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REVIEW

# Lung development: orchestrating the generation and regeneration of a complex organ

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## ABSTRACT

The respiratory system, which consists of the lungs, trachea and associated vasculature, is essential for terrestrial life. In recent years, extensive progress has been made in defining the temporal progression of lung development, and this has led to exciting discoveries, including the derivation of lung epithelium from pluripotent stem cells and the discovery of developmental pathways that are targets for new therapeutics. These discoveries have also provided new insights into the regenerative capacity of the respiratory system. This Review highlights recent advances in our understanding of lung development and regeneration, which will hopefully lead to better insights into both congenital and acquired lung diseases.


**KEY WORDS: Branching morphogenesis, Epigenetics, Lung, Regeneration**

## Introduction

The primary function of the lungs is to exchange oxygen in the external environment with carbon dioxide in the cardiovascular system. Although this process sounds straightforward, it is fraught with multiple barriers to its success. The entire airway, like the skin, is constantly exposed to the external environment and must cope with challenges including temperature, particulate matter and allergens. Terrestrial life has adapted to these and other challenges through the evolution of a complex respiratory system consisting of a multitude of cell lineages. These cellular components are constantly communicating with each other to exchange gas efficiently while preventing blood and fluid loss and dangerous infection from pathogens.

Lung development has been studied extensively in recent years, generating new insights into the origins of the different cell lineages that exist in the lung as well as the molecular pathways that regulate these lineages. This has led to novel insights into congenital lung diseases, lung abnormalities and acquired lung diseases (Box 1), including asthma and chronic obstructive pulmonary disease (COPD), and the lung's response to acute injury. In addition, these studies have revealed that some cell lineages within the mammalian respiratory system can regenerate after injury through the activation of stem/progenitor populations or through proliferation-induced cellular expansion. The molecular pathways that regulate these regenerative processes have been identified, raising the hope that these can be harnessed to promote lung regeneration in humans. Moreover, using the robust knowledge of pathways that regulate early lung development, lung epithelial cells can now be generated from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), providing an additional source of cells for disease modeling and potential cell-based therapies (Longmire et al., 2012; Mou et al., 2012). In this Review, we highlight recent advances in the basic understanding of lung development and regeneration with a focus on the cell biology of the developing lung epithelium, the role of endoderm progenitors, and the epigenetic regulation of lung development and regeneration. We also discuss how these advances have provided insight into both lung disease and the ability to repair and regenerate damaged lung tissue. Readers are encouraged to read other excellent recent reviews on this subject (Cardoso and Lü, 2006; Rawlins and Hogan, 2006; Morrisey and Hogan, 2010; Rock et al., 2010).

## The basics of lung development

The lungs, together with the trachea, arise from the anterior foregut endoderm, a tissue that generates multiple organs, including the respiratory system, esophagus, thyroid and liver (Fig. 1). Lung specification begins around embryonic day (E) 9.0 in the mouse as the transcription factor Nkx2.1 is expressed in endodermal cells on the ventral side of the anterior foregut. By E9.5, evagination of these epithelial cells results in the formation of the trachea and two lung buds and the beginning of the embryonic stage of lung development (E9.5-E12.5). During this stage, the trachea completes its separation from the esophagus. During the embryonic and pseudoglandular stages (E12.5-E16.5), the two lung buds undergo a highly regulated branching process called branching morphogenesis to generate a tree-like network of airways with thousands of terminal branches. This is followed by the canalicular (E16.5-E17.5) and saccular [E18.5-postnatal day (P) 5] stages, during which these terminal branches narrow and form clusters of epithelial sacs that will later develop into alveoli in preparation for respiration at birth. Finally, full maturation of the alveolus occurs during the alveolarization stage (P0-P14). During all stages of endodermal development, the lung mesoderm (or mesenchyme) develops and interacts with the lung endoderm to promote branching and differentiation and to generate the various lineages within the lung, including airway and vascular smooth muscle and pericytes.

Notably, the separation of the tracheal tube from the esophagus and the formation of the branching lung appear to be distinct developmental processes from the formation of the branching lung as studies have shown that evagination of the lung buds still occurs in the setting of tracheal agenesis (Domyan et al., 2011). The molecular pathways that control each of these stages of lung development have been a topic of extensive exploration in recent years. As we discuss below, multiple signaling pathways, including the Wnt, bone morphogenetic protein (Bmp), and fibroblast growth factor (Fgf) pathways, are implicated in regulating lung specification, branching and patterning.

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**Box 1. Congenital lung diseases, lung abnormalities and acquired lung diseases**

**Asthma.** Airway hyper-constriction occurring due to a variety of inducers including allergic and inflammatory responses. Although the causes of asthma remain unclear, the high prevalence in young children suggests a possible role for developmental cues in the susceptibility to asthma.

**Bronchopulmonary dysplasia.** Results from pulmonary immaturity at birth and often caused by oxygen therapy. Usually occurs with increased fibrosis in the lung.

**Chronic obstructive pulmonary disease (COPD).** A progressive disease often caused by tobacco smoking and air pollution. A role for epigenetic alterations, including HDAC activity, has been demonstrated.

**Congenital cystic adenomatoid malformation.** Congenital defects leading to the formation of small or large cysts within the lung. Can be successfully resected using surgery.

**Tracheal-esophageal fistula.** Tracheal defects leading to fusion of the improperly developed trachea with the esophagus. Many types can be corrected by surgery.

**Tracheal/lung agenesis.** Various types of defects that lead to partial or complete loss of pulmonary tissue.

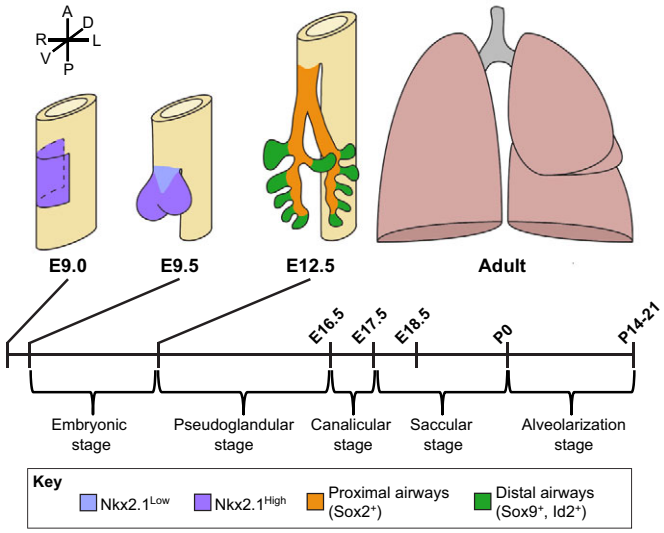


**Fig. 1. Overview of the stages of lung development.** Lung endoderm specification begins at ~E9.0 on the ventral side of the anterior foregut endoderm (yellow) where initiation of Nkx2.1 expression commences. By E9.5-E10.0, the formation of the trachea is observed and the embryonic stage of lung development begins and ends at E12.5. The other stages of lung development and the time period in which they occur are displayed. A, anterior; D, dorsal; L, left; P, posterior; R, right; V, ventral.

