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Patterning and Plasticity in Development of the Respiratory Lineage

Eric T. Domyan[1] and **Xin Sun**[1]

[1] Laboratory of Genetics, University of Wisconsin-Madison, Madison, WI 53706

Abstract

The mammalian respiratory lineage, consisting of the trachea and lung, originates from the ventral foregut in an early embryo. Reciprocal signaling interactions between the foregut epithelium and its associated mesenchyme guide development of the respiratory endoderm, from a naive sheet of cells to multiple cell types that line a functional organ. This review synthesizes current understanding of the early events in respiratory system development, focusing on three main topics: 1) specification of the respiratory system as a distinct organ of the endoderm, 2) patterning and differentiation of the nascent respiratory epithelium along its proximal-distal axis, and 3) plasticity of the respiratory cells during the process of development. This review also highlights areas in need of further study, including determining how early endoderm cells rapidly switch their responses to the same signaling cues during development, and how the general proximal-distal pattern of the lung is converted to fine-scale organization of multiple cell types along this axis.

Keywords

endoderm; respiratory system; patterning

Introduction

The image of a newborn being welcomed into the world with a spank from the doctor to jump start breathing serves well to demonstrate the importance of a functional gas-exchange system in postnatal life. The trachea and lungs of the respiratory lineage are composed of an endoderm-derived epithelial cells surrounded by a mesoderm-derived mesenchymal cells. During embryogenesis, reciprocal signaling interactions between these two cell lineages guide the patterning and subsequent development of a functional respiratory system (Shannon and Hyatt, 2004). While many of the molecular players in this process have been identified, significant gaps in our knowledge remain.

General cellular development is often modeled as a sequential process (Slack, 1991). First, cells are *specified* to a certain fate, but for a certain period of time remain labile to alter their fate in response to cues from their environment. Next, cells become *determined*, as they commit to a certain fate and are no longer able to alter their developmental trajectory in response to external cues. Third, cells *differentiate* to assume the morphology and function of the mature cell type. The purpose of this mini-review is to discuss what is known about these processes in respiratory development, both during the separation of the respiratory lineage from other endoderm-derived lineages, and during patterning of the respiratory system along its proximal-distal (P-D) axis. We will start by outlining the timing of these

Author for correspondence: Xin Sun, Phone: (608)265–5405, Fax: (608)262–2976, xsun@wisc.edu.

events as well as the signaling molecules involved. We will primarily discuss findings from mouse, but will also refer to results from other model organisms during similar stages of development. This review is not intended to be a comprehensive review of endoderm or lung development, and the interested reader is referred to other excellent reviews on these topics (Cardoso and Lu, 2006, Zorn and Wells, 2009, Morrisey and Hogan, 2010).

Prenatal development of the respiratory system: sequence of events

The embryonic endoderm emerges through the process of gastrulation at approximately embryonic day (E) 6.5-8.0 in the mouse (Zorn and Wells, 2009) (Fig. 1A). A recent study demonstrates that as definitive endoderm cells emerge from the epiblast layer, they intercalate among visceral endoderm cells, and that both populations contribute to the gut tube (Kwon et al., 2008). Subsequently at E8.5, the endoderm folds to produce a gut tube (Fig. 1B).

While the first morphological appearance of the respiratory primordium is not apparent until a pair of lung buds form at E9.5, respiratory specification likely occurs much earlier. The earliest known marker of the respiratory lineage is the transcription factor *Nkx2-1* (also known as *Thyroid Transcription Factor 1, Ttf1*), which is detectable in the ventral foregut by RT-PCR as early as E8.25, at the 8-somite stage (Lazzaro et al., 1991, Minoo et al., 1999, Serls et al., 2005) (Fig. 1B). While it is commonly used as a marker of the respiratory lineage, it is not exclusively expressed there, as it is also expressed in the developing thyroid and brain (Lazzaro et al., 1991). In *Nkx2-1* null mutant embryos, although primary lung buds form, the trachea is absent, suggesting that it is essential for the initiation of a subset of the respiratory system (Minoo et al., 1999). While the timing of *Nkx2-1* expression suggests that the respiratory lineage is first specified at E8.25, another study using an inducible lineage-labeling system suggests that progenitors of the distal respiratory system (peripheral lung tubules) may be set aside prior to or coincident with gastrulation (Perl et al., 2002). The identification and characterization of additional respiratory markers is needed to more accurately depict the early events of respiratory specification.

