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Lung Endoderm Morphogenesis: Gasping for Form and Function

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Abstract

The respiratory endoderm develops from a small cluster of cells located on the ventral anterior foregut. This population of progenitors generates the myriad epithelial lineages required for proper lung function in adults through a complex and delicately balanced series of developmental events controlled by many critical signaling and transcription factor pathways. In the past decade, understanding of this process has grown enormously, helped in part by cell lineage fate analysis and deep sequencing of the transcriptomes of various progenitors and differentiated cell types. This review explores how these new techniques, coupled with more traditional approaches, have provided a detailed picture of development of the epithelial lineages in the lung and insight into how aberrant development can lead to lung disease.

Keywords

branching morphogenesis; congenital lung disease; transcription factor; signaling; noncoding RNA

INTRODUCTION

To fuel aerobic metabolism and sustain terrestrial life, higher vertebrates have evolved complex systems to efficiently extract oxygen from and discard carbon dioxide into the environment. The challenges of this process are enormous, and to meet the high metabolic

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demands of mammals in particular, a massive surface area of contact between air and blood circulating in the cardiovascular system is required to permit adequate gas exchange. In human adults, the surface area of the respiratory system is approximately 80–100 square meters, approximately half the size of a tennis court (Colebatch & Ng 1992). With such a large amount of epithelial tissue exposed to the environment and in close proximity to the circulatory system, terrestrial organisms risk excessive fluid loss and exposure to noxious environmental agents and infectious organisms. Moreover, dysregulation in the development of the respiratory system can result in impaired gas exchange, dehydration, infection, or dysfunctional immune responses in response to noxious environmental insults.

Hundreds of millions of years of evolution have resulted in sophisticated and fascinating strategies to address these challenges. After budding from the foregut, the lung undergoes a carefully choreographed phase of branching morphogenesis, the first step in forming the elaborate, highly branched, intertwined networks of the airway tree and pulmonary vascular system. As outgrowth continues, specialized cell types are established and begin to mature, and additional airspaces are created and refined. Cell lineages are established to perform multiple functions, including secreting mucus to hydrate and moisturize the airways and clearing airways of particulate matter. In the distal alveolar region, epithelial lineages either form the thin gas-exchange interface with the cardiovascular system or produce the lipids and proteins needed to reduce surface tension to allow proper compliance during respiration. Many poorly defined cell populations within the pulmonary mesenchyme also make important contributions by providing blood supply (pulmonary vasculature), removing excess fluid (lymphatic system), regulating airway tone (airway smooth muscle), providing structural support (tracheal cartilage), supporting epithelial homeostasis, and directing repair and regeneration after injury. Additional tissue types, including nerves and immune cells, must also be integrated into this complex structure to assure a properly functioning respiratory system that is adequately protected from the outside world.

Given the complexity of the lung and the necessity that it function immediately after birth and continuously thereafter, it is not surprising that dysfunction of this organ is a common source of human disease. Disruption of lung development may lead a variety of pathologies, such as congenital cystic lung disease, pulmonary hypoplasia, or bronchopulmonary dysplasia. Invasion of this fragile space by pathogens can lead to infections that are local (e.g., pneumonia) or systemic (e.g., sepsis) at any age, and exposure to external agents (e.g., cigarette smoke, fine dust) can result in severe injury to the lung and diseases such as chronic obstructive pulmonary disease (COPD) or pulmonary fibrosis. Disruptions in the balance between cellular quiescence and proliferation may result in primary lung cancer, the leading cause of cancer deaths worldwide (US Cancer Stat. Work. Group 2014). Many of these lung diseases remain difficult to treat, owing to our incomplete understanding of the various cell populations within the lung; the mechanisms by which they coordinately develop; and the molecular mechanisms that direct normal developmental processes, maintain cellular quiescence during homeostasis, and allow the proper response to acute and chronic injury.

This review covers the seminal studies that have revolutionized our understanding of lung development, including important early developmental processes such as branching

morphogenesis, the role of the many cell lineages that constitute the lung, and the molecular pathways involved in the proper differentiation of these lineages. We primarily focus on the development of the lung endoderm and touch upon how this information has guided our understanding of how the respiratory system responds to disease and repairs itself after injury. This review builds upon and complements other recent and excellent reviews of lung morphogenesis, repair, and regeneration (Cardoso & Lü 2006, Herriges & Morrissey 2014, Hogan et al. 2014, Kotton & Morrissey 2014, Morrissey & Hogan 2010).

AN OVERVIEW OF LUNG DEVELOPMENT

The respiratory endoderm is specified in the anterior foregut endoderm, a tissue that generates multiple organs including the thyroid, esophagus, and liver. The respiratory endoderm generates all of the epithelial lineages that line the mature airways and alveoli of the lungs and trachea. Specification of the respiratory endoderm can be observed at approximately embryonic day (E)9.0 in mice, detected by expression of the transcription factor *Nkx2.1* on the ventral side of the anterior foregut. By E9.5–10, *Nkx2.1*⁺ endoderm cells protrude ventrally and form the primitive trachea and two lung buds, which will generate the left and right lobes of the lung. In E9.5–12.5, the trachea fully separates from the esophagus. During the next 4 days of mouse development (E12.5–16.5), the process of branching morphogenesis generates the extensive arborized network of airways that is critical for producing the large surface area for respiration. From E16.5 to approximately postnatal day (P)3–5, saccules develop at the distal end of these branches and come in close apposition to the codeveloping cardiopulmonary vasculature. The final alveolarization stage of lung development in mice (P3–14) results in a thin interface between distal alveolar epithelial cells and the vascular capillary plexus that is essential for highly efficient gas exchange to occur in high-metabolism animals such as mammals. In mice, the process of alveolarization does not begin until after birth, whereas in humans this process begins by 36 weeks of gestation (Figure 1). In the sections that follow, we examine the molecular pathways that drive each of these stages of lung development.

