that this is mediated through progenitors. Shu *et al.* (2005) demonstrated that proximal–distal lung patterning depends on Wnt/β-catenin signaling and is mediated, in part, through regulation of N-myc, Bmp-4, and FGF signaling. Potentiation of β-catenin signaling in proximal airway results in arrested differentiation of immature bronchiolar stem cells, but β-catenin is unnecessary for adult bronchiolar stem cell maintenance (Zemke *et al.*, 2009). Fortunately, reporters of Wnt pathway activity are highly active in distal lung epithelial cells. Recent studies suggested that Wnt signaling regulates proximal–distal patterning and progenitor proliferation independently, and that Wnt promotes distal airway fate at the expense of the proximal. (Mucenski *et al.*, 2003; Shu *et al.*, 2005). Shu and coworkers overexpressed Dickkopf-1 to inhibit Wnt pathway activity throughout developing epithelium: this expands proximal (conducting) airways at the expense of the distal, without effects on total levels of cell proliferation (Shu *et al.*, 2005).

Similarly, Mucenski *et al.* (2003) showed that lung-specific deletion of β-catenin abrogates distal epithelial differentiation. Notch signaling favors progenitor identity at the expense of differentiated phenotypes in different organs (Jadhav *et al.*, 2006; Mizutani *et al.*, 2007) and is also required for lung epithelial progenitors. Notch1 is highly expressed in distal epithelial progenitors during the pseudoglandular stage (Post *et al.*, 2000). Notch controls cell fates in developing airways (Tsao *et al.*, 2009), and arrests normal differentiation of distal lung progenitors before they initiate an alveolar program (Guseh *et al.*, 2009). Notch misexpression in the distal lung prevented the differentiation of alveolar cell types (Guseh *et al.*, 2009); expression of a constitutively active form of Notch3 throughout the developing lung epithelium prevents cell differentiation (Dang *et al.*, 2003).

Furthermore, BMP signaling is also required for lung epithelium development, probably by promoting distal and repressing proximal cell fate. Inactivation of Bmp signaling by overexpression of a dominant-negative BMP receptor, or BMP antagonists Gremlin or Noggin, results in proximalization of lung epithelium (Weaver *et al.*, 1999; Lu *et al.*, 2001). Thus, reduction of BMP or Wnt signaling causes lung proximalization phenotypes (Eblaghie *et al.*, 2006; Li *et al.*, 2002).

### 5.5. Emergence of specific cell types during lung organogenesis

At least 40 differentiated cell types emerge during lung organogenesis. Early trachea and esophagus are both lined with ciliated epithelium; following their septation, esophageal epithelium becomes squamous, while tracheal epithelium retains cilia. Primitive airway epithelium expresses several marker proteins including cGRP, Clara cell protein, and SP-A: its differentiation starts around E16 in mouse with emergence of pulmonary neurendocrine (PNE) cell rests, surrounded shortly after by Clara cells. In the periphery, AEC2 differentiation in E18 mouse is denoted by glycogen granules' disappearance and emergence of surfactant-containing lamellar bodies with increased SP-C expression.

In mature lung, epithelial lineages are arranged proximodistally along the airways. Cartilage lies outside the submucosa and decreases in amount as bronchial caliber decreases; it is absent from bronchioles. The two major epithelial cell types in proximal bronchi are pseudostratified ciliated columnar cells and mucous (goblet) cells. Both arise from BCs, but ciliated cells predominate. Goblet cells begin to mature around 13 weeks' gestation in humans (mature ciliated columnar cells are already present), express mucin markers (MUC5B, 5A, 5C), and release mucus granules into the airway which reduces drying and, through ciliary-driven cephalad mucus flow, cleanses the airway. In cystic fibrosis, mutation of the cystic fibrosis transmembrane conductance regulator (*Cftr*) gene disrupts expression of the encoded transmembrane $Na^+$ ion transporter protein leading to thick mucus that overwhelms ciliary clearance and increases susceptibility to infection. In chronic airway injury, goblet cell hyperplasia may follow repair or experimental epithelial IL-9 exposure; the latter increases epithelial lysozyme and mucus production (Vermeer *et al.*, 2003). IL-4, IL-13, and allergens enhance TGF-α release, which is a ligand for the EGFR that also stimulates goblet cell differentiation (Lordan *et al.*, 2002).

There are three types of cells in bronchial submucosal glands. Myoepithelial cells surround the gland, while mucous cells (pale cytoplasm) and serous cells (basophilic cytoplasm) produce mucins. These secreted mucins mix with lysozyme and IgA on airway surface.

Kulchitsky cells are also found next to bronchial glands, but their function is unclear. It is believed they are pulmonary neuroendocrine cells (PNECs) producing peptides such as serotonin and calcitonin. Their cytoplasmic extensions usually reach the airway lumen. Kulchitsky cells expressing gastrin-releasing peptide (GRP), calcitonin gene-related peptide (CGRP), and chromogranin may be related to small cell carcinoma and carcinoid tumors.

However, PNEC differentiate earlier by 10 weeks' human gestation and are the first fully differentiated murine airway epithelial cells.

Clara cells reside in distal bronchiolar airway epithelium (normally lacking mucous cells) and produce mucus-poor, watery secretion. They emerge during the 19th week in humans and appear to assist with clearance, detoxification, and surface tension reduction in small airways. Clara cell-specific protein (CC10, CCSP, or uteroglobin) and cytochrome P450 reductase CC10 can be used as Clara cell markers. Whilst normal mice feature few mucin-positive cells in the airway, mucus metaplasia is associated with numerous Clara cell-derived mucous cells with excess mucin production or reduced secretion (Evans *et al.*, 2004).

Most of the alveolar surface is covered by flat type I epithelial cells that are believed to be terminal differentiated and express several markers, such as T1a and aquaporin 5. T1a is developmentally regulated and encodes an apical membrane protein of unknown function. Absence of T1a protein blocks type I cell differentiation. Homozygous T1a null mice die at birth of respiratory failure with lungs that will not inflate normally (Ramirez *et al.*, 2003). Aquaporin 5 is a water-channel in type I epithelial cells. Recently, a knock in of Cre-ERT2 into the *Aqp5* locus has been reported (Borok, personal communication). These mice will be useful, not only to target gene deletion in type I cells but also for lineage tracing of the type I cells under development, injury, and repair.