### Specification of the lung endoderm in the anterior foregut

The first evidence of respiratory system specification in the anterior foregut endoderm is expression of the transcription factor Nkx2.1, which occurs at ~E9.0 in the mouse and 28 days gestation in the human. Studies have shown that Wnt signaling plays a crucial role in specifying Nkx2.1$^+$ respiratory endoderm progenitors during development. Wnt2 and Wnt2b are expressed in the ventral anterior mesoderm surrounding the region of the anterior foregut endoderm where Nkx2.1$^+$ respiratory endoderm progenitors are located (Goss et al., 2009) (Fig. 2A). In *Wnt2/2b* combined null mutants, there is a complete loss of Nkx2.1$^+$ lung endoderm progenitors and a failure to form the trachea or branching lung (Goss et al., 2009). This phenotype is recapitulated upon loss of β-catenin in the anterior foregut endoderm (Goss et al., 2009; Harris-Johnson et al., 2009). In addition, forced activation of Wnt/β-catenin signaling leads to an expansion of Nkx2.1$^+$ progenitors in the posterior gut, including the stomach, suggesting that Wnt is not only necessary but also sufficient to drive lung progenitor identity in foregut endoderm (Goss et al., 2009; Harris-Johnson et al., 2009).

Wnt signaling does not act alone in specifying lung fate; the ability of Wnt/β-catenin signaling to promote Nkx2.1$^+$ respiratory endoderm progenitor fate is dependent upon active Bmp signaling (Domyan et al., 2011). Bmp4 is expressed in the ventral mesenchyme surrounding the anterior foregut, and loss of Bmp signaling in the foregut endoderm through inactivation of the Bmp receptors Bmpr1a and Bmpr1b leads to tracheal agenesis with retention of the branching region of the lungs (Domyan et al., 2011). Bmp signaling appears to act by repressing the transcription factor Sox2, which allows for expression of Nkx2.1 in the presumptive lung endoderm (Domyan et al., 2011). Thus, early respiratory specification and development requires both Wnt and Bmp signaling (Fig. 2A).

Defects in this early process of tracheal separation from the foregut and development of the branching regions of the lung underlie many types of congenital lung disease (Box 1). Thus, a better understanding of how these early developmental processes are regulated to form the distinct regions of the respiratory system is needed for understanding and promoting this process in the context of pediatric respiratory regenerative therapies.

### Branching morphogenesis and epithelial organization of the lung

After the early budding of the main bronchi or airways, the lung buds extend into the surrounding mesenchyme and develop rapidly through a process called branching morphogenesis that is crucial for generating the highly arborized airway tree. Branching morphogenesis is essential for forming both the structural airways as well as the terminal alveolar compartments in which gas exchange occurs. Lung branching proceeds in a stereotypical fashion and most of the branching that occurs in early development is genetically hard-wired (Metzger et al., 2008). Although the molecular cues for forming new branch points are still somewhat unclear, signaling between the developing endoderm and mesoderm appears to be crucial for instigating new branch points in the developing airways. Fgf signaling, in particular Fgf10 signaling to Fgfr2 in the developing endoderm, is essential for branching morphogenesis, and loss of this pathway leads to complete abrogation of branching (Sekine et al., 1999; Ohuchi et al., 2000). Fgf10 expression occurs at specific regions in the distal lung mesenchyme and is thought to be regulated by other signaling pathways, including Bmp4 and sonic hedgehog (Shh), suggesting that a complex interplay of signaling molecules regulates new branch point formation and outgrowth (Bellusci et al., 1997a; Pepicelli et al., 1998; Weaver et al., 2000). Recent papers have presented models in which the Fgf10-Shh interaction is sufficient to promote much of the branching that occurs in the early lung (Hirashima et al., 2008; Cellière et al., 2012). However, Fgf10 also acts as a potent mitogen and its expression in the mesenchyme adjacent to the developing lung just prior to the formation of a new branch point has led many investigators to suggest that this mitogenic signal is important for instigating and initiating the outgrowth of new airway branches (Bellusci et al., 1997b; Park et al., 1998; Weaver et al., 2000). By contrast, recent studies indicate that such precise spatial expression may not be as important as the actual level of Fgf10 expression (Volckaert et al., 2013).

One of the most important and underexplored questions in early lung development is what drives changes in the shape of the epithelial sheet that comprises the airways during branching morphogenesis. Little is known about how this epithelial sheet bends to generate new

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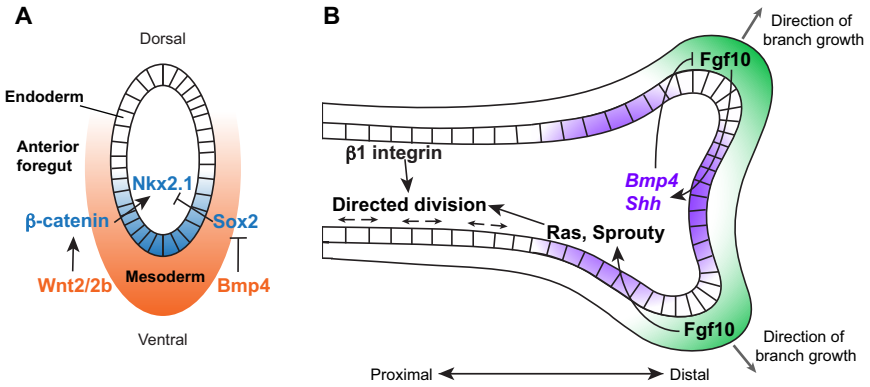


**Fig. 2. Specification and early development of the lung endoderm.** (A) The lung endoderm (marked by Nkx2.1 expression, blue) is first specified on the ventral side of the anterior foregut at E9.0. Wnt2/2b and Bmp4 signaling (indicated in orange) from the surrounding mesoderm is required for this specification and for patterning of the anterior foregut in a ventral-dorsal manner. Wnt2 and Wnt2b signal via β-catenin to promote the expression of Nkx2.1 whereas Bmp4 represses the inhibitory action of Sox2 on Nkx2.1 expression. (B) During lung development, Fgf10 is essential for branching morphogenesis and is expressed in the mesenchyme surrounding developing branch points (Fgf10 expression indicated in green). This expression is restricted by epithelial expression of genes such *Shh* and *Bmp4* (purple). Fgf10 also directs the orientation of epithelial cell division through regulation of Ras/Sprouty, setting up the appropriate direction of branch growth.

bud tips, although recent evidence has suggested that apical constriction plays an important role (Kim et al., 2013). Other reports suggest that changes in the orientation of cell division can alter the shape of the branching lung and drive extension of branching regions towards a source of mitogenic signals. For example, Ras/Sprouty activity, which acts 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 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 (Fig. 2B). Other pathways, such as the planar cell polarity (PCP) pathway, an arm of the Wnt signaling pathway, are likely to be important for regulating epithelial cell shape and consequently tube shape in the branching lung. Loss of multiple PCP components, including Scribbled, Celsr1 and Vangl2, has been shown to have subtle but important defects in lung epithelial development including early branching (Yates et al., 2010; Yates et al., 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.

Cell-matrix interactions also play important roles in generating airway epithelial tube structure. Cell matrix proteins, such as fibronectin, accumulate at sites of branch point constriction, suggesting that cell-matrix interactions are important for directing new branch point formation (Sakai et al., 2003). Fibronectin interacts with cells through several integrin receptors, including the β1 family of integrins, and the role of integrins in lung development has recently been examined. It is known that the proximal airways of the lung develop a pseudostratified epithelium whereas the distal airways form a single simplified epithelial lining. A recent study, however, showed that loss of β1 integrin in lung epithelium leads to the development of an extensive multilayered epithelium in the lung and a block in branching morphogenesis (Chen and Krasnow, 2012). Moreover, β1-deficient lung epithelia exhibited a loss of apical-basal cell polarity. These data suggest that one role for β1 integrin-mediated cell-matrix interactions in the lung is to restrict epithelial cell multilayering and promote proper apical-basal epithelial polarity. Interestingly, β1 integrins and other cell adhesion molecules, including E-cadherin (cadherin 1), are aberrantly expressed in certain congenital lung abnormalities, such as bronchopulmonary sequestration and congenital cystic adenomatoid malformations (Volpe et al., 2009). Further exploration of cell-matrix interactions during the development of the airways will probably uncover additional insights into how lung epithelial cells are organized to give rise to the appropriate architecture of the branched airway network.