At E9.5, two ventral lung evaginations establish the left and right lung buds (Fig. 2A). Concomitantly, the foregut anterior to the buds separates into two parallel tubes, a ventral trachea and dorsal esophagus. Following elongation of the primary lung buds into the main bronchi, multiple branches form following a stereotypical sequence (Metzger et al., 2008). The respiratory system is patterned along its proximal-distal (P-D) axis (Fig. 2). The proximal region gives rise to the trachea and major conductive airways, and the distal region gives rise to the smaller bronchioles and gas exchange units. Differentiation along the P-D axis begins at E14.5, the final result being cells of distinct morphologies and functions. In the mature lung, the proximal airways are lined primarily with ciliated, basal, neuroendocrine, and secretory cell types, while distal gas-exchange units are lined with type I and type II alveolar epithelial cells (AECs, also termed pneumocytes) (Fig. 3) (Cardoso and Lu, 2006,Crystal et al., 2008). At birth, the various cell types fulfill distinct roles including cleaning and moisturizing air, production of surfactant and facilitation of gas exchange.

Step-wise specification and dynamic responses to inductive cues

The respiratory system originates from a stereotypical location within the ventral foregut endoderm. In addition to the respiratory system, the foregut gives rise to an impressive array of organs, including the thymus, thyroid, esophagus, liver, pancreas, and stomach. The midgut and hindgut, in contrast, primarily give rise to the small and large intestine (Zorn and Wells, 2009). The first specification step is to distinguish the foregut from that of mid/hindgut. Several transcription factors have been implicated as indicators of endoderm

patterning: *Hhex and Sox2* are expressed in the foregut, while Cdxge nes are expressed in mid/hindgut (Fig. 1A) (Zorn and Wells, 2009). Multiple studies demonstrate that this foregut versus mid/hindgut pattern is established during gastrulation and shortly thereafter by ectodermal and mesodermal cues including BMP (Tiso et al., 2002, Matsushita et al., 2008), FGF (Davidson et al., 2000, Wells and Melton, 2000, Dessimoz et al., 2006, Li et al., 2008), WNT (McLin et al., 2007, Li et al., 2008), and retinoic acid (RA) (Bayha et al., 2009). For example, explant studies in chick have demonstrated that signals from Hensen's node or its derivatives specify ingressing endoderm cells as foregut as indicated by *Sox2* expression, and that BMP antagonists Noggin or Chordin are sufficient for this effect (Matsushita et al., 2008). Together, these data suggest that endoderm cells adopt an anterior fate unless programmed into more posterior fate through the combined action of secreted factors. In fact, it has been previously noted that the mechanism of endoderm patterning closely mirrors that of neural patterning, in which neural tissue has an anterior fate unless "posteriorized" by multiple signaling pathways (McLin et al., 2007). One exception to this pattern is the finding that Tgfβ family member *Nodal*, which is highly expressed in the early anterior primitive streak, is necessary for anterior fate (Vincent et al., 2003).

Following specification of the foregut, a second specification step towards respiratory identity is to partition the foregut into distinct organ compartments, including trachea/lung. Along the A-P axis of the developing foregut, studies have focused on cues that lead to differential specification of the respiratory versus liver and pancreas cell fates (Fig. 1B). Strong evidence suggests that FGF signaling plays a dosage-dependent role in this process (Wandzioch and Zaret, 2009,Serls et al., 2005,Jung et al., 1999, Calmont et al., 2006). For example, it has been shown that foregut endoderm cells from 2- to 5-somite stage embryos (E8.0-E8.5) adopt pancreatic fate (*Pdx1* expression) when cultured in the absence of FGF, adopthepatic fate (*Hhex* expression) when cultured in a low concentration of FGF, and adoptlung fate (*Nkx2-1* expression) when cultured in a high concentration of FGF (Serls et al., 2005). These and data from related studies suggest that increasing levels of FGF signal promote a progressively more anterior organ identity, from pancreas, to liver, to lung (Wandzioch and Zaret, 2009,Serls et al., 2005,Jung et al., 1999, Calmont et al., 2006). Remarkably, this is opposite to the finding during gastrulation where increasing levels of FGF promote posterior (mid/hindgut) identity(Dessimoz et al., 2006,Wells and Melton, 2000).