The cardiopulmonary vasculature forms simultaneously with the branching airways. The codevelopment of these two branching networks involves important epithelial-mesenchymal crosstalk, which is discussed in detail below. This crosstalk is mediated by many of the critical pathways active in embryogenesis, including Wnt, bone morphogenic protein (Bmp), sonic hedgehog (Shh), and fibroblast growth factor (Fgf) (Peng et al. 2013). Importantly, defects or interruptions at any point in these early developmental processes can lead to a broad array of congenital or neonatal lung diseases, from congenital cystic adenomatoid malformations to bronchopulmonary dysplasia (Figure 1).

Evolutionary Perspective on Lung Development

Although effective absorption of oxygen from the environment has always been an essential problem for complex organisms, the transition from aquatic to terrestrial life more than 375 million years ago demanded evolution of a complex organ structure dedicated to this task. Swim bladders have evolved several times during the evolution of fish, and many of the molecular pathways important for their development are conserved in early mammalian lung

development (Perry et al. 2001). The pioneering FoxA transcription factors, which are essential for proper specification of the foregut endoderm from which the lung arises, are conserved from humans to *Caenorhabditis elegans*; in the latter species, the homolog PHA-4 is expressed in all 80 pharyngeal cells and activates a pharyngeal gene program in a dose-dependent manner (Kalb et al. 1998, Pilon 2014). Despite gross differences in lung morphology, studies of the molecular pathways directing initial lung specification and early development in frog, mouse, and human demonstrate remarkable conservation of the core pathways and transcription factors (e.g., the Wnt/ β -catenin, Fgf, and Bmp pathways and the transcription factor Nkx2.1) (Rankin et al. 2015). Even proteins involved in adult lung function, such as the surfactant proteins, are conserved in the swim bladders of fish (Daniels et al. 2004). As lung development proceeds, however, key differences begin to appear, including adaptations designed to meet the highly varied metabolic needs and environmental demands encountered by various species. Even the mouse lung, which at a very superficial glance bears great similarity to the human lung and has been extensively used as a universal model of mammalian lung development and pulmonary disease, exhibits key differences from human lungs in structure and cell-type distribution. These differences are important in our understanding and use of this model system to study normal human lung development and disease. For example, the medium-sized intralobar airway branches (intrapulmonary airways) in humans contain cartilage, smooth muscle, and mucus-secreting goblet cells extending deep into the lung, whereas the mouse intralobar airway branches lack both cartilage and goblet cells and are surrounded by a thinner ring of smooth muscle. These anatomic and cellular differences could have important implications for modeling a disorder, such as asthma, characterized by airway constriction due to smooth muscle contraction and excessive mucus production due to goblet cell hyperplasia.

Specification of Lung Within the Anterior Foregut Endoderm

Specification of the lung begins within a narrow region of the ventral foregut endoderm at approximately E9–9.5 in mouse and 28 days gestation in human. Signals from surrounding tissues, including the notochord and splanchnic mesoderm, begin to establish a dorsal-ventral pattern in the foregut endoderm necessary for expression of the earliest known marker of lung, the transcription factor Nkx2.1 (Figure 2*a*). The Wnt ligands Wnt2 and Wnt2b are expressed in the ventral mesoderm surrounding the anterior foregut and activate canonical Wnt signaling to promote expression of Nkx2.1 ventrally, where the future trachea and lung develop, and restrict Sox2 expression to the distal side of the foregut endoderm, site of the future esophagus (Figure 2*a*). Disruption of Wnt signaling during this critical window results in a lack of Nkx2.1 expression, absence of tracheal morphogenesis, and complete lung agenesis (Goss et al. 2009, Harris-Johnson et al. 2009). Similarly, Bmp4 is expressed in the ventral mesoderm, and the Bmp antagonist Noggin is expressed from the notochord on the dorsal side, signaling to the foregut endoderm through the Bmp receptors Bmpr-1a and -1b. This helps establish the dorsal-ventral gradient of Bmp signaling required to promote proper placement of the lung along the proximal-distal axis of the foregut (Domyan et al. 2011). Fgfs expressed from the ventral mesoderm further augment early specification and patterning of the respiratory endoderm, in part by promoting lung outgrowth (Figure 2*a*).

By E10 in the mouse, two lung buds have begun to form and extend caudally. At this time, the tracheoesophageal folds form and eventually fuse to generate a tracheal-esophageal septum, separating the trachea from the esophagus. This highly dynamic process involves cell proliferation and dramatic changes in cell shape mediated by cytoskeletal rearrangements. Disruption at any step in this process can lead to congenital malformations of the foregut, such as tracheoesophageal fistula, with devastating consequences for neonates (Figure 1).