### Progenitor cells in the developing lung endoderm

During branching morphogenesis, the lung endoderm also begins to develop distinct cell lineages along its proximal-distal axis. Sox2 expression marks the proximal endoderm progenitor lineage whereas the combined expression of Sox9 and the transcriptional regulator Id2 marks the distal endoderm progenitor lineage (Fig. 3). Importantly, these two populations have distinct fates: the proximal progenitors give rise to airway neuroendocrine cells, secretory cells, ciliated cells and mucosal cells, whereas the distal progenitors give rise to type 1 and type 2 alveolar epithelial cells (AEC1 and AEC2). Studies have shown that Sox2 is necessary for the differentiation of proximal progenitors into their various progeny; loss of Sox2 expression leads to loss of the mature secretory and ciliated lineages in the lung airways (Que et al., 2009; Tompkins et al., 2011). The precise molecular pathways required for the formation and differentiation of distal Sox9/Id2 progenitors are poorly understood. Inhibition of both Wnt/β-catenin signaling and Bmp signaling results in a loss of distal lung epithelial lineages (Weaver et al., 1999; Lu et al., 2001; Mucenski et al., 2003). In addition, transcription factors, such as N-myc (Mycn) and Foxp1/2, appear to play important roles in the differentiation of distal endoderm progenitors (Okubo et al., 2005; Shu et al., 2007).

Recent lineage-tracing studies have provided key insights into the potency of $Sox2^{+}$ and $Sox9^{+}/Id2^{+}$ progenitors. For example, the generation of an $Id2^{creERT2}$ line has allowed the assessment of distal progenitors in a carefully defined temporal manner, demonstrating that they are multipotent up to E13.5 (Rawlins et al., 2009). $Id2^{+}$ cells can generate both alveolar and airway epithelial derivatives up to this time but become increasingly restricted such that they are only able to generate alveolar epithelial lineages in late gestation.

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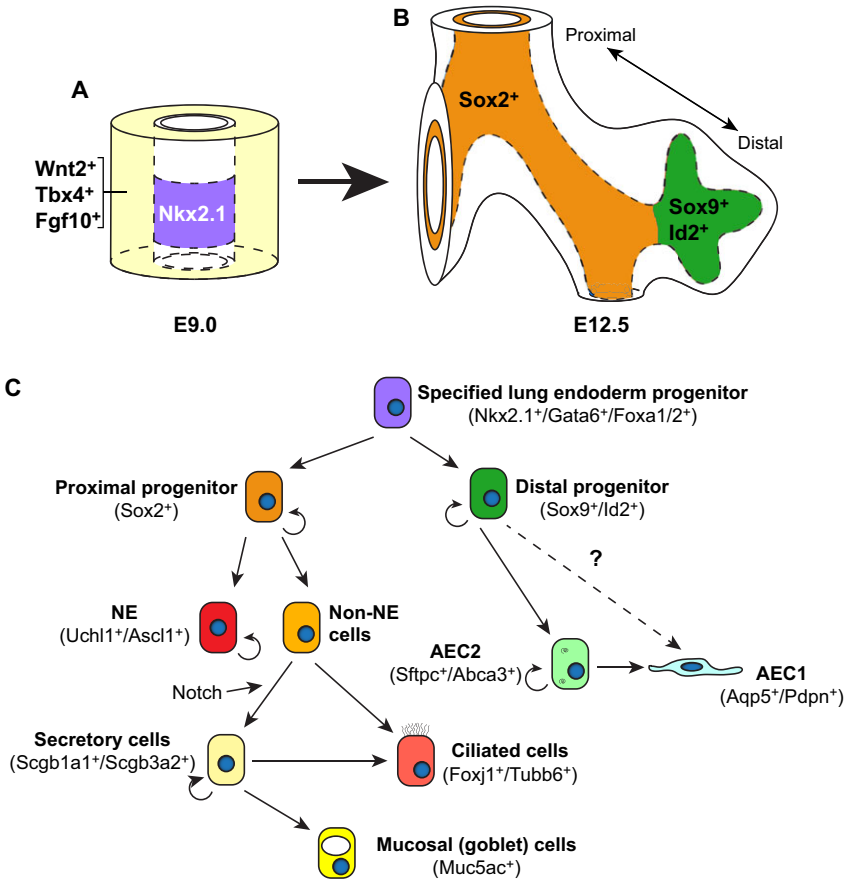


**Fig. 3. Progenitors and cell lineages within the developing lung.** (A) Early Nkx2.1$^+$ lung endoderm progenitors (purple) are surrounded by lung mesenchyme (yellow), which is marked by the expression of Wnt2, Tbx4 and Fgf10. (B,C) This Nkx2.1-positive endoderm gives rise to both Sox2$^+$ proximal progenitors and Sox9$^+$/Id2$^+$ distal progenitors, which in turn give rise to distinct sets of differentiated cells. The proximal progenitors give rise to neuroendocrine (NE) cells, secretory cells, ciliated cells and mucosal cells that all populate the conducting airways. By contrast, the distal progenitors give rise to type I and type 2 alveolar epithelial cells (AEC1 and AEC2) that populate the alveoli. Markers expressed in each cell type are indicated in parentheses.

Such studies suggest that the early distal lung endoderm is both multipotent and highly plastic in its developmental potential. What regulates this temporal change in developmental potential is still unclear but is likely to involve changes in the expression and activity of both signaling pathways and transcription factors that occur during this time of lung development. Of note, recent studies demonstrated an important role for Sox9 in the early branching process as well as promoting alveolar epithelial differentiation (Chang et al., 2013; Rockich et al., 2013). Similar lineage-tracing studies have not been carried out for proximal endoderm progenitors marked by Sox2 expression but the generation of a Sox2creERT (Arnold et al., 2011) allows for such studies to be performed. Additional markers of both proximal and distal endoderm progenitors are also needed to expand upon these findings and help further define these populations. Importantly, the level of heterogeneity within the two progenitor populations is still unclear. Such information will be crucial for generating mature lung epithelial lineages from ESCs and iPSCs for use in pharmacological studies as well as future cell replacement therapies.

### Progenitor cells in the developing lung mesoderm

Although the origins of the various epithelial lineages in the lung have been explored extensively in recent years, little is understood about the origins of the multiple mesodermal cell lineages of the lung. The lung mesoderm is an important source of paracrine signals such as Fgf10 and Wnt2 (Bellusci et al., 1997b; Sekine et al., 1999; Weaver et al., 2000; Goss et al., 2009). These signals are essential for multiple processes during lung development, including the patterning of early endoderm progenitors, epithelial proliferation, and differentiation. Previous studies using tissue recombination experiments demonstrated that distal lung mesenchyme can induce branching in tracheal endoderm (Shannon, 1994; Shannon et al., 1998). Thus, the lung mesoderm plays an important instructive role in promoting proper lung endoderm development. The mature lung contains many mesodermal derivatives, including airway smooth muscle, vascular smooth muscle, endothelial and mesothelial cells, as well as multiple less well-understood cell types, such as pericytes, alveolar fibroblasts, and lipofibroblasts (Fig. 4). However, our ability to characterize the differentiation of these mature lineages from early mesodermal progenitors is hampered by the dearth in our understanding of the gene expression patterns in these various lineages. A recently generated *Fgf10$^{cre\text{-}ERT2}$* allele, which appears to be useful for tracking Fgf10$^+$ cells in the lung mesoderm, may provide important insight into lung mesodermal differentiation (El Agha et al., 2012). However, additional markers are required to help sort out the heterogeneity and respective capacity of the mesoderm to generate these differentiated lineages.