There are additional examples for distinct endoderm responses to the same signal at consecutive time periods. As mentioned above, at gastrula stage during foregut specification, overexpression of WNT leads to inhibition of foregut markers including *Nkx2-1* (McClin and Zorn). A short time later during specification of the respiratory lineage (E8.25-E9.0 in mouse), inactivation of either WNT ligands (*Wnt2/2b*) or the key WNT effector *β-Catenin* lead to either loss or reduction of *Nkx2-1*, while forced activation of WNT/β-Catenin signaling via ectopic expression of a dominant-stable allele of *β-Catenin* in the ventral stomach promotes *Nkx2-1* expression (Goss et al., 2009, Harris-Johnson et al., 2009). In contrast, at later stages of development(E18.5), forced activation of WNT/β-Catenin signaling via expression of a β-Catenin/LEF fusion protein causes lung epithelium to transdifferentiate into intestinal epithelium (Okubo and Hogan, 2004). Therefore, similar to FGF, it appears that WNT/β-Catenin can either promote or inhibit the respiratory fate depending on the context (timing, tissue site and perhaps level) of signaling.

This dynamic endoderm response to signaling is not restricted to the respiratory lineage. In the developing mouse liver and pancreas, BMP promotes liver over pancreas at 3-4 somites, but produces the opposite response at 5-6 somites (Wandzioch and Zaret, 2009). How the transcriptional regulatory circuitry is "rewired" in just a few hours remains an important question to be answered in developmental biology. Interestingly, TGFβ signaling plays an

indirect role in permitting pancreatic development, by restraining the specification of cells competent to respond until they move into the BMP-inductive region at 5-6 somites (Wandzioch and Zaret, 2009).

Compared to A-P patterning of the foregut endoderm, relatively little is known regarding dorsal-ventral (D-V) patterning of this tissue. Opposite to respiratory precursors in the ventral foregut endoderm, the cells of the dorsal foregut will give rise to the esophagus (Fig. 1B). It is important to note that folding of the endoderm to form the gut tube brings cells from distinct regions along the A-P axis into close apposition along the D-V axis. Fate mapping studies in chick demonstrate that cells at the anterior end of Hensen's node of mid-primitive streak stage embryos ultimately contribute to the ventral midline of the foregut, thus giving rise to the respiratory primordia (Matsushita et al., 2008). In contrast, cells located more posteriorly in the mid-primitive streak stage embryos will contribute to the dorsal midline of the foregut, thus giving rise to the esophagus. These data suggest that prospective-dorsal and prospective-ventral endoderm cells ingress at different times, and follow different migratory routes during gastrulation (Rosenquist, 1971,Kimura et al., 2006,Matsushita et al., 2008). These data have led some researchers to propose that separation of the respiratory and digestive tracts should most accurately be viewed as separate morphogenetic programs executed independently by two pre-patterned semi-circular canals, rather than patterning and division of a single tube (Brown and James, 2009). More consideration need to be paid to the morphogenetic history of the dorsal versus ventral endoderm cells to better understand their pre-existing molecular differences leading up to D-V patterning of the foregut.

Different transcription factor genes mark the dorsal versus ventral endoderm cells in a common foregut tube (Fig. 1B, 2A). *Sox2*, initially expressed throughout the entire foregut, is downregulated in the respiratory domain by E9.0 (Sherwood et al., 2009), coincident with the upregulation of *Nkx2-1* in this domain. In addition to being markers, *Nkx2-1* and *Sox2* are necessary for formation of the ventral trachea and dorsal esophagus, respectively (Minoo et al., 1999,Que et al., 2007). Loss-of-function mutations of one transcription factor causes upregulation of the other along the entire D-V axis (Que et al., 2007), suggesting that they inhibit each other's expression.