Extension and Outgrowth of the Lung Bud

Initial outgrowth of the lung bud, and subsequent growth of the branching lung tips, is driven by multiple pathways, including the Fgf receptor pathway. The Fgf10 ligand is expressed in the mesenchyme and signals to Fgfr1/2 in the endoderm to direct branching morphogenesis (Figure 2*b*). Disruption of this pathway leads to a loss of branching, though lung specification and separation of the trachea from the esophagus still occur (Min et al. 1998, Sekine et al. 1999). Careful control over the timing and expression of Fgf10 is provided by a complex interplay of multiple signaling pathways, including retinoic acid (RA), Shh, Wnt, and BMP/Tgf- β signaling (Figure 2*b*). RA is produced in the mesenchyme of the foregut region prior to lung specification, as early as E8 (Malpel et al. 2000). Synthesis of bioactive RA from retinaldehyde is controlled by the retinaldehyde dehydrogenases (RALDHs or ALDH1A1–3). ALDH1A2 is developmentally regulated and appears to be the major isoform involved in embryogenesis, including patterning of the early foregut (Malpel et al. 2000). RA produced by ALDH1A2 in the mesoderm, in turn, signals to the mesoderm itself and the adjacent endoderm. Signaling through RA receptor β (RAR β) in the mesoderm, in particular, regulates Fgf10 expression to modulate lung bud outgrowth (Desai et al. 2004, 2006). Disruption of RA production during this timeframe via deletion of ALDH1A2 results in severe anomalies of the foregut, including the stomach and liver, and in failure of lung bud outgrowth owing to defective Fgf10 signaling (Wang et al. 2006).

Shh signaling mediated by the effectors Gli1, Gli2, and Gli3 in the mesoderm is also an important regulator of initial lung bud outgrowth. Loss of both Gli2 and Gli3 leads to tracheal and pulmonary agenesis (Hui et al. 1994, Motoyama et al. 1998). Tbx2, Tbx3, and Tbx4 are similarly coexpressed with Fgf10 in the mesenchyme and may also function to positively regulate Fgf10 expression (Chapman et al. 1996, Sakiyama et al. 2003).

Branching Morphogenesis

Shortly after the lung buds have formed and separated from the esophagus, they begin a dynamic phase of lung development involving outgrowth and branching into the surrounding mesenchyme. The result of this next phase of lung development is the generation of a highly arborized structure that lays the foundation for providing the large surface area required for efficient gas exchange. The program of branching morphogenesis is remarkably consistent between animals and across various inbred strains of mice, indicating that it is largely genetically hardwired. The branching process can be divided into at least three relatively simple subroutines (Metzger et al. 2008). In the first subroutine, smaller branches form in rows along the length of and around the circumference of the parent branch, like small shoots growing out of the side of a large tree branch. In the second two subroutines, the tip

of each branch grows outward and bifurcates. In planar bifurcation, two or more branches occur within the same plane. In orthogonal bifurcation, each branch occurs at a 90° angle to the previous branch.

A complex interplay of signaling factors carefully coordinates the process of bud outgrowth, extension, and bifurcation. Normal branching requires both positive, stimulating signals and a host of negative signals providing feedback to prevent ectopic budding and overgrowth of the developing lung bud. Signaling by Fgf10 from the mesenchyme to Fgfr1/2 in the distal tip epithelium continues to be a major driver of outgrowth of the lung buds during branching morphogenesis, as it was for initial outgrowth of the primary lung bud from the foregut epithelium. Shh signaling provides an important layer of control over this process, in complex and seemingly contradictory ways. Shh is highly expressed in the distal epithelium of the lung bud and signals to the mesenchyme, where it activates patched (Ptch1)/smoothened (Smo) and their transcriptional effectors Gli1, Gli2, and Gli3. This in turn downregulates Fgf10 expression as the lung bud expands (Bellusci et al. 1997, Lebeche et al. 1999). However, at the very tip of the bud, where Shh levels are highest, Shh signaling also leads to induction of hedgehog-interacting protein (Hhip). Hhip inhibits Shh signaling by sequestering the Shh ligand, which in turn decreases repression of Fgf10 at the expanding tip (Chuang et al. 2003). Additional complexity is generated by the ability of Gli3 to stimulate the expression of Foxf1 in the mesenchyme, which in turn promotes Fgf10 expression (Li et al. 2004). Recently, Chang et al. (2013) have proposed that alveolar differentiation antagonizes the lung branching program. In this model, regulators of the distal branch point endoderm, including Sox9, suppress alveolar differentiation while promoting early branching. Such a model may help explain the switch from an early branching mode of lung development to a mode of epithelial differentiation toward the end of gestation.

Wnt and Bmp signaling play equally important, but complex and intertwined, roles in regulating branching morphogenesis. Canonical Wnt signaling occurs when one of several Wnt ligands binds to a Frizzled (Fzd) receptor, causing β -catenin, normally found in the cytoplasm, to translocate to the nucleus and affect gene expression. In the branching lung, nuclear β -catenin is predominantly observed in the distal epithelium, where it is activated by ligands such as Wnt2/2b, which are secreted by the mesenchyme, and Wnt7b, which is generated in the epithelium (De Langhe et al. 2005, Okubo & Hogan 2004) (Figure 2c). Disruption of canonical Wnt signaling during lung development by conditional deletion of β -catenin or overexpression of the Wnt inhibitor Dickkopf1 severely impairs branching morphogenesis and decreases epithelial differentiation (Mucenski et al. 2003, Shu et al. 2005). This occurs at least in part through regulation of Fgfr2 and Bmp4 in the lung epithelium (Rajagopal et al. 2008, Shu et al. 2005). Whereas Fgfr2 expression promotes outgrowth of the lung bud, autocrine signaling by Bmp4 in the epithelium appears to limit Fgf10-mediated budding (Lebeche et al. 1999, Weaver et al. 2000). Further crosstalk exists with the Shh pathway, wherein Shh can stimulate expression of Wnt2 and Bmp4 in the mesenchyme (Pepicelli et al. 1998).