The origin of the many cell lineages that comprise the pulmonary vasculature is also unclear and has remained a point of controversy, with data supporting both angiogenic and vasculogenic processes involved in generating the complex vasculature tree of the lungs (deMello et al., 1997; Schachtner et al., 2000; Anderson-Berry et al., 2005; Parera et al., 2005). However, evidence from lineage-tracing experiments has begun to address this controversy. A recent study used the expression of Wnt2 in the region of ventral mesenchyme surrounding the developing lung buds to examine the cell fate of these progenitors during cardiopulmonary development (Peng et al., 2013). This study showed that, prior to specification of the lung, Wnt2$^+$ cells are able to generate most of the mesoderm/mesenchymal lineages within the lung, including airway smooth muscle (ASM), vascular smooth muscle (VSM), proximal endothelium and Pdgfrβ$^+$/Ng2 (Cspg4)$^+$ pericyte-like cells (Fig. 4).

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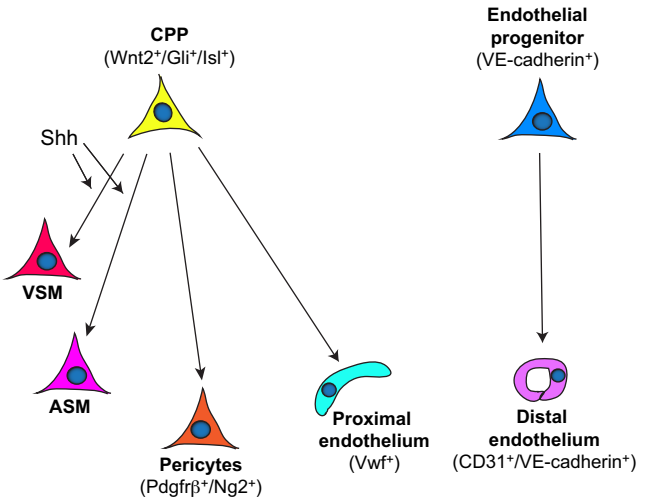


**Fig. 4. The origin of the pulmonary vasculature and other lung mesodermal derivatives.** The majority of lung mesodermal lineages, including vascular smooth muscle (VSM), airway smooth muscle (ASM), pericytes and proximal endothelium are derived from $Wnt2^+$ cardiopulmonary progenitors (CPPs). Hedgehog signaling regulates the differentiation of CPPs into smooth muscle. By contrast, distal vascular endothelial cells that generate the majority of the alveolar vascular plexus are derived from VE-$cadherin^+$ progenitors. Thus, the pulmonary vasculature is generated using a multi-lineage approach. Markers expressed in each cell type are indicated in parentheses.

Interestingly, $Wnt2^+$ cells at this time also generate cardiomyocytes and endocardial cells within the inflow tract of the heart, demonstrating a lineage connection between the cardiac and pulmonary mesoderm. $Wnt2^+$ cells also express Gli1 and Isl1, and together these factors demarcate a cardiopulmonary progenitor (CPP) that orchestrates pulmonary and cardiac development, and the development of which is regulated by Hedgehog signaling. Interestingly, CPPs do not generate the majority of the endothelium in the distal alveolar vasculature. Rather, this endothelium appears to arise from VE-cadherin (cadherin 5)$^+$ endothelial cells in the trunk vasculature that exist prior to lung formation, pointing to an angiogenic process for generating the distal vascular endothelium of the lung. Moreover, the combined action of CPPs and VE-$cadherin^+$ endothelium points to a dual origin model for formation of the cardiopulmonary vasculature.

Single cell clonal analysis shows that CPPs can clonally generate ASM and VSM as well as proximal endothelium, revealing that these lineages are related in early development and that CPPs are multipotent at the time of lung specification. Despite these new findings, many questions remain on how diverse the early lung mesoderm is and whether CPPs generate all smooth muscle or whether there are other origins for this lineage. Given the ventral location of CPPs during development, other mesoderm precursors located in a more dorsal region of the embryo could also contribute to smooth muscle lineages in the lung. Pathways other than Hedgehog known to regulate smooth muscle development in the lung include Wnt and Bmp (Bragg et al., 2001; Lu et al., 2001; Frank et al., 2005; Goss et al., 2011). Much work still needs to be performed to characterize the various mesoderm linages in the lung, and additional tools including new markers of smooth muscle lineages and sublineages along with novel lines for lineage tracing will be necessary to further delineate similarities and differences in these lineages in the lung, both during development and in their response to injury and disease.

## Molecular mechanisms underlying lung development

Many of the signaling pathways and transcription factors that regulate lung epithelial and mesoderm development are also expressed and active in other tissues. In addition, a number of epigenetic regulators and non-coding RNAs have been identified as key regulators of lung development. As we discuss below, this suggests that the anterior foregut endoderm uses a combinatorial approach to specify and drive differentiation of lung cell lineages.

### Transcription factors dictate proper lung epithelial cell fate and differentiation

A host of transcription factors have been implicated in the regulation of lung endoderm specification and differentiation, including members of the homeobox factor family (Nkx2.1), the Gata transcription factor family (Gata6), the forkhead box transcription factor family (Foxa1/2, Foxp1/2/4, Foxj1) and the Ets transcription factor family (Etv4/5, Npas, Elf5, Spdef). Some of these, including Fox and Gata factors, are known to act as pioneer transcription factors, which can insert themselves into compacted chromatin to initiate lineage-specific gene expression programs (Cirillo et al., 2002). Although loss of many of these genes does not completely abrogate lung specification, most are required for subsequent epithelial differentiation and branching morphogenesis. Furthermore, the lack of a phenotype upon deletion of some of these genes is likely to be due to their existence in large families containing many redundant members. As a result, our understanding of precisely how these factors regulate lung development programs is still relatively unclear.

Nkx2.1 is a hallmark transcription factor expressed in the lung and thyroid epithelium as well as in the forebrain. Nkx2.1 null lungs do not branch and exhibit decreased expression of many crucial lung genes, including the AEC2 marker *Sftpc* and the secretory cell marker *Scgb1a1* (Kimura et al., 1996). Recent evidence has indicated that Nkx2.1 is also a crucial tumor suppressor in lung cancer; loss of Nkx2.1 leads to increased adenocarcinoma formation in part through conversion of Nkx2.1-deficient cells into an intestinal fate (Winslow et al., 2011). The phenotype observed in $Nkx2.1^{-/-}$ mutants may thus be generated in part through loss of lung identity and activation of a more posterior gut fate (Snyder et al., 2013). These data suggest that Nkx2.1 lies at the center of a transcriptional network that is essential for imposing lung epithelial cell fate through both activating and repressive mechanisms.

Like many transcription factors, Nkx2.1 interacts with additional transcriptional regulators to effect gene expression. One of these is Gata6, which is a member of the Gata family of zinc finger proteins and is highly expressed in the developing endoderm and vascular smooth muscle of the lung (Morrisey et al., 1996). Lung epithelial-specific loss of Gata6 during development leads to defective epithelial differentiation and increased proliferation, suggesting a developmental block at a more primitive progenitor state, whereas postnatal loss of Gata6 in lung epithelium leads to defective airway epithelial regeneration after injury (Zhang et al., 2008). Importantly, mice heterozygous for both *Gata6* and *Nkx2.1* have defects in lung epithelial cell differentiation (Zhang et al., 2007), further supporting a cooperative interaction between Nkx2.1 and Gata6 in regulating lung epithelial cell gene expression.