Multiple signaling pathways have been implicated in D-V patterning of the foregut tube, including BMP (Que et al., 2006, Li et al., 2007, Li et al., 2008), SHH (Litingtung et al., 1998) and FGF (Que et al., 2007) (Fig. 1B). For example, *Bmp4* is expressed in the ventral mesenchyme surrounding the undivided foregut tube while *Noggin*, which encodes a BMP antagonist, is expressed in the dorsal foregut endoderm (Que et al., 2006, Li et al., 2007). Loss- and gain-of-BMP pathway mutants show tracheal agenesis and esophageal atresia, respectively, suggesting that proper balance of BMP signaling is essential for the formation of the two distinct lineages (Que et al., 2006, Li et al., 2007, Li et al., 2008). *Shh* is expressed in the ventral foregut endoderm, and *Shh* mutant embryos show defects in tracheal-esophageal separation (Litingtung et al., 1998). While it is not currently known whether SHH and BMP interact during D-V patterning of the foregut, at later stages of lung development, SHH secreted by lung epithelium induces *Bmp4* in the mesenchyme (Weaver et al., 2003). A similar relationship could then at least partially explain the overlap in *Shh* and *Bmp* mutant phenotypes in the foregut. Evidence suggests that cells remain responsive to D-V signaling cues for a significant amount of time after the initial establishment of pattern. At E11.0, which is after the separation of the esophagus from the trachea, culturing the esophagus in the presence of exogenous FGF10 leads to downregulation of digestive markers and upregulation of respiratory markers (Que et al., 2007).

As morphogenesis proceeds, the nascent lung buds elongate and branch. At the same time, they are patterned along the P-D axis, resulting in the differentiation of distinct cell types (Fig. 2,3). We next turn our attention to what is known about how this process occurs.

P-D patterning of the respiratory system

Compared to respiratory specification, more is known about how the respiratory system is patterned along its P-D axis. We will discuss two stages of P-D patterning: distinction between the trachea (proximal) versus lung (distal); and within the lung, distinction between the airway epithelium (proximal) versus alveolar epithelium (distal). Perl *et al.* found that by using an inducible SpC-driven Cre system, they could genetically label distal lung epithelium without labeling tracheal and bronchial cells before E8.5 (Perl et al., 2002). Consistent with this, a more recent study using an inducible *Id2-CreER*^{T2} has shown that cells in the distal tip of the elongating lung branch at E11.5 ultimately contribute to all lung epithelium, but not tracheal epithelium cell types. These data suggest that precursors for the trachea versus lung are separated perhaps as early as when the respiratory lineage is set aside from most other foregut-derived organ progenitors. Data from knockout mice show that a number of genes are necessary for trachea but not initial lung bud formation, including *Nkx2-1* (Minoo et al., 1999), *Shh* (Litingtung et al., 1998), and *Bmp4* (Li et al., 2008). Conversely, *Fgf10* and its cognate receptor *Fgfr2* are essential for lung but not trachea formation (Min et al., 1998, Arman et al., 1999, Sekine et al., 1999, De Moerlooze et al., 2000). These results support the concept that distinct genetic programs promote trachea versus lung development.

For P-D patterning within the lung, a large number of genes have been identified that are preferentially expressed in the distal epithelium, with a smaller number preferentially expressed in the proximal epithelium (Liu and Hogan, 2002, Lu et al., 2004). Many of the same factors that regulate patterning of foregut endoderm are redeployed for the growing respiratory system (Fig. 2B). Interestingly, the opposing gradients of *Sox2* and *Nkx2-1* along the D-V axis of the separating esophagus and trachea at E10.0 are subsequently observed along the P-D axis of the elongating lung at E11.5 (Gontan et al., 2008, Que et al., 2009). During lung branching morphogenesis *Sox2* inhibits branching and promotes proximal cell fate, while *Nkx2-1* is required for branching and distal cell fate (Minoo et al., 1999, Yuan et al., 2000, Gontan et al., 2008, Que et al., 2009, Tompkins et al., 2009). Familiar signaling pathways are critical for P-D patterning of the branching lung as compared to earlier lung developmental events, although distinct ligands and receptors may be deployed at different stages. During respiratory specification (E8.25-E9.0), FGF1 and FGF2 from the cardiac mesenchyme signal to FGFR1 and FGFR4 in the endoderm (Serls et al., 2005). Starting from the initiation of lung budding however (E9.5 onwards), FGF10 from the mesenchyme surrounding the nascent lung buds signals to FGFR2 in the endoderm to drive outgrowth of lung buds and elongation of epithelial branches (Bellusci et al., 1997, Sekine et al., 1999, Weaver et al., 2000) (Figure 2A, C). Whether the employment of distinct FGF ligands and receptors in specification versus budding/branching is functionally relevant deserves further study.