Several factors provide negative feedback on bud outgrowth in a more direct fashion. The sprouty (Spry) proteins inhibit Fgf and Egf signaling, and Spry2, which is induced in the lung epithelium in response to Fgf10, inhibits Fgf10-mediated signaling through Fgfr2

(Mailleux et al. 2001; Metzger et al. 2008; Tefft et al. 1999, 2002). In vitro and ex vivo data suggest that Tgf- β 1 accumulates in the mesenchyme of the proximal stalk region between buds and inhibits branching morphogenesis via induction of extracellular matrix components, such as collagens and fibronectin (Heine et al. 1990, Serra et al. 1994, Zhou et al. 1996). However, loss of Tgf- β 1 in vivo does not appear to result in branching defects, suggesting possible redundancy with other ligands expressed in the lung, including Tgf- β 2 and Tgf- β 3 (Letterio et al. 1994).

The role of the extracellular matrix (ECM) and cell-matrix interactions in regulating branching morphogenesis is likely of high importance, although little is understood about how the growing epithelium interacts with the underlying ECM. Fibronectin accumulates at sites of branch point constriction, suggesting that such cell-ECM and cell-matrix interactions are required for formation of new branch points (Sakai et al. 2003). A recent report shows that one of the integrins that interacts with fibronectin, β 1 integrin, is required for formation of a properly structured epithelial lining in the developing lung airways. Loss of β 1 integrin in the developing lung epithelium leads to an extensive multilayered epithelium and a block in branching morphogenesis (Chen & Krasnow 2012). This β 1-deficient lung epithelium exhibits a loss of apical-basal cell polarity, with cells growing in multiple layers. This study suggests that β 1 integrin restricts epithelial cell multilayering and is required for proper epithelial polarity during lung development. Such a role is likely important in human lung development, as β 1 integrin is aberrantly expressed in patients with bronchopulmonary sequestration and congenital cystic adenomatoid malformations (Volpe et al. 2009).

The significance of cell-matrix interactions in branching morphogenesis highlights the importance that epithelial remodeling has in lung bud outgrowth. Interestingly, gene expression analysis in epithelial buds at the earliest stages of branching demonstrates upregulation of genes involved in cell rearrangement and migration, inflammatory processes, lipid metabolism, proteolysis, and metastatic behavior, but not cell proliferation (Lu et al. 2005). To form a three-dimensional (3D) tubular structure from a simple spherical cluster of epithelium requires dynamic changes in cell shape. The nature of these cell-shape changes, and the mechanisms by which these changes occur, is only just beginning to be explored. Constriction at the apical surface of cells by actin-myosin is essential in this process (Kim et al. 2013). Conditional deletion of the Wnt receptor Fzd2 in the lung results in the formation of distal cysts, owing to a failure to form new domain branches. Signaling through Fzd2 regulates Rho signaling to control cell shape and apical constriction during branching (Kadzic et al. 2014). Other studies have highlighted the importance of the orientation of cell division to lung branching. Ras/Spry activity, which occurs downstream of Fgf signaling, regulates spindle-pole orientation, and activation of Kras randomizes the direction of spindle-pole formation, leading to lack of directionality in epithelial cell proliferation in the developing lung (Tang et al. 2009). This leads to a loss of new branch points associated with dilation of the developing airway epithelium, indicating that Fgf signaling plays an important role in regulating the orientation of cell division along the proximal-distal axis of the developing airways. Other pathways, such as the planar cell polarity (PCP) pathway, an arm of the Wnt signaling pathway, are likely important for regulating epithelial cell shape and consequently tube shape in the branching lung. Loss of multiple PCP components, including scribble, Celsr1, and Vangl2, results in subtle but important defects in lung

epithelial development, including early branching (Yates et al. 2010, 2013). Whether these act up- or downstream of other components of Wnt signaling is unknown. More work is needed to understand the effects of epithelial cell behavior on early lung branching and the molecular pathways that regulate this important aspect of lung morphogenesis.

Preparing for Gas Exchange: Canalicular, Saccular, and Alveolar Development

By E16.5 in mice, thousands of branched airway tubes have formed and the lung begins to increase the space available for gas exchange, ensuring that these spaces are in immediate proximity to the pulmonary vasculature so that gas exchange occurs efficiently. At this stage, the terminal bronchioles open into the respiratory bronchioles and widening tips known as the terminal sacs. These dilated tips will continue to expand into sacs known as primary alveoli, in a process called sacculatation. The processes of branching morphogenesis and alveolar differentiation are thought to be antagonistic, leading to the termination of branching morphogenesis and the initiation of an epithelial differentiation mode (Chang et al. 2013). The walls of these developing alveoli are known as primary septae and are in close contact with the developing vascular capillary plexus. Complex signaling between the epithelium, mesenchyme, and vasculature, as well as interactions with the ECM, is critical to normal development of the alveolar spaces. In later stages of alveolar development, secondary septae extend as ridges and further divide the airspaces to increase surface area. Myofibroblast progenitors and endothelial cells migrate into these ridges, with subsequent deposition of matrix proteins, including elastin. This process is at least in part regulated by Pdgfr- α (Bostrom et al. 1996, Lindahl et al. 1997).