The forkhead box (Fox) family of proteins is a highly conserved and ancient transcription factor family that contains dozens of members and plays important roles in several aspects of lung development. Foxa1 and Foxa2 are key regulators of endoderm identity and development. Whereas Foxa1 is not essential for lung development, Foxa2 is crucial for late epithelial differentiation in the lung (Wan et al., 2004a; Wan et al., 2004b; Wan et al., 2005). In

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addition, the combined loss of Foxa1 and Foxa2 leads to severe defects in lung epithelial cell differentiation, including loss of the AEC2 markers Sftpc and Sftpb and the airway epithelial markers for the secretory (Scgb1a1) and ciliated (Foxj1) epithelial lineages (Wan et al., 2005). Other Fox transcription factors also play important roles in lung epithelial differentiation. Foxj1 is required for differentiation of proximal $Sox2^{+}$ progenitors into ciliated epithelium, and overexpression of Foxj1 throughout the developing lung epithelium leads to ectopic formation of ciliated epithelium (Chen et al., 1998; Tichelaar et al., 1999; Whitsett and Tichelaar, 1999). Foxp1, Foxp2 and Foxp4 are all expressed in overlapping patterns within the developing and postnatal lung; Foxp1 and Foxp4 expression is widely observed in both proximal and distal epithelial lineages whereas Foxp2 expression is confined primarily to distal endoderm and later on is observed in alveolar epithelium. As with Foxa1/2, the Foxp1/2/4 subfamily acts in a highly redundant manner. Loss of Foxp2 alone leads to alveolarization defects and death by three weeks after birth (Shu et al., 2007). However, loss of a single *Foxp1* allele in addition to both *Foxp2* alleles results in a more dramatic inhibition of lung epithelial development, including decreased expression of the homeodomain protein Hopx and N-myc (Shu et al., 2007).

Members of the Ets family of transcription factors also regulate lung epithelial development. Both Etv5 and Npas are essential for lung alveolarization (Liu et al., 2003; Zhou et al., 2009), and overexpression of the Ets factor Elf5 leads to defective branching morphogenesis and a block in alveolar and airway epithelial differentiation (Metzger et al., 2008b). In addition, the Ets factor Spedf plays a key role in the differentiation of lung goblet cells, which are found scattered throughout human airways but in rodents are generally absent unless the airways are injured. Spdef expression induces development of goblet cells in mouse airways and genetic deletion of *Spdef* in mice inhibits goblet cell formation upon airway injury or in asthma models (Park et al., 2007; Chen et al., 2009).

Although genetic loss- and gain-of-function studies have elucidated many roles for transcriptional regulators, there is still much to learn about how the factors described above, as well as many others, regulate the specification and differentiation of the myriad of epithelial and mesodermal lineages in the lung. Newer techniques, including ChIP-seq and RNA-seq, will need to be employed to determine where these factors bind in the genome of the various cell lineages at different times during development. Moreover, precise transcriptome analysis after gain- and loss-of-function in these cell lineages will provide better insight into how haploinsufficiency in factors such as Nkx2.1 lead to variable human lung respiratory distress (Pohlenz et al., 2002; Moya et al., 2006; Carré et al., 2009).

### Epigenetic regulators of lung epithelial cell fate and differentiation

Recent studies have also identified epigenetic mechanisms, such as histone modifications, as key regulators of lung development (Fig. 5A). Histones are modified through acetylation, methylation, phosphorylation and ubiquitylation at specific amino acids along the histone tail. Of these marks, histone acetylation plays an important role in regulating lung development and function. Histone acetyltransferases (HATs) mediate acetylation of the histone tail, which promotes gene transcription, and histone deacetylases (HDACs) remove the acetyl group, leading to gene silencing. Although HATs and HDACs are largely studied for their ability to modify histones, they can also target a wide array of proteins, including other epigenetic regulators and transcription factors (Choudhary et al., 2009). The effect of these modifications on protein function widens the potential influence of HDACs and HATs and complicates the interpretation of how these proteins regulate lung development.

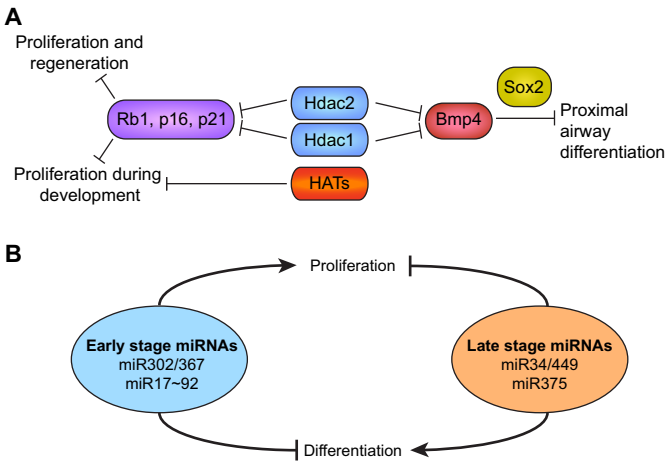


**Fig. 5. Epigenetic factors regulate lung development and regeneration.** (A) Hdac1/2 activity promotes proliferation during development and regeneration through inhibition of tumor suppressors, such as Rb1, p16 and p21. During development, Hdac1/2 also promote proximal airway differentiation through inhibition of Bmp4. (B) miRNAs that are expressed early in lung development (miR302/367 and miR17~92) promote proliferation and inhibit differentiation of lung epithelium, whereas those expressed at later stages (miR34/449 and miR375) inhibit proliferation and promote differentiation.

Histone acetylation was first shown to play an important role in the lung through the analysis of lung diseases such as COPD and asthma (Ito et al., 2002; Ito et al., 2006; Banerjee et al., 2012). However, recent work indicates that a proper balance between HDAC and HAT activity is also necessary for embryonic lung development. Loss of Hdac1 and Hdac2 in the lung epithelium results in reduced expression of Sox2 and prevents development of multiple proximal cell types (Wang et al., 2013c). This change in Sox2 expression is in part mediated by increased expression of Bmp4, a direct target of Hdac1/2, which also contributes to the severe branching defects observed in Hdac1/2 mutants. Hdac1 and Hdac2 also promote endoderm progenitor proliferation during lung development and airway regeneration in part through repression of the tumor suppressors retinoblastoma 1 (Rb1), p16 (Cdkn2a) and p21 (Cdkn1a) (Wang et al., 2013c). It has also been shown that hyperoxia during neonatal development results in decreased Hdac1/2 activity, leading to alveolar hyperplasia and disrupted alveolarization (Londhe et al., 2011; Zhu et al., 2012).

In addition to their roles as histone deacetylases, HDACs may influence lung development through interactions with other non-histone proteins. Recent data has shown that Hopx, which can repress gene transcription, does not bind directly to DNA but rather binds to Hdac2 and is broadly expressed after E13.5 in the developing lung epithelium (Yin et al., 2006). Approximately 25% of live born Hopx null animals die within 24 hours owing to increased surfactant expression and disrupted alveolarization (Yin et al., 2006). These data suggest that Hopx-HDAC complexes regulate lung epithelial maturation, which could provide a new nodal point for therapeutic intervention for enhancing epithelial maturation in preterm infants or after lung injury.