In addition to acting as a chemoattractant for lung epithelial branching, FGF signaling plays a critical role in lung P-D patterning. Loss of FGF signaling through conditional deletion of *Fgf10* or *Fgfr2* after lung budding results in expansion of proximal marker *Sox2* and downregulation of distal marker *Sox9* (Abler et al., 2009). Overexpression of *Fgf10* promotes distal fate while inhibiting terminal differentiation of lung epithelium (Nyeng et al., 2008). A similar paradigm for FGF signaling exists in P-D patterning of the limb, suggesting that this may be an evolutionarily-conserved function of FGF (Tabin and Wolpert, 2007, Mariani et al., 2008).

Upstream of *Fgf10*, while the comprehensive mechanism remains to be uncovered, several genes have been identified that control the dynamic and spatially-restricted pattern of *Fgf10* expression. During lung branching morphogenesis, RA inhibits the expression of *Fgf10* (Malpel et al., 2000) (Fig. 2B). Interestingly, the effects of RA on *Fgf10* expression are stage-dependent. Opposite to its impact on *Fgf10* expression at branching stage, RA promotes the initial expression of *Fgf10* at budding stage, likely through repressing TGFβ signaling (Desai et al., 2004, Chen et al., 2007, Chen et al., 2010) (Fig. 2A). The mechanism by which the relationship between RA and FGF signaling changes in such a short period of time is unknown. The transcription factors *Tbx4* and *Tbx5* also promote *Fgf10* expression, but since they are expressed ubiquitously in the mesenchyme, they likely act with additional factors to achieve the spatially restricted *Fgf10* expression (Cebra-Thomas et al., 2003, Sakiyama et al., 2003).

Downstream of *Fgf10*, it induces additional signaling pathways that promote distal cell fate (Fig. 2B). For example, *Bmp4*, which is induced by FGF10 in the distal epithelium starting from E11.0, acts to promote distal and restrict proximal fate (Weaver et al., 1999,Hyatt et al., 2002,Hyatt et al., 2004,Eblaghie et al., 2006,Sun et al., 2008). This epithelial expression of *Bmp4* is in addition to its earlier-initiated expression in the mesenchyme (Que et al., 2006,Li et al., 2007), although the significance of this additional site of expression is not known. Reduction of BMP signaling through overexpression of a dominant-negative receptor *dnBmpr1b* or secreted antagonist *Xnoggin* results in expansion of proximal cell markers and downregulation of distal markers (Weaver et al., 1999), and loss of BMP receptor *Bmpr1a* results in defects in distal lung development (Eblaghie et al., 2006,Sun et al., 2008).

Following their critical role in respiratory lineage specification mentioned above, *Wnt2* and *Wnt2b*, two canonical Wnt genes, continue to be expressed in distal lung mesenchyme during lung branching morphogenesis (Yin et al., 2008, Yi et al., 2009) (Fig. 2B). Similar to FGF, loss-of-function studies demonstrate that endogenous levels of WNT/β-Catenin signaling are essential to promote distal lung fate (Mucenski et al., 2003, Shu et al., 2005), while gain-of-function studies suggest that WNT/β-Catenin signaling are sufficient to prevent differentiation (Reynolds et al., 2008). Upstream of WNT, its activity is promoted by RA through repression of WNT antagonist *Dickkopf* (*Dkk*) (Chen et al., 2010) (Fig. 2A). Downstream of WNT, its ability to promote distal fate and inhibit differentiation is mediated, at least in part, though its regulation of *Fgfr2*, *Bmp4* and *Mycn* expression in the distal epithelium (Okubo et al., 2005, Shu et al., 2005, Cox et al., 2007) (Fig. 2B).

In addition to canonical WNT/β-Catenin pathway, there is evidence that non-canonical WNT, such as WNT5a signaling also plays a role in lung P-D patterning. *Wnt5a* is expressed most highly in the distal epithelium and mesenchyme (Li et al., 2002) (Fig. 2B). Loss of *Wnt5a* results in expansion of the distal epithelium and inhibition of differentiation, as well as increased expression of *Bmp4* and *Fgf10*. This raises the possibility that WNT5a may control P-D patterning by inhibiting the expression of these distal-promoting factors.