Compared with our understanding of the signaling pathways directing branching morphogenesis, the exact molecular mechanisms directing alveologenesis remain poorly understood, despite the profound medical implications of disruptions at this stage of lung development. Infants born as early as 23–25 weeks gestation, prior to completion of alveologenesis, are now routinely cared for in newborn intensive care units across the country. A combination of ventilator injury, oxygen toxicity, infection, and inadequate nutrition can lead to impaired alveolar and pulmonary vascular development, with lifelong morbidities in those infants who survive (Patel et al. 2015, Vaucher et al. 2012). Other more rare disorders may also be seen, such as alveolar capillary dysplasia (ACD) with misalignment of the pulmonary veins, which is characterized by failure of formation and ingrowth of the alveolar capillaries, impaired alveolar development, and pulmonary veins abnormally positioned next to the pulmonary arteries (MacMahon 1948). In roughly 66% of ACD cases, deletions or point mutations involving the transcription factor *Foxf1* can be identified (Sen et al. 2013). Further characterizing the molecular mechanisms directing normal alveologenesis, the lung's response to injury during this stage of development, and strategies to mitigate arrest of alveolar development and differentiation in response to injury, remains a major challenge for the field of pulmonary biology, with profound implications for pediatric healthcare.

LUNG EPITHELIAL CELL LINEAGES AND PROGENITOR CELL POPULATIONS

Concordant with branching morphogenesis, the lung develops various endoderm progenitors along the proximal-distal axis of the developing airways. Prior to this proximal-distal patterning, the lung endoderm broadly expresses several transcriptional regulators, including *Nkx2.1*, *Gata6*, and *Foxa1/2* (Figure 3). These progenitors are responsible for generating the distinct epithelial lineages required for proper respiratory function in the adult mammal. The proximal lung epithelium is generated from *Sox2*-expressing endoderm progenitors that differentiate into ciliated, secretory, and basal cell lineages during development (Figure 3). In the distal compartment, *Sox9/Id2*-expressing endoderm progenitors generate the critical alveolar epithelial lineages known as type 1 and 2 alveolar epithelial cells (AEC1 and AEC2) (Figure 3). Lineage tracing of the *Sox9*⁺/*Id2*⁺ cell population using the *Id2*^{creERT2} mouse line has demonstrated that this cell population is multipotent and capable of generating both airway and alveolar epithelial cell lineages until E13.5, after which time this population becomes progressively more restricted in its potential. At later time points, the *Sox9*⁺/*Id2*⁺ cell population is only capable of generating distal alveolar cell lineages (Rawlins et al. 2009). A complex array of factors, including canonical Wnt, BMP, and Fgf signaling, is thought to be critical for proliferation and differentiation of *Sox9/Id2*-expressing distal progenitors (Bellusci et al. 1997, Desai et al. 2004, Lu et al. 2005, Mucenski et al. 2003, Rawlins et al. 2009, Shu et al. 2005, Weaver et al. 2000). The precise roles of additional transcription factors known to mark this population, including *Sox9*, *Id2*, *Foxp1/2*, *N-myc*, *Etv4/5*, and members of the Iroquois protein family (*Irx1*, *Irx2*, *Irx3*), have been difficult to elucidate owing to the extensive redundancy of these factors with highly related family members expressed in the lung (Cardoso & Lü 2006, Okubo et al. 2005, Shu et al. 2007).

Development of Proximal Airway Lineages

As the distal tip of the lung bud continues to proliferate and extend forward, the progeny of the multipotent progenitor cells left behind in the stalk begin to downregulate *Sox9* and upregulate the transcription factor *Sox2*. In addition to marking proximal endoderm progenitors within the developing lung, *Sox2* regulates the subsequent differentiation of these cells into mature lineages (Que et al. 2009). The first evidence of differentiation arises at E14.5, with the scattered appearance of *Dll1* in the upper airways, marking the future neuroendocrine (NE) cell population (Post et al. 2000) (Figure 3). At roughly the same time, *Foxj1*-expressing cells appear, marking the multiciliated cell lineage (Rawlins et al. 2007). The transcription factor *Foxj1* is required for formation of the multiciliated cells from *Sox2*⁺ progenitors. Loss of *Foxj1* leads to a lack of ciliated epithelium, and pan-epithelial expression of *Foxj1* in the distal lung endoderm leads to ectopic formation of ciliated cells (Chen et al. 1998, Tichelaar et al. 1999). By E15.5, differentiation of the secretory cell lineage can be observed using *Scgb1a1* expression (Rawlins et al. 2007) (Figure 3).

Notch signaling plays a key role in differentiation of the proximal airway epithelium, including establishing and maintaining a proper balance between the various differentiated cell types. Early in development, chemical inhibition of Notch results in expansion of distal

progenitor cells at the expense of their proximal counterparts (Tsao et al. 2008). Deletion of the Notch target genes *Hes1* or *Mash1* (*Ascl1*) results in increased or decreased numbers of NE cells, respectively (Kageyama et al. 2008). Later in development, Notch signaling appears to modulate the balance between the secretory cell lineage versus the ciliated and NE lineages, with increased Notch signaling favoring the secretory cell state. Inactivation of Notch signaling, through conditional deletion of the genes for *Pofut1* (which encodes *O*-fucosyltransferase, necessary for Notch signaling) or *Rbpjk* (which encodes a transcriptional effector of Notch signaling), increases the number of ciliated and NE cells and decreases the number of secretory cells (Tsao et al. 2009). By contrast, artificially increasing Notch signaling results in expansion of the secretory cell compartment (Guseh et al. 2009).

Notch signaling also plays a critical role in directing the differentiation of basal cells in the adult lung. Activation of Notch in keratin-5-expressing basal cells promotes the secretory cell fate, whereas inhibition of Notch favors differentiation toward the ciliated cell lineage (Guseh et al. 2009).