Although histone acetylation is known to play an important role in the lung, little is known about the roles of other epigenetic complexes during lung development. The methyltransferases Suv39H1 and Suv39H2, which induce transcriptional silencing through histone H3 lysine 9 methylation, directly repress the

expression of the surfactant protein SP-A (Sftpa1) during hypoxia (Benlhabib and Mendelson, 2011). Suv39H1 and Suv39H2 are also highly expressed in early lung development, suggesting that they may inhibit SP-A transcription until later in development. During pulmonary fibrosis, DNA methylation by Dnmt1 represses transcription of miR17~92, a microRNA cluster that regulates lung development (Dakhlallah et al., 2013). Likewise, Dnmt1 mediates the progression of lung cancer through methylation of various promoter regions (Tang et al., 2009; Dakhlallah et al., 2013). As is the case for HDACs and HATs, the roles for these epigenetic factors in disease states strongly suggests a role for these factors during development that will need to be determined in further studies.

#### microRNA-mediated control of lung development

microRNAs (miRNAs) are a class of small (~22 nucleotides in length) non-coding RNAs that suppress gene translation, most commonly through promoting mRNA degradation or disrupting mRNA translation. miRNAs themselves are transcribed as part of larger precursor transcripts that often contain clusters of miRNAs. The miRNA clusters are spliced to form pre-miRNA hairpins in the nucleus, which are then exported to the cytoplasm and processed by the enzyme Dicer to form double-stranded RNA (dsRNA). Studies have shown that the crucial miRNA-processing proteins argonaute 1-4 and Dicer are expressed within the developing lung epithelium and mesoderm (Lü et al., 2005). Loss of Dicer in the lung epithelium results in severe defects in branching and epithelial structure leading to perinatal lethality (Harris et al., 2006). Several studies have identified >100 miRNAs that are differentially regulated during lung development; however, for most of these miRNAs little is known about how or if they regulate lung development (Williams et al., 2007; Bhaskaran et al., 2009; Dong et al., 2010).

The miRNAs that peak early in lung development during the pseudoglandular stage generally promote proliferation and inhibit differentiation (Fig. 5B). Expression of the miR17~92 cluster, for example, is high early in development and decreases as development progresses (Lu et al., 2007; Carraro et al., 2009). This cluster promotes proliferation, in part through repressing Rbl2 expression, and loss of this cluster leads to perinatal lethality and severely hypoplastic lungs (Lu et al., 2007; Ventura et al., 2008). By contrast, miR17~92 overexpression in the lung epithelium induces hyperproliferation and inhibits differentiation of both proximal and distal epithelial progenitor cells (Lu et al., 2007). Important for its role in regulating proliferation, miR17~92 is a direct target of the oncogenes c-Myc (Myc) and E2F1-3 (O'Donnell et al., 2005; Sylvestre et al., 2007; Woods et al., 2007). miR17~92 is functionally redundant with its paralogs miR106b-25 and miR106a-363, and although loss of just the miR17~92 cluster has no effect on branching, knockdown of miR17, miR20a and miR106b *in vitro* leads to reduced E-cadherin expression and defects in terminal branching (Ventura et al., 2008; Carraro et al., 2009). This knockdown also disrupts Bmp4 and β-catenin signaling (Carraro et al., 2009).

The miR302-367 cluster also regulates the balance between lung epithelial proliferation and differentiation (Tian et al., 2011). This cluster is expressed primarily in the embryonic lung epithelium prior to E15, during which time it promotes the proliferation of both proximal and distal lung progenitors but prevents their subsequent differentiation (Fig. 5B). The ability of miR302-367 to promote proliferation is in part due to this cluster directly repressing expression of the tumor suppressors Rbl2 and Cdkn1a (Tian et al., 2011).

In the later stages of lung development, there is a switch from proliferation to differentiation that is supported by a shift in the expression and function of miRNAs. For example, miR449/34 is highly expressed late in development and suppresses proliferation through promoting p53 (Trp53) acetylation and activation (Lizé et al., 2010a; Lizé et al., 2010b). This cluster also promotes multiciliogenesis and is further upregulated following birth or exposure to air, suggesting that it might play an important role in the postnatal maturation of airway epithelial cells (Lizé et al., 2010a; Marcet et al., 2011). This regulation of multiciliogenesis is in part mediated by inhibition of Notch1 and Dll1, which repress ciliated cell differentiation (Marcet et al., 2011). miR375 is another late stage miRNA that is upregulated just prior to birth in AEC2 cells and remains highly expressed postnatally (Wang et al., 2013b). If AEC2 cells are cultured on plastic plates, miR375 levels decrease, inducing trans-differentiation of AEC2 into AEC1 through upregulation of β-catenin signaling (Wang et al., 2013b). A similar trans-differentiation process occurs during development, although it is unclear whether miR375 similarly regulates this process *in vivo* through inhibition of β-catenin signaling.

#### The role of long non-coding RNAs in the lung

Long non-coding RNAs (lncRNAs) are a recently identified class of non-coding RNAs that are 200 nucleotides or longer and interact with proteins, RNA and DNA to regulate a wide variety of processes (Batista and Chang, 2013; Ulitsky and Bartel, 2013). Hundreds of lncRNAs are expressed in the lung, including lncRNAs that regulate developmental processes such as differentiation and proliferation in other contexts (Askarian-Amiri et al., 2011; Cabili et al., 2011; Kretz et al., 2012). Although there is no direct evidence indicating that lncRNAs regulate lung development, the deletion of a genomic locus containing lncRNAs upstream of *Foxf1* is associated with lung developmental disorders normally associated with *Foxf1* mutations, including pulmonary vascular defects (Szafranski et al., 2013). This suggests that these lncRNAs regulate Foxf1 activity during lung development and that they may be part of a larger group of lncRNAs influencing lung development as a whole. A major hurdle for future studies looking at the role of lncRNAs in lung development will be the generation of reliable *in vivo* models. Many lncRNAs overlap enhancer regions, protein-coding genes, or promoters for other genes, making it difficult to genetically inactivate a single lncRNA without potentially affecting another genomic element. However, the importance of such reliable *in vivo* models is emphasized by recent studies of the lncRNA Malat1 (metastasis-associated lung adenocarcinoma transcript 1); although early studies showed that Malat1 is expressed at high levels in the developing lung and suggested that it promotes the lung cancer proliferation and metastasis, the analysis of a mouse *Malat1* knockout showed that it is not required for normal lung development (Schmidt et al., 2011; Eissmann et al., 2012; Zhang et al., 2012; Gutschner et al., 2013).

### Lung developmental pathways in injury and regeneration

The ability of tissues to respond to injury can be divided into three basic categories along an injury response spectrum (Fig. 6A). At one end of this spectrum are tissues that have a well-delineated stem/progenitor cell hierarchy and exhibit constant turnover during the life of the organism. This includes the skin, the intestine and the hematopoietic system. At the other end of this spectrum are tissues such as the heart and brain that contain ill-defined stem/progenitor cells and respond very poorly after injury, generally leaving behind only scar tissue. In between these two extremes are tissues that do not exhibit constant turnover and have either poorly or partially defined stem/progenitor cell lineages but can respond robustly after injury to regenerate damaged cells. The lung, liver and pancreas are in this category and these tissues can be described as having a