Similar to *Wnt5a*, additional genes implicated in promoting proximal cell fate do so through antagonism of one or more distalizing signals (Fig. 2B). *Spry2*, which is induced by FGF10 in the distal epithelium, feeds back to limit FGF activity, thereby reducing the expression of distal markers (Tefft et al., 1999,Mailleux et al., 2001). A negative feedback loop also limits the amount of BMP signaling, as BMP4 induces expression of its antagonist *Noggin* in the proximal mesenchyme (Weaver et al., 2003). Overexpression of *Noggin* in the distal lung epithelium promotes proximal cell fate, suggesting that Nogginrestricts the domain of distal cell fate promoted by BMP4 (Weaver et al., 1999).

Unique among signals important for P-D patterning of the respiratory system, Notch and its transmembrane ligands act through cell-cell contacts, at a finer scale compared to the secreted signals mentioned above. A number of Notch ligands are expressed in the distal epithelium of the lung during early development (Post et al., 2000), and appear to be downstream of FGF10 signaling (Tsao et al., 2008). Chemical inhibition of Notch signaling results in overexpansion of distal fate and loss of proximal fate in cultured lung explants, suggesting that Notch functions to restrict distal fate (Tsao et al., 2008). Notch signaling pathway also plays a role in lung epithelial cell differentiation as discussed below.

Differentiation of the respiratory epithelium

Following broad-stroke patterning of the respiratory system, fine-scale differentiation of the respiratory epithelium proceeds in a P-D direction, starting with the appearance of neuroendocrine cells in the proximal airway at E14.5, and ending with the differentiation of type I and type II AECs in the distal epithelium shortly before birth (Fig. 3) (Morrisey and Hogan, 2010). Although a number of transcription factors necessary for formation of particular cell types in the lung have been identified (Chen et al., 1998,Wan et al., 2004,Wan et al., 2008,Chen et al., 2009,Que et al., 2009), few signaling pathways have been implicated in differentiation of cells within either the proximal or distal compartments of the respiratory system.

Notch signaling has recently been shown to be involved in the fate choice among cells in the proximal lung (Fig. 3). The Notch target *Hes1* is expressed in the trachea, bronchi, and bronchiolar airways, but is absent from the distal saccules at E18.5 (Guseh et al., 2009). Loss-of-function Notch pathway mutants show an absence of Clara cells and overabundance of ciliated and neuroendocrine cells in proximal airways (Tsao et al., 2009,Morimoto et al., 2010), while gain-of-function mutants have fewer ciliated cells and overabundance of goblet cells (Guseh et al., 2009). Together, these data suggest that Notch signaling functions in the proximal lung to promote Clara and goblet cell fate at the expense of ciliated and neuroendocrine fate. The TGFβ type I receptor *Activin like kinase 5* (*Alk5*) is also expressed in the bronchiolar epithelium, and inactivation of *Alk5* results in loss of Clara cells while leaving differentiation of other cell types relatively unperturbed (Xing et al., 2010). This raises the possibility that TGFβ may operate after Notch to promote differentiation of Clara cells once their fate has been determined. Subsequent experiments demonstrate that TGFβ promotes Clara cell differentiation in a SMAD-independent fashion, through upregulation of pERK and pAKT. It remains possible that other signaling pathways function in lung epithelial cell differentiation, and their roles in this late process may be obscured by the requirements for these signals in earlier lung developmental events. Late and cell type-specific inactivation of these pathway members may reveal additional signaling components essential for epithelial cell differentiation.

Plasticity of the respiratory lineage

The issue of respiratory epithelium plasticity can be considered on several levels: commitment to respiratory versus other (e.g. digestive) cell fate, commitment to trachea versus lung cell fate, and commitment to one versus another terminally differentiated cell fate. Lineage analysis and *Nkx2-1* expression data suggest that the respiratory progenitors are specified by E8.25, if not earlier (Perl et al., 2002, Lazzaro et al., 1991, Minoo et al., 1999, Serls et al., 2005). However, determination of respiratory cell fate occurs surprisingly late in development. At lung branching stage (E11.0 and later), ectopic activation of WNT can cause lung epithelium to transdifferentiate into intestine (Okubo and Hogan, 2004), while exogenous FGF can cause esophageal epithelium to transdifferentiate into respiratory epithelium (Que et al., 2007). These results suggest that cells remain competent to switch

between the respiratory and digestive fates even after the morphological appearance of distinct structures.