Whilst type I epithelial cells cover more than 95% of the alveolar surface area, they account for only 40% of total AECs: the other 60% are rounded type II pneumocytes. These plump or cuboidal cells can regenerate and replace type I cells post injury and have finely stippled cytoplasm and surface microvilli. They manufacture surfactant phospholipids and proteins that modulate alveolar surface tension, such that despite varied size, alveoli remain open and end-expiratory atelectasis is reduced. Surfactant protein C (*SftpC*) is a commonly used type II cell marker. The four surfactant proteins, SP-A, B, C, and D play critical roles: SP-A and SP-D participate in airway host defense; SP-B and SP-C contribute to surfactant's surface tension reduction (Whitsett *et al.*, 2002).

Macrophages, although a small percentage of alveolar cells, are a major sentinel of host defense and derived primarily from blood monocytes; once in the lung, their turnover is extremely slow.

### 5.6. Stem and progenitor cells in the postnatal respiratory system

Stem and progenitor cells presumably help repair damaged lung, but identification of such cells remains problematic. The large surface area, numerous branches, and folded topography suggest lung harbors several stem or progenitor cell types. In trachea and bronchi, certain BCs and mucous-gland duct cells are believed to be stem/progenitor cells. Clara-like cells and type II pneumocytes are also thought to function as stem/progenitor cells in bronchioles and alveoli, respectively. Another population of stem/progenitor cells apparently lies at the bronchoalveolar duct junction (Kim *et al.*, 2005). They have been proposed to function as bipotential precursors of both the SP-C- and CC10-expressing cell lineages.

It has recently been reported that bone marrow-derived mesenchymal stem cells can differentiate into airway epithelial cells and alveolar type I pneumocytes, particularly post-injury. By contrast, *in vitro* cell culture indicates Syrian hamster fetal lung epithelial M3E3/C3 cells differentiate into Clara cells and type II pneumocytes under different culture conditions. Whether CCSP-expressing cells with pre-Clara cell phenotypes are stem cells for the entire respiratory tract remains to be determined. In addition, the concept of a pluripotent

stem cell for the whole lung needs to be further investigated due to the great differences between identified stem cell or progenitor candidates in proximal bronchi and distal alveoli. Recently, we discovered lung contains cells with stem or progenitor characteristics that can be FACS sorted from adult rat and mouse lung and which are relatively apoptosis-resistant and perhaps responsible for post-injury alveolar repopulation. Another such population sorted as "side cells" on FACS may repopulate several tissues including bone marrow.

ASM derives from at least two progenitor populations: one comes from the lung periphery and arises from *Fgf10*-expressing cells in subepithelial mesenchyme. By virtue of FGF10 expression, these cells first help mediate epithelial branching; however, as the airway elongates into peripheral mesenchyme, these progenitors remain to lie along the more proximal stalk of the distal bud. Here they differentiate into ASM, most probably under paracrine induction of BMP4 and Sonic hedgehog from the adjacent epithelium (DeLanghe *et al.*, 2006). The second ASM progenitor population arises around the proximal large airways (Shan *et al.*, 2008) and appears to meet with the distally-derived counterparts beyond major lobar and segmental branches. It is speculated that whilst size of such ASM progenitor populations are determined during embryonic airway branching, they may nevertheless determine susceptibility to later BPD and asthma. Moreover, maternal smoking may dysregulate ASM progenitors and their progeny via the cholinergic-agonist, nicotine.

### 5.7. Potential strategies to protect lung progenitors

Both FGF7 and inosine treatment ameliorate DNA damage in AECs, as well as enhancing mitochondrial protection and the ability of AEC to migrate and repair in an *in vitro* scratch assay (Buckley *et al.*, 1997). FGF7 has also been evaluated by others *in vivo* as a treatment to enhance resistance to alveolar injury in animal models (Plantier *et al.*, 2007; Ray *et al.*, 2003). Also, FGF10 has a protective effect against lung injury and fibrosis (Gupte *et al.*, 2009). We have also shown that inosine has protective properties against oxygen injury, including glutathione repletion, mitochondrial protection, decreased apoptosis, and increased VEGF expression (Buckley *et al.*, 2005). Thus, it appears that protection or enhancement of alveolar progenitor cell function may be a viable therapeutic option that could possibly be evaluated in clinical trials of lung progenitor cell protection using small molecules such as inosine or FGF7 or FGF10.

## 6. Postnatal and Adult Lung

### 6.1. The transition to air breathing

Maturation of the surfactant system is one of two key steps to prepare fetal lung for air breathing. During the last 8 weeks of human gestation, fetal lung glycogen is converted into surfactant phospolipids, the most important of which is disaturated phosphatidylcholine (DSPC). This maturation is under the control of, and can be stimulated by, corticosteroids since it is blocked in mice with null mutations of glucocorticoid receptors or corticotrophin-releasing hormone. Human mutations have been found, such as surfactant protein B, that adversely affect stability of surfactant and hence the ability to maintain lung inflation.

The transition to air breathing occurs rapidly in mature neonatal lung. Immediately following severance of the umbilical circulation, a spike in catecholamine levels switches off chloride secretion and stimulates sodium/potassium ATPase (Brown *et al.*, 1983; Olver and Strang, 1974; Olver *et al.*, 1986). This replaces tracheal fluid production with its rapid absorption into lung interestiitum (and thence to lymphatic and capillary circulations). Null mutation of Na/K ATPase in mice leads to failure to absorb fetal lung liquid, which causes significant respiratory distress and even neonatal lethality (Hummler *et al.*, 1996). In humans delayed lung liquid absorption manifests as transient tachypnea of the newborn.

### 6.2. Lung aging and involution

From middle age in normal humans, an inexorable decline in lung function supervenes (illustrated by FEV1). By 120 years, FEV1 resembles that end-stage COPD in a younger person; hence, degenerating lung function appears currently to limit human life expectancy (Fletcher and Peto, 1977; Shi, W. and Warburton, D. 2010). Whilst some genetic mutations and/or environmental exposures fundamentally disrupt lung development and result in pre- or perinatal death, less critical leions may only be manifest as lung disease in infancy, childhood, or beyond. For example, minor genetic changes such as DNA polymorphisms may have very subtle impacts on lung organogenesis with apparently normal neonatal phenotype. Nevertheless, such lungs may have abnormal responses to subsequent environmental injury (e.g., cigarette smoke or vehicular pollution) that degrade lung anatomy and physiology faster than normal and predispose to, for example, COPD (Figure 3.10). Therefore, by understanding, protecting, and re-entraining developmental processes, amelioration or reversal of lung degeneration may permit enhanced duration and quality of life.