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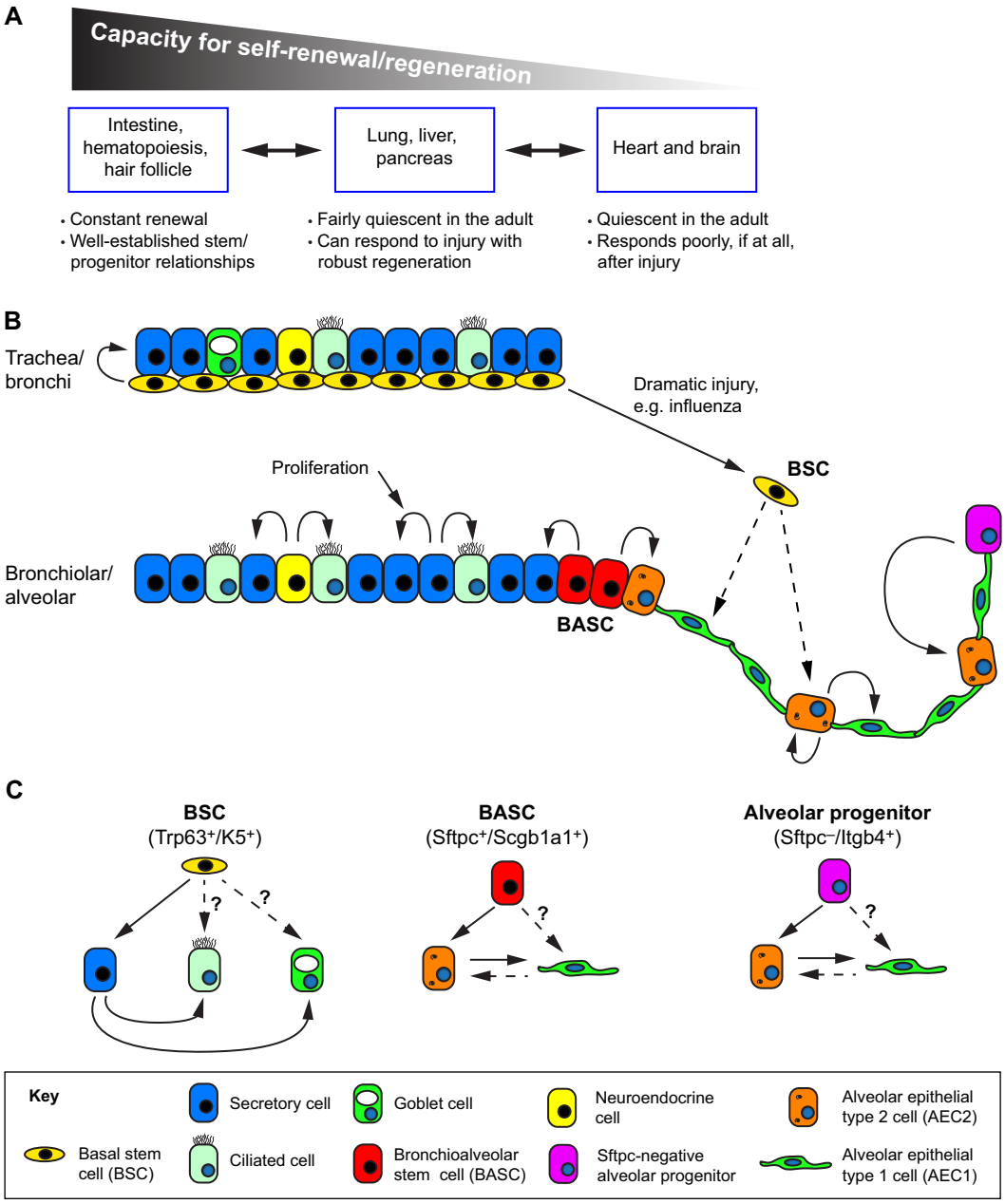


**Fig. 6. Regional progenitors contribute to lung repair and regeneration.** (A) Different organs have different capacities for self-renewal and regeneration. The lung, although largely quiescent in the adult, is capable of regenerating in response to injury. (B) Basal stem cells (BSCs) can regenerate both secretory and ciliated epithelium of the trachea and proximal bronchi. Some evidence indicates that BSCs can also generate alveolar epithelium after extreme injury. In the bronchiolar region, secretory cells can regenerate themselves and ciliated cells after injury. Neuroendocrine cells can also regenerate secretory and ciliated cells after loss through injury, although this capacity maybe limited. Bronchioalveolar stem cells (BASCs) lie at the border between the bronchiolar and alveolar regions and are marked by co-expression of Sftpc and Scgb1a1. BASCs are thought to contribute to both bronchiolar and alveolar regions after injury. Within the alveolar region, Sftpc$^+$ AEC2 cells have been shown to self-renew and differentiate into AEC1 cells after injury and during homeostasis. (C) Relationship between the various stem/progenitor lineages and their differentiated progeny. Relationships that are predicted to possibly occur but have not yet been demonstrated are shown with dashed arrows.

facultative regenerative response. This regenerative capacity has prompted studies into the cells and factors that mediate lung regeneration, as well as attempts to harness this regenerative response to produce lung progenitors *in vitro*.

### General mechanisms and pathways of lung regeneration

Although the lung has extensive abilities to repair and regenerative damaged cells after injury or disease, little is understood about the cell lineages involved and the pathways that regulate this process. As in other tissues, attention has been paid to pathways that play an important role in lung specification and differentiation during development. Other mechanisms, including activation of proliferation in a spatial- and temporal-specific fashion, are also likely to be important players. Indeed, pneumonectomy-induced injury, in which ~50% of the lung is removed, leads to a global increase in cell proliferation and re-establishment of overall respiratory capacity. Evidence exists for both expansion of certain stem/progenitor populations and coordinated proliferation of cell

lineages to expand existing lung tissue (Voswinckel et al., 2004; Hoffman et al., 2010; Eisenhauer et al., 2013). However, the underlying mechanisms related to such a dramatic increase in lung size and capacity still remain unclear.

#### Cell types involved in the regenerative response

The fully mature lung, similar to the developing lung, has several distinct niches that are populated by different stem/progenitor populations important for the response of the tissue to injury and disease (Fig. 6B,C). The trachea and main stem bronchi are lined with a pseudostratified epithelium that is underlined with a population of cells expressing both the transcription factor Trp63 and the cytokeratin Krt5, which are known as basal stem cells (BSCs). BSCs are capable of regenerating both the secretory and ciliated epithelium lining the trachea after injury and this process is controlled in part by Notch signaling, which promotes secretory fate and inhibits the ciliated fate (Rock et al., 2009; Rock et al., 2011b). Recent evidence also points to a potential role for $Trp63^{+}/Krt5^{+}$ BSCs in generating distal alveolar epithelium; following severe lung injury through influenza infection, $Trp63^{+}/Krt5^{+}$ 'pods' appear to emanate from the large airways and migrate to more peripheral regions of the lung where they are thought to differentiate into alveolar epithelium (Kumar et al., 2011). This ability of BSCs to move great distances indicates highly active tissue remodeling in the context of lung regeneration. Such studies also suggest that stem/progenitor cells that are distant to the site of damage can contribute significantly to repair and regeneration. However, this study does not distinguish between the activation of a defined stem/progenitor population such as the BSC and the activation of a repair/regeneration gene expression program that triggers Trp63 and Krt5 expression in non-BSC cell lineages. The full contribution of these activated BSC $Trp63^{+}/Krt5^{+}$ pods to injury repair is also unclear. Additional studies, such as live imaging of BSCs during the repair process and precise lineage tracing, will be needed to distinguish between these various possibilities.