On trachea versus lung determination, in spite of the evidence suggesting early separation of trachea versus lung progenitors, tracheal epithelium from E13 rat lungs (similar to E11 in mouse) can form buds and express markers of distal lung fates when grafted adjacent to mesenchyme from the distal lung, while distal lung epithelium will fail to branch and will express markers of proximal respiratory fate when grafted adjacent to proximal lung epithelium (Shannon, 1994, Shannon et al., 1998). Interestingly, lung mesenchyme from near full-term embryos shows similar inducing capability as mesenchyme from E13 rat embryos (Deimling et al., 2007). At the molecular level, explant culture studies indicate that FGF, often in combination with other factors in the medium, is capable of reprogramming tracheal epithelium into a distal lung fate (Shannon et al., 1999, Ohtsuka et al., 2001, Hyatt et al., 2004).

Within the developing lung during cell differentiation, several recent studies suggest that proximal and distal lung epithelial cells in late-gestation lungs show different levels of phenotypic stability (Deimling et al., 2007). Tissue recombination experiments indicate that when recombined with mesenchyme from skin or intestine, lung epithelium from near-term rat embryos continues to express Clara cell marker *Clara cell secretory protein* (also known as *Scgb1a1*), but downregulates type II AEC markers *surfactant protein A, B and C* (*SpA, SpB,* and *SpC*). These data suggest that Clara cells maintain their characteristics in the absence of promoting signals from lung mesenchyme, but type II AECs still require factors from distal lung mesenchyme to maintain their characteristics. An additional study demonstrates that ectopic expression of *Sox17* in the distal airways of adult mice reprograms a subset of AECs into Clara and ciliated cells (Lange et al., 2009) suggesting that plasticity may be retained in at least a subset of respiratory cells into adulthood.

Despite evidence for a proximal-to-distal sequence of cell fate commitment(Rawlins et al., 2009a), there appear to be pockets of cells in the adult lung epithelium that remain labile. For example, basal cells within the trachea and bronchial epithelium remain capable of generating multiple cell types during homeostatic or repair conditions (Rock et al., 2009) (Fig. 3). More distally at the bronchioalveolar duct junction, bronchioalveolar stem cells (BASCs) have been identified to exhibit multipotency and the ability to self-renewal (Kim et al., 2005, Nolen-Walston et al., 2008, Zhang et al., 2008). Determining the identity, location, and hierarchical organization of adult pulmonary stem/progenitor cells is an area of active research. For a more comprehensive review on this topic, see Rawlins in this issue.

Perspectives: from phenomenology to hypothesis

The formation of a functional respiratory epithelium from ventral foregut endoderm consists of a series of intricately organized events, of which we are only beginning to understand. We have identified many of the signaling pathways and transcription factors involved in respiratory development, but we know little about how they are integrated with each other, and what cis-regulatory modules mediate this integration. These are questions that pertain to each of the developmental events discussed here. For example, during respiratory specification, the earliest known marker of the respiratory lineage, *Nkx2-1*, is not strictly essential for respiratory specification (Kimura et al., 1999), suggesting that it is only one of several factors involved in this process. Tracing upstream, the transcription factor genes currently known to directly activate the expression of *Nkx2-1: Foxa2* (Ikeda et al., 1996), *Gata6* (Shaw-White et al., 1999), and *Sp1* and *Sp3* (Li et al., 2000), all show expression patterns broader than *Nkx2-1*, suggesting that either these factors function together to drive *Nkx2-1* expression in their shared domain, or that additional factors are involved. Whether

and how known signaling pathways may participate in regulating the expression or activity of these transcription factors are questions that require more investigation (Vincent et al., 2003). For the goal of elucidating the gene regulatory networks that govern the various stages of respiratory development, we can perhaps learn from prior successful studies whereby combining gene expression/perturbation analyses and identification of relevant cis-regulatory modules, researchers have successfully developed explanatory models for endomesoderm formation in the sea urchin embryo (Ben-Tabou de-Leon and Davidson, 2009, Peter and Davidson, 2010). Similar approaches, while likely more challenging in the complex mammalian respiratory system, would prove fruitful in advancing our understanding of lung/trachea development.

Two additional open questions are: how do cells alter their responses to the same signaling cues in periods as short as a few hours (Davidson et al., 2000, Serls et al., 2005, Wandzioch and Zaret, 2009); and how do cells maintain their plasticity to respond to inducing signals for a long period of time after organ establishment (Okubo and Hogan, 2004, Que et al., 2007)? These seemingly divergent behaviors may in fact be dictated by similar epigenetic regulation of the responsible transcriptome. Whether and how the known genetic network is integrated with potential epigenetic mechanisms is a fruitful direction to explore. Given its vital importance starting at first breath, reconstructing the formation and maintenance of a healthy respiratory system has been and will continue to be a rewarding theme of study.