## 7. Conclusions

Appreciating that distal lung mesenchyme could trigger epithelial airway development has stimulated the search for controls of lung development. Given the mortality and morbidity of lung disease at all stages of life, lung regeneration is a global therapeutic priority. To achieve such goals, clinicians and scientists need to decipher how the lung is formed. Whilst this understanding began with histological analyses, advances in biology have allowed the “molecular embryology” of the lung to be elucidated. In parallel with this progress, lessons from human lung maldevelopment illustrate the importance of mechanical forces to normal lung growth. Such forces encompass both extrinsic factors (thoracic size, FBMs) and intrinsic ones (lung fluid, airway peristalsis, endogenous airway occlusions). Attempting to weave these diverse influences to facilitate regenerative lung growth appears a daunting task. Nevertheless, there are reasons for optimism: first, following Alan Turing’s insight, complex (lung) morphogenesis may arise via simple iterative biochemical signaling; secondly, Benoit Mandelbrot illustrated that simple mathematics can be applied to generate apparently complex form; thirdly, D’Arcy Thompson made clear that the set of genetically possible forms are vastly constrained by fundamental physical constraints; fourth, despite huge uncertainties about the regulation of lung development, regenerative medicine has already allowed transplantation of autologous tissue-engineered airway to aid patients. Hence, despite the structural complexity of the lung, its organogenesis is governed by simpler routines more readily susceptible to discovery and therapeutic exploitation. In pursuing the latter, we may similarly be reassured that physical constraints limit the possible structures we may engineer. Finally, despite all that we do not know, clinically important aspects of pulmonary regeneration can already be achieved. The challenge for the future will be the generation of more complex and vascularized structures that can ultimately support and/or replace impaired lung function.

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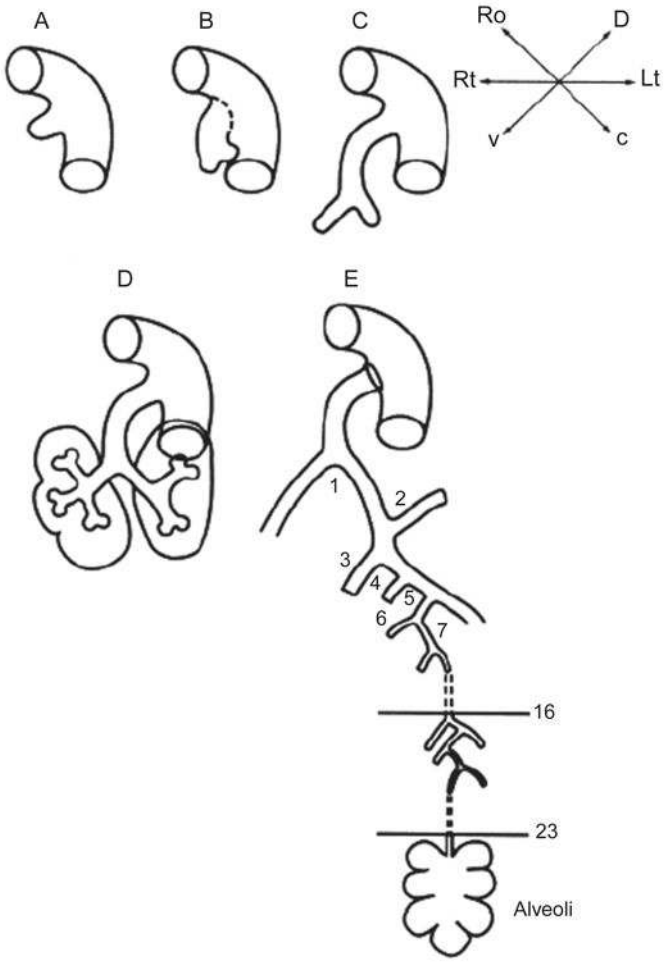


**Figure 3.1.**

(A) The primitive lung anlage emerges as the laryngotracheal groove from the ventral surface of the primitive foregut at 5 weeks' gestation in the human. (B) The primitive trachea separates dorsoventrally from the primitive esophagus as the two primary bronchial branches arise from the lateral aspects of the laryngotracheal groove at 5 or 6 weeks' gestation in the human. (C) The embryonic larynx and trachea with the two primary bronchial branches are separated dorsoventrally from the embryonic esophagus at 6 weeks in the human. (D) The primitive lobar bronchi branching from the primary bronchi at 7 weeks in the human. (E) A schematic rendering of the airway at term in the human. The stereotypical first 16 airway generations are complete by 16 weeks in humans; between 16 and 24 weeks, further branching is nonstereotyped. Alveolarization begins about 20 weeks in humans and is complete by 7 years of age at the earliest. (After West, Burri, Warburton, and others).

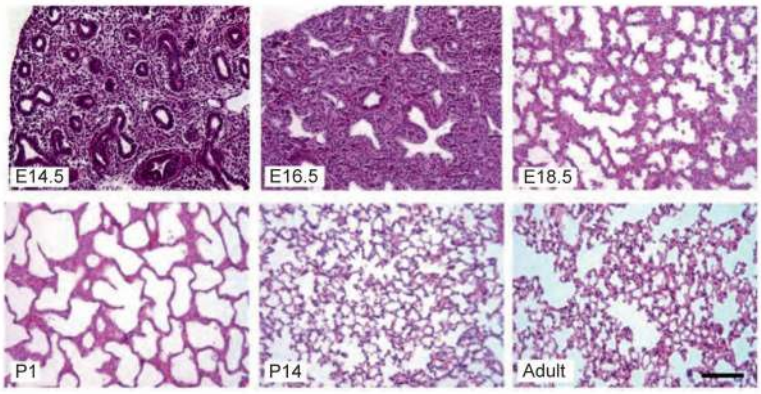


**Figure 3.2.**

Histology of mouse lung at characteristic stages of development. Embryonic mouse lung develops from pseudoglandular stage (E14.5) to canalicular stage (E16.5) and further terminal sac stage (E18.5 and P1). Neonatal mouse lungs undergo alveolarization, resulting in the formation of many septa (P14). Finally, a mature honeycomb-like structure with alveoli surrounding alveolar ducts conferring normal respiratory structure and function is formed, as observed in the adult. Scale bar: 100 μm.