Further down the respiratory tract, the bronchiolar region of the rodent lungs is lined with a single-layer epithelium consisting of both secretory and ciliated lineages. BSCs also line this region of the airway tract in humans but their presence has not been well documented in rodent lungs (Chilosi et al., 2002; Sheikh et al., 2004). There is evidence that, in the bronchiolar region, a sub-population of secretory cells that express Scgb1a1 but lack expression of Cyp2f2 can act as facultative progenitor cells. The bronchiolar epithelium, like much of the rest of the lung, is quiescent until injured. Using a model of naphthalene-induced depletion of airway secretory cells, several reports have shown that this $Scgb1a1^{+}/Cyp2f2^{-}$ population is spared from napthalene toxicity and expands rapidly and re-epithelializes the damaged airways (Reynolds et al., 2000; Giangreco et al., 2002; Reynolds et al., 2002). Cell proliferation plays an important role in this injury response but it remains unclear whether $Scgb1a1^{+}/Cyp2f2^{-}$ cells go through a process of de-differentiation to re-enter the cell cycle in order for the population to expand before re-differentiating. Neuroendocrine cells are also able to generate secretory and ciliated cells after napthalane-based injury. However, depletion of neuroendocrine cells does not impede secretory cell regeneration, suggesting a limited contribution of this lineage to normal airway regeneration (Song et al., 2012).

Stem/progenitors also exist in the alveolar niche, which consists of AEC1 and AEC2 cells, vascular endothelium, and associated mesenchymal lineages. Both historical as well as recent cell lineage tracing evidence shows that AEC2 cells can self-renew and act as progenitors for AEC1 cells during normal homeostatic turnover as well as after epithelial loss due to injury (Evans et al., 1973; Barkauskas et al., 2013). Diptheria toxin-mediated depletion of $Sftpc^{+}$ AEC2 cells leads to clonal expansion of these cells to repopulate injured alveolar airspaces (Barkauskas et al., 2013). Interestingly, these clones expand significantly beyond a single alveolar unit, suggesting that AEC2 cells, like BSCs described above, can migrate significant distances to repopulate and repair damaged lung tissue. $Sftpc^{+}$ AEC2 cells can also be propagated in culture but only in the presence of $Pdgf\alpha^{+}$ mesenchyme, indicating that the principle of epithelial-mesenchymal cross-talk that occurs in lung development also plays an important role in adult stem/progenitor-mediated regeneration (Barkauskas et al., 2013).

There are at least two other potentially important stem/progenitor populations in the adult lung: bronchioalveolar stem cells (BASCs), which reside at the junction of the bronchiolar and alveolar regions, and an Sftpc-negative α6/β4 integrin-positive ($Sftpc^{-}/\beta4^{+}$) alveolar stem cell population. BASCs are marked by expression of both secretory (Scgb1a1) and AEC2 (Sftpc) markers. Studies have indicated that BASCs can repopulate both the secretory and alveolar regions and can act as the cell of origin for lung adenocarcinomas (Kim et al., 2005; Rock et al., 2011a). However, the use of lineage tracing reveals a more complicated story. Although $Scgb1a1^{+}$ BASCs can generate $Sftpc^{+}$ AEC2 cells under dramatic injury settings, it remains somewhat unclear to what extent these cells contribute towards injury repair (Rock et al., 2011a). In contrast to BASCs, $Sftpc^{-}/\beta4^{+}$ cells reside in the alveolar region of the lung and can convert to Sftpc-expressing cells after injury (Chapman et al., 2011). Moreover, isolated $Sftpc^{-}/\beta4^{+}$cells can generate lung organoids in a kidney capsule assay and can expand clonally in cell culture. As with BASCs, it remains unclear to what extent $Sftpc^{-}/\beta4^{+}$ cells contribute towards injury repair but future studies using lineage-tracing techniques and additional injury models should help resolve this question.

#### Generating lung cell lineages *in vitro*

The generation of endodermal derivatives, such as the lung endoderm, from ESCs or iPSCs has remained difficult compared with both mesodermal and neural cell lineages. However, using some of the important developmental pathways described above, several recent studies have demonstrated that ESCs and iPSCs can be induced to differentiate into anterior foregut endoderm, which can then generate lung epithelial cell lineages including both $Sftpc^{+}$ AEC2 and $Scgb1a1^{+}$ secretory cell lineages (Longmire et al., 2012; Mou et al., 2012; Huang et al., 2013). These studies utilized several of the known molecular pathways important for lung development, including Fgf, Wnt and Bmp. Importantly, the finding that posterior and dorsal fate can be inhibited by inhibiting Bmp signaling allowed for researchers to promote anterior foregut endoderm over posterior gut endoderm in differentiating pluripotent stem cells (Green et al., 2011). Moreover, the careful application of specific activators and inhibitors of developmental pathways in a precise temporal fashion was required to promote lung epithelial cell fate. Although the cell lineages from these experiments are immature, the finding that it is possible to generate lung epithelial lineages from ESCs and iPSCs does allow for an extensive new line of investigation using human-derived cells, which will have an important impact on the study of diseases such as cystic fibrosis and asthma. Recent studies have also described the use of decellularized matrices that can provide useful assays for lung cell lineage differentiation capabilities (Ott et al., 2010; Petersen et al., 2010). Such assays combined with the ongoing optimization of lung epithelial differentiation from ESCs and iPSCs

should allow for a better understanding of the cell plasticity and lineage fidelity inherit in lung epithelial and mesenchymal lineages.

## Conclusions

Our understanding of lung development and its impact on lung regeneration has grown dramatically in recent years. As in other tissues, this increased understanding will undoubtedly have an impact on human health. However, the field still needs more information regarding the fate and capacity of early lung endoderm progenitors to generate the various epithelial lineages of the adult lung. Moreover, whether the pathways that govern the differentiation of these progenitors during development are re-activated upon injury and regeneration is unclear in many cases. This may require the invention of new injury and regeneration models. Alternatively, a more careful consideration of current models could improve our understanding of the molecular responses to the lung after injury. Ultimately, this information may be able to help premature infants that suffer from lung deficiencies and congenital abnormalities.

One of the greatest deficits in our knowledge of lung development is our understanding of the potency and heterogeneity of early lung mesodermal progenitors, in particular their capacity to generate the various smooth muscle and endothelial lineages in the lung. Several new marker genes have been identified in recent years and there have been important lineage-tracing studies using these markers. However, how the early multipotent mesoderm differentiates into different sublineages and ultimately into fully differentiated smooth muscle, various fibroblast sublineages, and endothelium is almost a complete black box. Such questions are also important in the postnatal regenerating lung where inappropriate repair could lead to increased fibrosis due to runaway mesenchymal expansion. Much work is needed to increase our understanding of this aspect of lung development and homeostasis to the same level that exists for the epithelial lineages. The ability to perform genomic and transcriptomic analyses at the single cell level is likely to generate an increased understanding of the cellular heterogeneity in both the mesodermal and epithelial lineages of the developing lung. As the field generates new marker genes from these genomic and transcriptomic analyses, new mouse lines can be generated to help advance our understanding of these questions. Importantly, recent leaps in genome-editing techniques open the possibility of using multiple cell-tagging/lineage-tracing techniques to allow for more precise lineage identification through the tracking of multiple marker genes simultaneously (Wang et al., 2013a; Yang et al., 2013).

Ultimately, an increased understanding of lung development should lead to better insights not only into neonatal lung disorders but also into diseases such as asthma and COPD. Studies using human lung tissue are difficult but the ability to generate lung epithelial cells from human pluripotent stem cells will help overcome some of these hurdles. Moreover, as therapeutically attractive pathways are identified in rodent models, there needs to be an emphasis on examining larger animal models for which lung physiology and structure more closely resemble that of humans. New genome-editing techniques may make it possible to generate large animal genetic models of pulmonary disease to perform mechanistic studies in a physiological setting that closely resembles the human respiratory system. Such studies would harness the best of basic science and translational research to benefit those afflicted with debilitating pulmonary diseases.

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### Competing interests

The authors declare no competing financial interests.

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