Development of Distal Epithelial Cell Lineages

As development proceeds between E16.5 and 18.5, distal tip multipotent progenitors begin to generate differentiated alveolar epithelium. AEC1 cells form a thin barrier between the alveolar airspace and closely approximated blood-filled capillaries. These cells are marked by the expression of Aquaporin 5 (Aqp5), Podoplanin (Pdpn), advanced glycosylation end-product receptor (Ager), and the transcription factor Hopx (Figure 3). AEC2 cells, by contrast, are cuboidal cells that serve as a major manufacturing and recycling lineage of the alveoli, producing surfactant proteins such as Sftpc and lipids critical for reducing surface tension, as well as proteins and peptides to aid innate immunity. The precise molecular pathways by which the Sox9⁺/Id2⁺ multipotent progenitor cells of the distal tip lead to mature AEC1 and AEC2 cells and the developmental pathways that these cells follow during this progression remain poorly understood. Recent work using RNA-seq to analyze the transcriptome of individual cells suggests that distal tip progenitors contain bipotent progenitor cells late in gestation that express both AEC1 (Pdpn) and AEC2 (Sftpc) markers (Treutlein et al. 2014). According to this model, these bipotent progenitors can differentiate into either AEC1 or AEC2 cells as development proceeds by promoting the program of one lineage while downregulating the other lineage's gene expression program. Additional lineage-tracing studies, along with a more complete assessment of the transcriptome and epigenetic changes that occur during late epithelial differentiation in the lung, are needed to confirm this model and understand its importance during alveologenesis.

TRANSCRIPTIONAL REGULATION OF LUNG DEVELOPMENT AND DIFFERENTIATION

Work over the past two decades has been remarkably successful in identifying and characterizing many transcription factors that are essential to the initial specification and subsequent differentiation of the lung epithelium (Ang & Rossant 1994, Kalinichenko et al. 2001, Morrissey et al. 1998). Understanding the mechanisms by which the expression of these important regulatory transcription factors is carefully refined during lung

differentiation is essential to a full understanding of lung organogenesis. Moreover, subtle perturbations of these key pathways are likely to be important in human disease. Pulmonary agenesis and widespread congenital lung disease are rare events, suggesting that lung development is tightly regulated with multiple backup mechanisms for controlling critical gene expression pathways. More common human pulmonary diseases, including those leading to severe morbidity and even mortality, are characterized by alterations in the delicate balance between the various cell populations composing the lung, including proliferation of the vascular smooth muscle in familial pulmonary hypertension and goblet cell hyperplasia in asthma and COPD. Since completion of the human genome sequencing project in 2004 and the subsequent launch of the ENCODE project, it has become clear that many mechanisms carefully regulate proper gene expression and that we are only just beginning to understand them (ENCODE Proj. Consort. 2004). A more complete understanding of these mechanisms should provide critical insight into congenital human lung diseases.

Noncoding RNAs: From Small to Large

Although it has long been recognized that the DNA sequence of genes encoding proteins accounts for only a minority of the genome, recent data suggest that as much as 60–80% of the genome is transcribed. This means that thousands of noncoding RNAs are expressed in cells but never translated into proteins (Cech & Steitz 2014, Derrien et al. 2012). The functional significance of these noncoding RNAs is just beginning to be explored. Noncoding RNAs have been divided into two major groups based on size: Transcripts greater than 200 base pairs (bp) in length are referred to as long noncoding RNAs (lncRNAs), and those fewer than 200 bp are called small ncRNAs. Small ncRNAs are further subdivided into microRNAs (miRNAs), small nucleolar RNAs, Piwi-interacting RNAs, and small nuclear RNAs. Of the small ncRNAs, only the role of miRNAs during lung development has been explored in a significant manner. miRNAs are processed from longer, immature RNA transcripts (pri- or pre-miRNAs) by the Drosha and Dicer complexes. Inhibiting miRNA processing within the lung epithelium by conditionally deleting Dicer severely disrupts branching morphogenesis and lung epithelial development (Harris et al. 2006). Moreover, germline mutations within the *Dicer* gene have been identified in familial cases of the rare pediatric lung malignancy pleuropulmonary blastoma (Hill et al. 2009).

The miR-17-92 cluster is expressed at high levels early during lung development and is progressively downregulated as differentiation proceeds (Carraro et al. 2009, Lu et al. 2007). Loss of this cluster leads to severe pulmonary hypoplasia owing to decreased epithelial proliferation, whereas overexpression increases cellular proliferation and decreases differentiation of both proximal and distal epithelial progenitors (Lu et al. 2007, Ventura et al. 2008). miR-17-92 is a direct target of the oncogenes c-Myc and E2F1-3 and is thought to promote proliferation, at least in part, by repressing expression of the transcriptional repressor Rbl2 (O'Donnell et al. 2005, Sylvestre et al. 2007, Ventura et al. 2008, Woods et al. 2007). Like many miRNAs and miRNA clusters, miR-17-92 exhibits functional redundancy with other miRNAs, including miR106b-25 and miR106a-363 (Meenhuis et al. 2011). Combined loss of miR-17-92, miR20a, and miR106b leads to a much more severe phenotype than loss of miR-17-92 alone, with defects in branching morphogenesis, altered

E-cadherin expression, and disrupted Bmp4 and Wnt signaling (Carraro et al. 2009, Ventura et al. 2008). The miR-302-367 cluster is also expressed in early lung development and promotes proliferation and inhibits differentiation, in part, through repression of the tumor suppressors Rbl2 and Cdkn1a (Tian et al. 2011).

Additional miRNA clusters play roles in later stages of development, when cellular proliferation slows and differentiation accelerates. miR-34/449 suppresses proliferation by activating p53 and promotes differentiation toward the multiciliated cell lineage by inhibiting Notch1 and Dll1 (Lize et al. 2010a,b; Marcet et al. 2011). Similarly, expression of miR375 in AEC2s increases just prior to birth, and transdifferentiation of AEC2s to AEC1s in culture is associated with decreased expression of miR375 (Wang et al. 2013a).