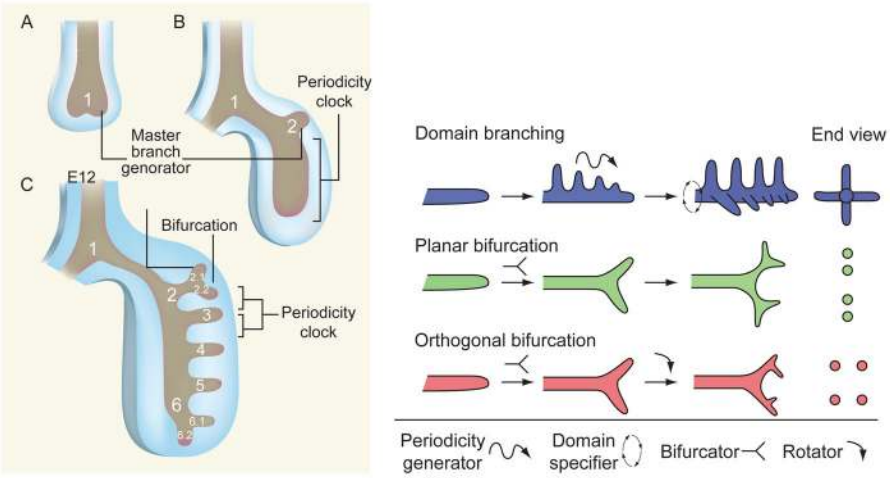


**Figure 3.3.**

How the airways can form in a sequential manner by reiterating a few, relatively simple sets of genetic instructions. In the upper panel, which is drawn after Warburton (2008), a master branch generator, a periodicity clock, and a bifurcator program are shown as controlling the layout of the mainstem and lobar branches. At embryonic day (E) 10.5, (A) the primary bronchial branch (1) forms, followed by (B) the development of the left upper-lobe branch (2) by E11, and then (C) the first two segmental branches of the left upper-lobe branch (2.2 and 2.3) form and the subsequent formation of branches 3–6 occurs by E12. The master branch generator is active throughout these events, and the inferred sites of action of the periodicity clock and bifurcator subroutines are shown. Then, in the lower panel, following the views of Metzger et al., (2008), a series of inferred genetic subroutines are shown, all driven by one master branch generator, shown as giving rise to domain or "bottle brush" branching along the lateral proximodistal axis of the main stem bronchi, which can then be rotated at right angles to give rise to a second rank of branches. Then, in subsequent rounds of branching, arising from the tips of the primary and secondary branches, it is shown how the same relatively simple periodicity generator, domain specifier, bifurcator, and rotator subroutines can give rise to apparently more complex patterns of peripheral branching to achieve an ever larger number of space filling terminal branches.

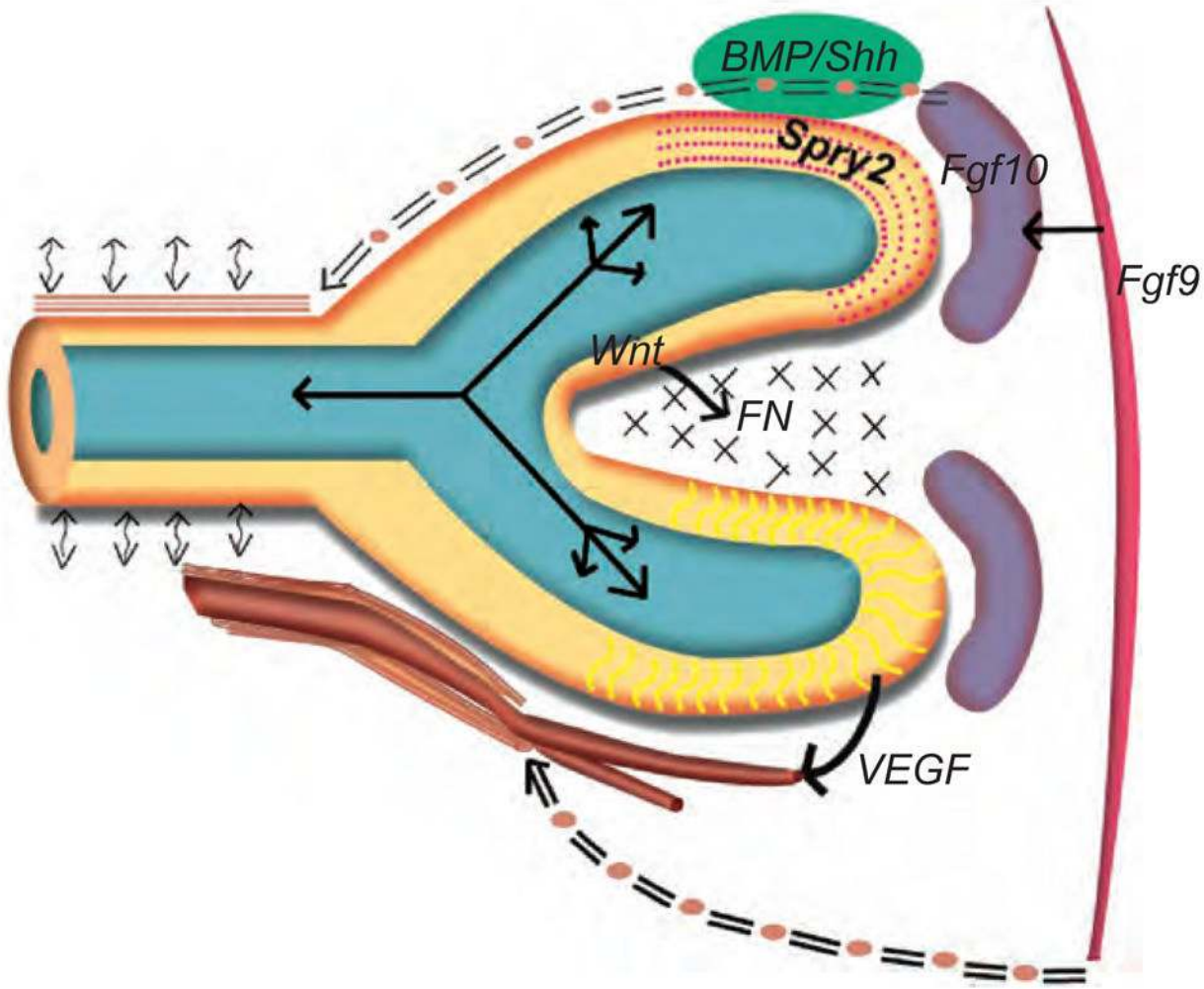


**Figure 3.4.**

Schematic illustration of the variety of biochemical and biomechanical regulators of lung growth. The epithelium of the end-bud (yellow) encloses a fluid filled lumen (blue-green) in which oscillatory fluid flows are depicted (solid bidirectional arrows) that result from periodic peristaltic contractions (bi-directional curvilinear arrows) of airway smooth muscle (ASM: sample shown in brown running parallel and above the main epithelial trunk). The ASM derives from the $FGF10^{+}$ precursor pool (seen in purple on the right), and these ASM progenitors are seen becoming more proximal (double-lined "=" at the top of the epithelial outline) relative to the growing epithelium. Examples of key biochemical signaling are given: FGF9 from the mesothelium (far right) regulates the $FGF10^{+}$ mesenchyme (purple), which in turn interacts locally with epithelial Sprouty (SPRY2), BMP4, and Sonic Hedgehog (SHH) signaling (the latter two epithelial signals are shown in green and, due to space constraint, adjacent to the schematized epithelium). Epithelial Wnt signaling regulates fibronectin (FN) elaboration (shown as "xx") in the cleft between epithelial branches. Epithelial VEGF signals to developing vasculature shown at the base of the figure. These vessels attract vascular smooth muscle precursors from the mesothelium (shown as "=" at the base of the figure). (See Color Insert.)

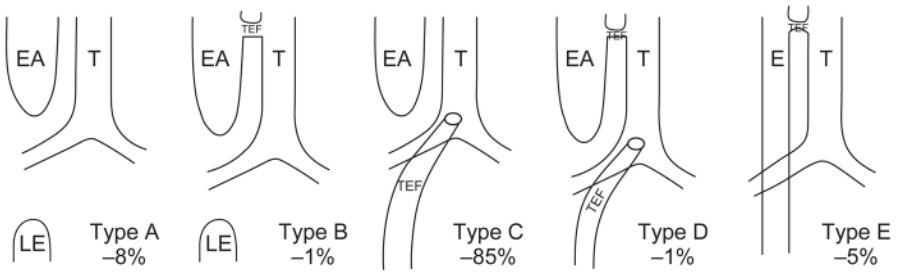


**Figure 3.5.**

Subtypes of esophageal atresia (EA) and/or tracheo(T)–esophageal (E) fistula (TEF) with percentage frequency amongst EA-TEF cases. Type A: 'Pure' EA without TEF. Lower esophagus shown (LE); Type B: EA with TEF from proximal esophageal pouch but without any distal TEF; Type C: EA with distal TEF only (the most common variant); Type D: EA with both proximal TEF and distal TEF; Type E: TEF in the absence of EA. Laryngotracheal clefts (not shown here) are still rarer anomalies in which trachea and esophagus form a single lumen for a variable length. In severe variants, a combined tracheo-esophagus connects to the stomach whilst also giving rise to the main bronchi.

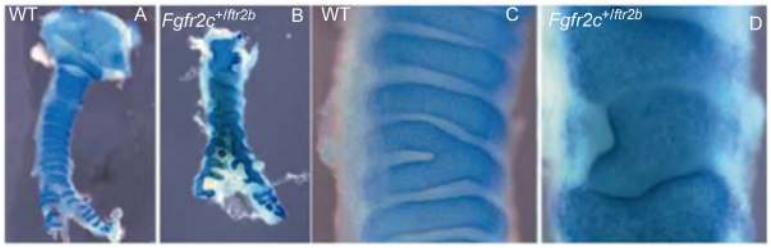


**Figure 3.6.**

Excessive mesenchymal FGF signaling leads to overgrowth of tracheal rings. Wild-type and mutant tracheas are stained with Alcian blue. (A) Wild-type trachea at P0 exhibiting regular cartilage rings separated by noncartilaginous mesenchyme; (B) $Fgfr2c^{+/Fgfr2b}$ trachea at P0 showing excessive growth of the cartilage with absence of noncartilaginous mesenchyme; (C, D) high magnification of A and B, respectively. (See Color Insert.)

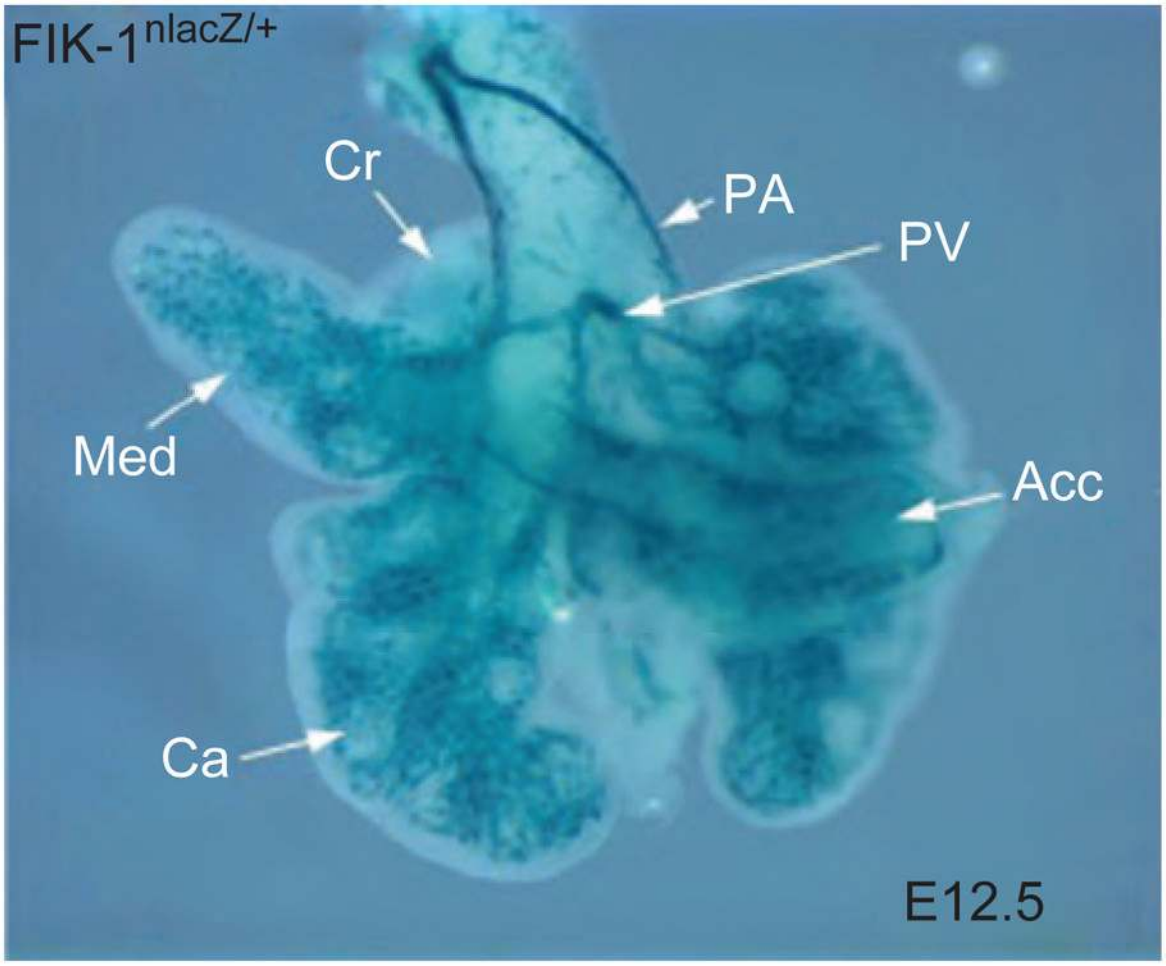


**Figure 3.7.**
Vascular endothelial development in E12.5 mouse lung is shown in whole mount as a blue signal resulting from transgenic expression of Flk1-β-galactosidase (Flk-$1^{nLacZ/+}$): pulmonary artery (PA); pulmonary vein (PV); cranial lobe (Cr); medial lobe (Med); caudal lobe (Ca); accessory lobe (Acc). (See Color Insert.)