The role of lncRNAs during lung development has been less explored. Recently, hundreds of lncRNAs were identified in the lung, many of which exhibit characteristic temporal and spatial patterns during development (Herriges et al. 2014). An important subset of these includes lncRNAs located adjacent to master regulator transcription factors; the former appear to regulate the latter in *cis*. For example, loss of the lncRNA *Nanci*, which is located 2 kb downstream of *Nkx2.1*, results in decreased expression of *Nkx2.1* and a lung phenotype similar to that caused by haploinsufficiency of *Nkx2.1* (Herriges et al. 2014). Another lncRNA, *Fendrr*, is located next to *Foxf1*. Unlike *Nanci*, *Fendrr* is upstream and antisense to *Foxf1*, with the two genes sharing the same promoter elements. Although the close proximity of the *Foxf1* promoter to *Fendrr* makes the function of this lncRNA particularly challenging to study using standard knockout mouse models, work from several groups suggests that *Fendrr* plays an essential role in mesoderm development (Grote & Herrmann 2013, Grote et al. 2013, Sauvageau et al. 2013). Moreover, genomic deletions involving a cluster of lncRNAs several hundred kilobases upstream of *Foxf1* have been implicated in the minority of patients with ACD who have no identifiable mutations within the *Foxf1* gene itself (Szafranski et al. 2013). lncRNAs as a group are highly diverse and function through a wide variety of mechanisms, including regulating neighboring genes in *cis* and distant genes in *trans* (Batista & Chang 2013, Ulitsky & Bartel 2013). For example, the lncRNA *MALAT1* modulates gene expression to promote cell proliferation and metastasis in several types of cancer, including lung cancer (Gutschner et al. 2013, Schmidt et al. 2011). However, knockout mouse models have demonstrated that this lncRNA is dispensable for normal pulmonary development (Eissmann et al. 2012, Zhang et al. 2012). This finding highlights the evidence that some lncRNAs have subtle effects during normal development and homeostasis but play critical roles during physiologic stress or disease states.

DNA Methylation and Histone Modifications

Chemical modifications of DNA and the histone proteins that support it have emerged as incredibly important regulators of gene expression (Kellis et al. 2014). DNA can be directly modified by attaching a methyl group to cytosine residues, and the amino acids of histone protein tails can be marked with a wide variety of chemical modifications, including methylation, acetylation, phosphorylation, and ubiquitylation. Each chemical signature confers a particular message to the cell, directing gene expression and establishing a particular cell state that may be modified or reversed with additional chemical modifications.

The role that this complex epigenetic program plays in directing normal lung development is only just beginning to be explored. During lung development, one of the best-studied epigenetic marks is histone acetylation. A group of proteins called histone acetyltransferases (HATs) adds acetyl groups to the tails of histones, which promotes gene expression. Histone deacetylases (HDACs) remove these acetyl groups, silencing genes. Conditional loss of the class I Hdac proteins, Hdac1 and Hdac2, in early lung endoderm severely disrupts lung development, evidenced by impaired ability to form proximal airway cell lineages. This defect results, at least in part, from derepression of Bmp4 expression, which leads to decreased expression of Sox2 (Wang et al. 2013b). Loss of Hdac1 and Hdac2 also leads to decreased proliferation via derepression of the tumor suppressors retinoblastoma 1 (Rb1), p16 (Cdkn2a), and p21 (Cdkn1a) (Wang et al. 2013b). Altered histone acetylation has been observed in association with several disease states in humans, including COPD and asthma, and in animal models of hyperoxic lung injury (Banerjee et al. 2012; Ito et al. 2002, 2006; Londhe et al. 2011; Zhu et al. 2012). Although acetylation and deacetylation of histones appear to be the primary functions of HATs and HDACs, these proteins, like other histone-modifying complexes, may play additional independent roles within the cell. Such roles may include interacting with and modifying nonhistone proteins, including transcription factors (Choudhary et al. 2009). The transcription factor Hopx represses gene expression by binding to HDAC2, without binding DNA directly. Loss of Hopx disrupts alveolarization and increases surfactant production, with a 25% neonatal mortality rate in mice owing to respiratory failure (Yin et al. 2006).

The polycomb repressive complex (PRC) plays an essential role during normal development by silencing genes as differentiation proceeds. Conditional loss of the PRC2 complex subunit Ezh2 in developing lung endoderm leads to the ectopic and premature appearance of p63⁺ basal cells along the entire length of the mouse airway. Decreased numbers of mature secretory cells are observed in these mutants, and cells that appear morphologically similar and express a partial basal cell expression program are observed in their place. This suggests that silencing of gene expression by the PRC2 complex is essential to proper airway epithelial patterning and differentiation during development (Snitow et al. 2015). Evidence that a variety of additional epigenetic complexes play important roles in lung development and homeostasis is beginning to emerge from an array of sources. Hypoxic lung injury results in the suppression of surfactant protein SP-A (Sftpa1) expression via Suv39H1- and Suv39H2-mediated methylation of H3K9 (Benlhabib & Mendelson 2011). Repression of gene expression via direct methylation of DNA by DNA methyltransferase 1 (Dnmt1) has been observed in pulmonary fibrosis and lung cancer (Dakhlallah et al. 2013, Tang et al. 2009). How epigenetic chemical modifications to DNA, histones, and even RNA itself direct normal lung development, confer memory of the differentiated cell state, and, when disrupted, lead to human pulmonary diseases remain important areas for future investigation.