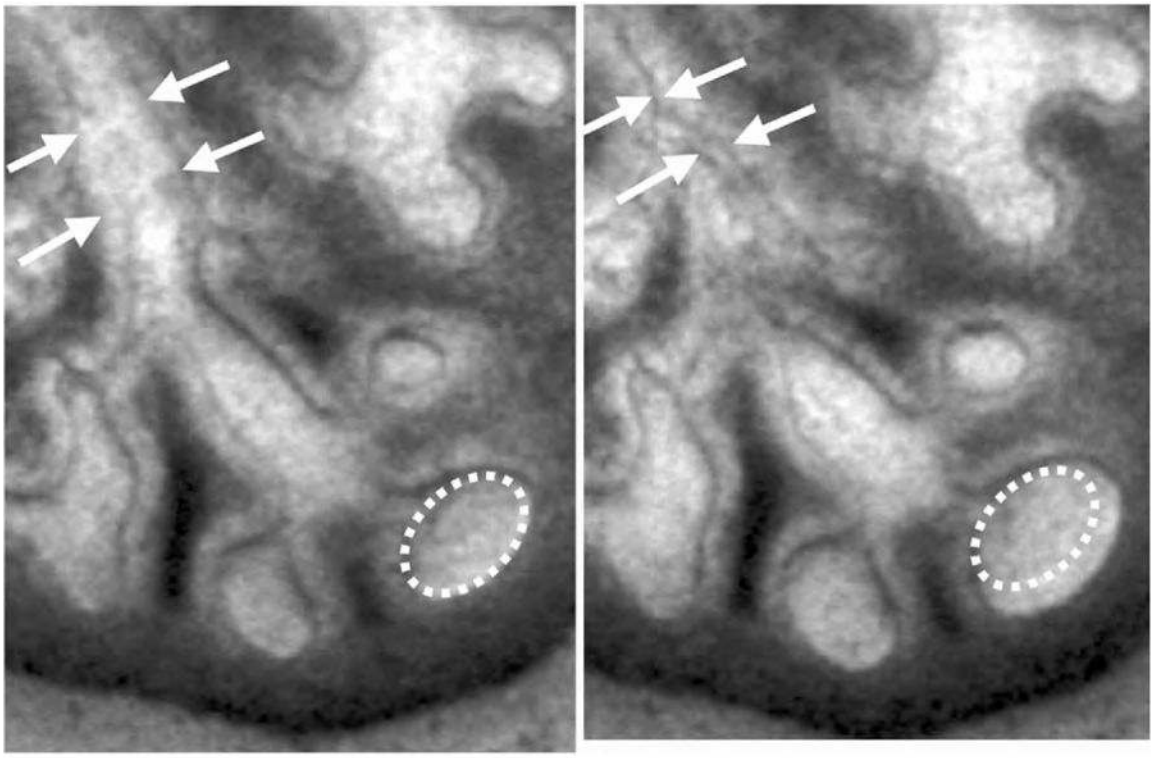

**Figure 3.8.**
Peristalsis occludes airway proximally whilst distending it distally. Sequential photomicrographs of cultured embryonic lung: in both panels the arrows outline proximal airway (open in the top picture but occluded by contraction in the lower one). The peristaltic airway occlusion's distal effect is shown in a terminal bud, which rapidly rhythmically increases in size (the same ovoid outline is applied to both pictures). This distension and relaxation recurs with each wave.

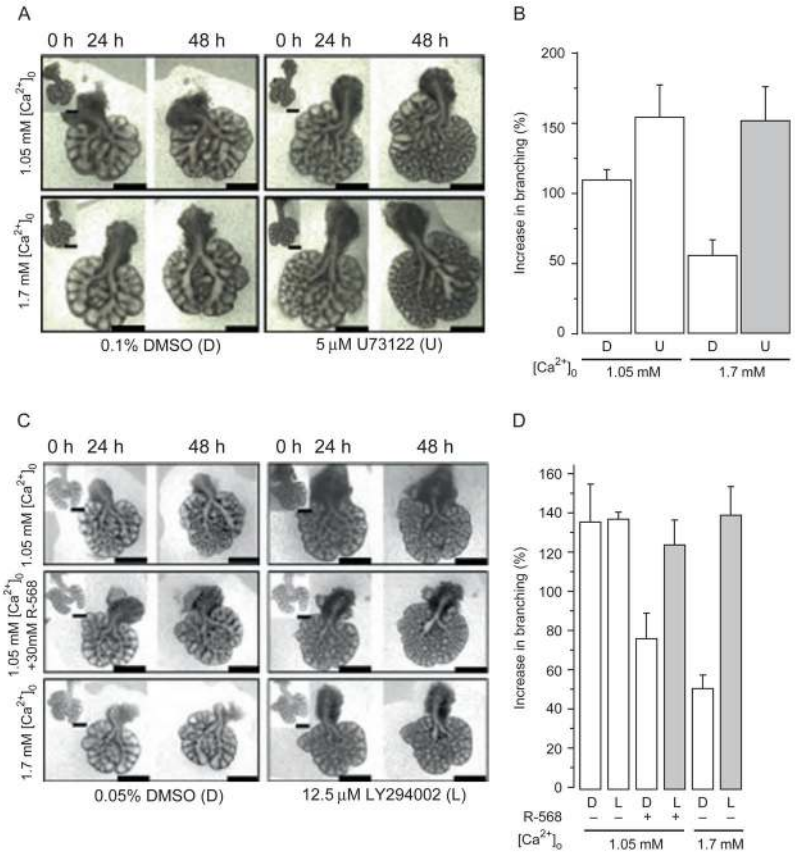


**Figure 3.9.**

CaSR-evoked inhibition of branching and its dependence on Pulmonary lymphangitiic carcinomatosis (PLC) and phosphoinositide 3 ($PI_3$) kinase signaling. Effect on branching of 1.05 mM (A, upper panels) or 1.7 mM (A, lower panels) $Ca^{2+}_o$ in the absence (0.1% DMSO vehicle control; A left panels) or presence (A, right panels) of 5 μM of the PLC inhibitor, U73122. Quantification of branching at 48 h in the four conditions is shown in (B). Inhibition of PLC rescues suppression of branching evoked by 1.7 mM $Ca^{2+}_o$. Bars = 700 μm. Effect on branching of 1.05 mM $Ca^{2+}_o$ (C, upper panels), 1.05 mM $Ca^{2+}_o$ plus 30 nM R-568 (C, middle panels), and 1.7 mM $Ca^{2+}_o$ (C, lower panels) in the absence (0.05% DMSO vehicle control) or presence of 12.5 μM of the PI3K inhibitor, LY294002. Bars = 750 μm. Quantification of branching at 48 h in the six conditions is shown in (D). The calcimimetic R-568 mimics the suppressive effect of high $Ca^{2+}_o$ on branching, further implicating CaSR in the process. Note $PI_3$ kinase inhibition rescues suppression of branching, whether evoked by high $Ca^{2+}_o$ or calcimimetic. Adapted from Finney et al. (2008).

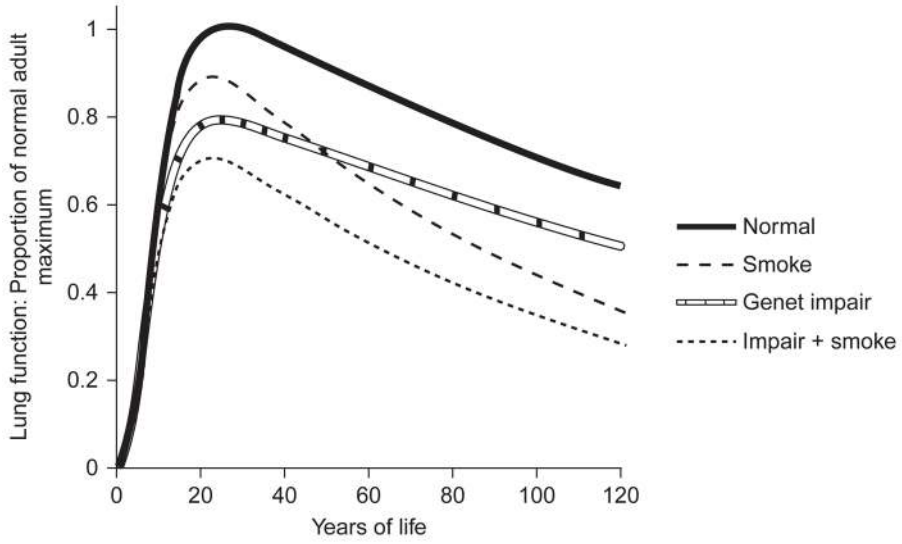


**Figure 3.10.**

Smoking and genetics synergize to degrade lung function with age (modified after Fletcher and Peto, 1977; Shi, W. and Warburton, D. 2010). Wild-type lungs "grown in room air" achieve greatest capacity and remain healthy despite age-related degradation. Smoking exacerbates degradation of lung function even in the healthy. Genetic alterations decrease the potential to develop maximal lung capacity compared to wild type and smoking exacerbates lung degradation still further. A 0.5% loss of lung function per year is assumed for normal lungs and double that for smokers. Lungs with a genetic defect were assumed to have 95% of the growth rate of a normal lung. Growth rate is considered as a process of doublings, but the rate of doublings declines from maturity exponentially with age. This is expressed in a simple differential equation for modeling lung function;

$L(t)$:rate of change of lung function=rate of increase of lung function−rate of decline of lung function; $dL/dt = r \star \exp(-\text{mat} \star t) \star L - a \star L$

, where mat is the maturation coefficient, r is the doubling coefficient, and a is the loss-of-function coefficient.

**Table 3.1**

Examples of mutations in mouse giving a reported lung and/or tracheal phenotype

| Gene symbol | Gene name | Expression pattern | Phenotype | Reference |
|---|---|---|---|---|
| Signaling molecule | | | | |
| Egfr | Epidermal growth factor receptor | Epithelium and mesenchyme | Impaired branching and deficient alveolization | Miettinen et *al.* (1997) |
| Fgf18 | Fibroblast growth factor 18 | Mesenchyme | Deficient alveolization | Usui *et al.* (2004) |
| Fgf9 | Fibroblast growth factor 9 | Epithelium and pleura | Impaired branching, reduced mesenchyme | Colvin *et al.* (2001) |
| Greml | Gremlin 1 | Epithelium and mesenchyme | Deficient alveolization | Michos *et al.* (2004) |
| Hipl | Huntingtin-interacting protein 1 | Mesenchyme | Impaired branching | Chuang et *al.* (2003) |
| Shh | Sonic hedgehog | Epithelium | Impaired branching, tracheoesophageal fistula | Litingtung *et al.* (1998) |
| Tgfb3 | Transforming growth factor, β3 | Epithelium and pleura | Impaired branching | Kaartinen *et al.* (1995) |
| Wnt7b | Wingless-related MMTV integration site 7B | Epithelium | Vascular defect, reduced mesenchyme | Shu *et al.* (2002) |
| Catnnb1 | β-Catenin | Epithelium | Impaired branching, proximal/distal specification | Mucenski *et al.* (2003) |
| Ltbp4 | Latent transforming growth factor β binding protein 4 | Not reported | Pulmonary emphysema | Sterner-Kock *et al.* (2002) |
| Wnt5a | Wingless-related MMTV integration site 5A | Mesenchyme and epithelium | Increased branching, tracheal defect | Li *et al.* (2002) |
| Fgfl0 | Fibroblast growth factor 10 | Mesenchyme | Lung agenesis | Sekine *et al.* (1999) |
| Fgfr2b | Fibroblast growth factor receptor 2b | Epithelium | Lung agenesis | De Moerlooze *et al.* (2000) |
| Fgf8 | Fibroblast growth factor 8 | Not reported | Right pulmonary isomerism | Fischer *et al.* (2002) |
| TAB1 | TGF-B activated kinase-1 binding protein-1 | Epithelium | Lung dysmorphogenesis | Komatsu *et al.* (2002) |
| Acvr2b | Activin receptor IIB | Not reported | Right pulmonary isomerism | Oh and Li (1997) |
| Nodal | Nodal | Not reported | Right pulmonary isomerism | Lowe *et al.* (2001) |
| Lefty1 | Left right determination factor 1 | Not reported | Left pulmonary isomerism | Meno *et al.* (1998) |
| Traf4 | Tnf receptor associated factor 4 | Not reported | Tracheal defect | Shiels *et al.* (2000) |
| Fgfr3/Fgfr4 | Fibroblast growth factor receptor 3/4 | Epithelium and mesenchyme | Defective elastin production, alveolarization defect | Weinstein *et al.* (1998) |
| Nog | Noggin | Mesenchyme | Lobation defect | Weaver *et al.* (2003) |
| Pitx-2 | Paired-like homeodomain transcription factor 2 | Not reported | Bilateral isomerism | Kitamura *et al.* (1999) |
| Dermo1 | Twist homolog 2 | Mesenchyme | Impaired branching | De Langhe *et al.* (2008) |
| BMP4 | Bone morphogenic protein 4 | Epithelium and mesenchyme | Abnormal lung morphogenesis with cystic terminal sacs | Bellusci *et al.* (1996) |
| Igf1r | Insulin-like growth factor 1 receptor | Not reported | Impaired development | Liu *et al.* (1993). |
| Notch2/3 | Notch gene homolog 2/3 | Epithelium | Defective myofibroblast differentiation, alveolarization defect | Xu *et al.* (2009) |