Emerging Mechanisms of Gene Regulation During Lung Development

Although it has been recognized for some time that histone modifications can modulate gene expression by altering local chromatin architecture, only with the recent advent of new technologies such as chromatin conformation capture (3C) and its variants has the impact of the higher-order 3D structure of chromatin within the nucleus begun to be explored (Gomez-

Diaz & Corces 2014). Chromosomes are packed within the nucleus into specific territories, and the specific arrangement of chromatin fibers within each region is nonrandom (Bauer et al. 2012, Bickmore 2013, Lieberman-Aiden et al. 2009, Nora et al. 2013). Although one hypothesis is that this phenomenon is merely an inconsequential by-product of evolution, increasing evidence is emerging that the precise topological organization of chromatin within the nucleus has functional consequences. The genomes of mouse and human embryonic stem cells are topologically distinct from those of more differentiated cells, with low levels of transcription globally, low levels of long-range chromosomal interactions, and disorganized heterochromatin (de Wit et al. 2013, Denholtz&Plath 2012, Efroni et al. 2008). As differentiation proceeds, the genomes of these cells are dynamically reorganized. The genome is compartmentalized into very specific interaction domains that are correlated with precisely controlled changes in gene expression (Apostolou et al. 2013, Peric-Hupkes et al. 2010, Wang et al. 2012, Wei et al. 2013, Zhang et al. 2013). How the topological organization of chromatin within lung epithelial cells is similarly reorganized during lung development, and how this impacts lung differentiation and the response to lung injury and repair during regeneration, remains completely unexplored. The significance of higher-order 3D chromatin structure in human disease also remains largely unexplored, though a recent study of gene expression in fibroblast lines derived from twins discordant for trisomy 21 provides exciting insight into this question. Differentially expressed genes were organized into domains that corresponded to chromatin domains associated with the nuclear membrane (lamin-associated domains). Moreover, these gene expression dysregulated domains corresponded to altered H3K4me3marks, and expression of the syntenic genomic regions was also disrupted in a mouse model of Down syndrome (Letourneau et al. 2014). Whether similar domain-specific alterations in gene expression or chromatin architecture are a feature of lung development or human pulmonary diseases remains unexplored.

SUMMARY AND FUTURE DIRECTIONS

The study of lung development has resulted in a dramatic expansion of our knowledge about how the respiratory system is formed as well as how it responds to injury. The mouse model system has proven very useful in establishing the basic signaling pathways and transcriptional complexes that are required for proper lung morphogenesis. However, as our understanding of these basic mechanisms in mouse has grown, so has the importance of determining whether these mechanisms play similar roles in the human respiratory system. Our increased understanding of the pathways that are critical for specification and differentiation of the early lung endoderm has led to important advances in the differentiation of both mouse and human pluripotent stem cells into lung epithelium (Green et al. 2011; Huang et al. 2014, 2015; Longmire et al. 2012; Mou et al. 2012). Such human culture model systems will likely provide important insight into both similarities and differences between rodent and human lung cell lineage differentiation.

Given the morphological and cellular differences between the mouse and human lung, important differences in the developmental processes required are likely to exist. The development of cell type-specific isolation techniques coupled with newer, more sensitive transcriptome analysis methods, including single-cell RNA-seq, should help define the differentiation pathways active in developing lung-cell lineages in humans. Moreover, recent

advances in ex vivo human lung organoid culture systems provide a unique opportunity to define mechanisms important for human lung cell differentiation and self-renewal (Barkauskas et al. 2013, Lee et al. 2014). With these newest techniques in hand, developments in our understanding of disease processes that affect the respiratory system will hopefully result in better diagnostics and eventually new therapies for both pediatric and adult lung disease.

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Figure 1. Timeline of mouse and human pulmonary development, showing diseases associated with developmental defects in the pulmonary system. Mouse and human gestation periods, along with landmark events during pulmonary development, are shown. The time periods when defects in human development can lead to congenital lung disease are noted at the bottom. Abbreviations: E, embryonic day; P, postnatal day; TE, tracheoesophageal fistula.

Figure 2.

Signaling interaction networks important for pulmonary endoderm development. (a) Wnt, Bmp, and Fgf signaling controls initial specification of the pulmonary endoderm, marked by expression of the transcription factor Nkx2.1. Noggin, secreted from the notochord, helps to establish a dorsal-ventral pattern of Bmp signaling to the foregut, resulting in high levels of Sox2 dorsally and Nkx2.1 ventrally. (b) During branching morphogenesis, a feedback loop between Shh and Fgf signaling promotes outgrowth and new branch formation. (c) Wnt signaling arising from either the mesoderm (Wnt2/2b) or early epithelium (Wnt7b) promotes outgrowth and differentiation of lung epithelium in part by activating Fgf signaling. Gene products are color coordinated with the lung diagram to indicate their localization to the epithelium or mesenchyme.

Figure 3.

Endoderm progenitors in pulmonary development. The early lung endoderm expresses a myriad of transcriptional regulators, including Nkx2.1, Gata6, and Foxa1/2. The distal branching tip endoderm, which remains multipotent up to embryonic day (E)13.5, expresses N-myc, Sox9, Id2, and Foxp1/2. These distal tip progenitors continue to express Sox9 and Id2 after E13.5 but become restricted to the alveolar fate and generate type 1 and 2 alveolar epithelial cells (AECs), directly or indirectly through a bipotent progenitor. The proximal Sox2⁺ progenitors generate neuroendocrine (NE), secretory, and multiciliated cells.