| Gene symbol | Gene name | Expression pattern | Phenotype | Reference |
|---|---|---|---|---|
| PDGFa | Platelet derived growth factor a | Epithelium | Defective myofibroblast elastin production, alveolarization defect | Bostrom *et al.* (2002) |
| Timp3 | Tissue inhibitor of metalloproteinase 3 | Mesenchyme | Reduced number of bronchioles and attenuated alveogenesis | Gill *et al.* (2003) |
| Transcription factor | | | | |
| Cebpa | CCAAT/enhancer binding protein (C/ EBP), α | Epithelium | Hyperproliferation of type II cells | Sugahara *et al.* (2001) |
| Foxa1/Foxa2 | Forkhead box A1/A2 | Epithelium | Impaired branching, reduced smooth muscle | Wan *et al.* (2005) |
| Foxf1a | Forkhead box F1a | Mesenchyme | Impaired branching, lobation defect | Lim *et al.* (2002) |
| Hoxa5 | Homeobox A5 | Mesenchyme | Impaired branching, tracheal defect | Aubin *et al.* (1997) |
| Klf2 | Kruppel-like factor 2 (lung) | Not reported | Impaired sacculation | Wani *et al.* (1999) |
| Mycn | Neuroblastoma myc- related oncogene 1 | Epithelium | Impaired branching | Moens *et al.* (1992) |
| Trp63 | Transformation- related protein 63 | Epithelium | Tracheobronchial defect | Daniely *et al.* (2004) |
| Titf1 | Thyroid transcription factor 1 | Epithelium | Loss of distal lung fate, impaired branching, tracheoesophageal fistula | Kimura *et al.* (1996) |
| Nfib | Nuclear factor I/B | Epithelium and mesenchyme | Sacculation defect | Steele-Perkins *et al.* (2005) |
| Sox11 | SRY-box-containing gene 11 | Epithelium | Hypoplastic lung | Sock *et al.* (2004) |
| Tcf21 | Transcription factor 21 (Pod1) | Mesenchyme | Impaired branching | Quaggin *et al.* (1999) |
| Rarb/Rara | Retinoic acid receptor α/β | Epithelium and mesenchyme | Left lung agenesis and right lung hypoplasia | Mendelsohn *et al.* (1994) |
| Pitx2 | Paired-like homeodomain transcription factor 2 | Mesenchyme | Right pulmonary isomerism | Lin *et al.* (1999) |
| Foxj1 | Forkhead box J1 | Epithelium | Left– right asymmetry, loss of ciliated cells | Brody *et al.* (2000) |
| Gata6 | GATA-binding protein 6 | Epithelium | Impaired sacculation | Yang *et al.* (2002) |
| Gli2/Gli3 | GLI-Kruppel family member GLI2/ GLI3 | Mesenchyme | Lung agenesis | Motoyama *et al.* (1998) |
| Ascl1 | Achaete-scute complex homolog- like 1 | Neuroendocrine cells | Loss of neuroendocrine cells | Ito *et al.* (2000) |
| Erm | Ets variant gene 5 | Epithelium | Impaired type I cell formation | Liu and Hogan (2002), Liu *et al.* (2003) |
| Wnt2/2b | Wingless-related MMTV integration site 2/2b | Mesenchyme | Complete lung agenesis | Goss *et al.* (2009)<br>Harris- Johson *et al.* (2009) |
| Alk3 | Aurora-like kinase | Epithelium | Retardation of lung branching, reduced cell proliferation and differentiation | Sun *et al.* (2008) |
| Others | | | | |
| Eln | Elastin | Mesenchyme | Deficient alveolization | Wendel *et al.* (2000) |
| Lmnb1 | Lamin B1 | Epithelium and mesenchyme | Deficient alveolization | Vergnes *et al.* (2004) |
| Lama5 | Laminin α 5 | Epithelium and pleura | Defective lobation | Nguyen *et al.* (2002) |

| Gene symbol | Gene name | Expression pattern | Phenotype | Reference |
|---|---|---|---|---|
| Pcaf | p300/CBP-associated factor | Epithelium and mesenchyme | Defective proximal and distal epithelial cell differentiation | Shikama *et al.* (2003) |
| Adam17 | A disintegrin and metallopeptidase domain 17 | Epithelium | Impaired epithelial differentiation, impaired branching | Zhao *et al.* (2001), Peschon *et al.* (1998) |
| Crh | Corticotropin- releasing hormone | Epithelium | Defective epithelial and mesenchymal maturation | Muglia *et al.* (1999) |
| Pthlh | Parathyroid hormone- like peptide | Epithelium | Deficient alveolization | Rubin *et al.* (2004) |
| Itga3 | Integrin α3 | Epithelium | Impaired branching | Kreidberg *et al.* (1996) |
| Cutl1 | Cut-like 1 | Epithelium | Impaired epithelial differentiation | Ellis *et al.* (2001) |
| RXRa | Retinoic X receptor alpha | Epithelium and mesenchyme | Decrease in alveolar surface area and alveolar number | McGowan *et al.* (2000) |
| Tmem16a | Transmembrane protein 16a | Epithelium | Abnormal tracheal cartilages resulting in tracheomegaly | Rock *et al.* (2008) |
| TACE | Tumor necrosis factor- α converting enzyme | Not reported | Failure to form saccular structures | Zhao *et al.* (2001) |
| PDGFa | Platelet derived growth factor a | Epithelium | Defective myofibroblast elastin production, alveolarization defect | Bostrom *et al.* (2002) |
| Na/K ATPase | Sodium/ Potassium ATPase | Epithelium | Failure to absorbfetal lung liquid, causes significant respiratory distress and neonatal lethality | Hummel *et al.* |
| Lfng | Lunatic Fringe | Epithelium | Impaired myofibroblast differentiation and alveogenesis | Xu *et al.* (2009) |