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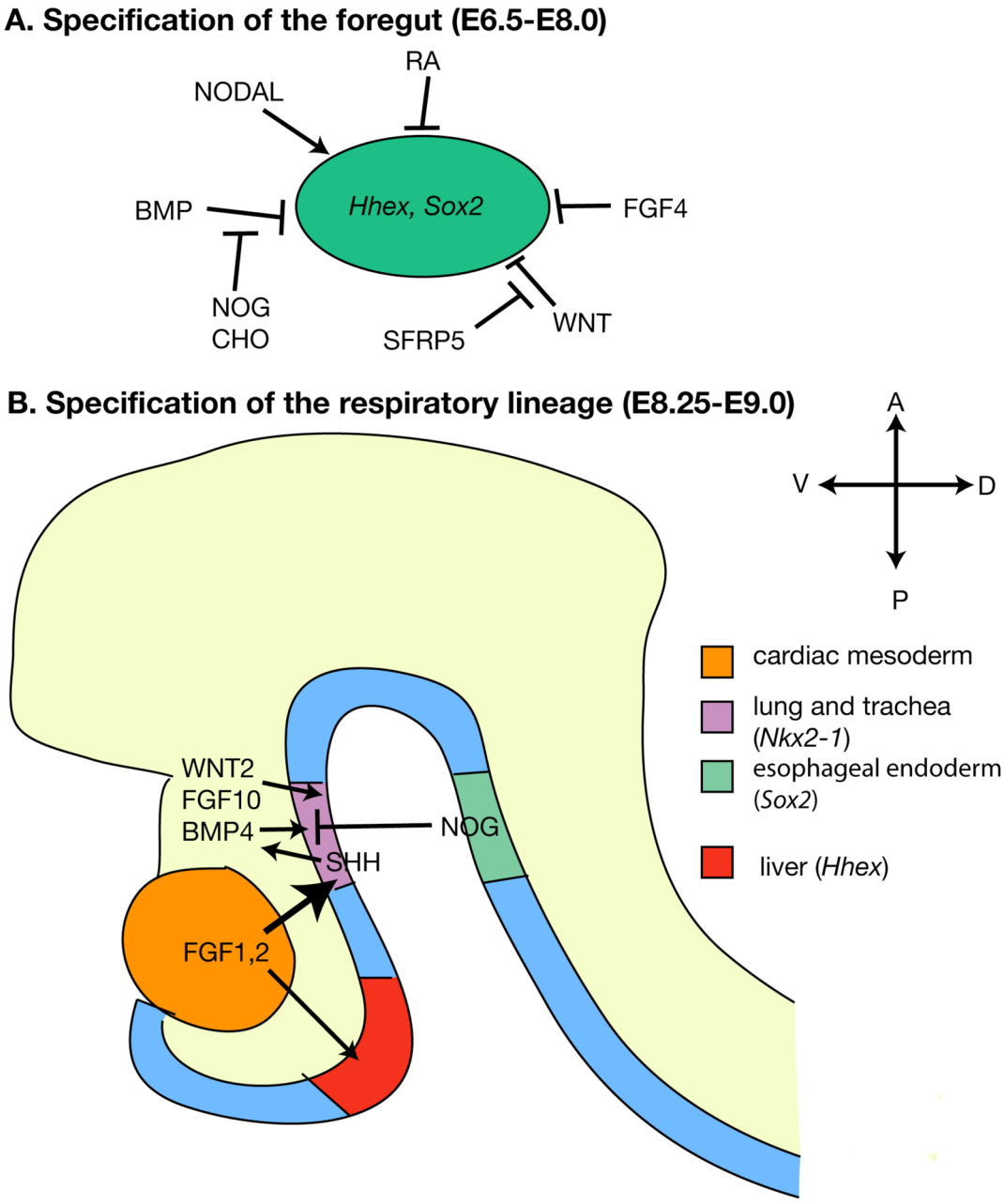

Figure 1. Signaling interactions during step-wise specification of the respiratory lineage

Schematic depictions of key secreted ligands and transcription factors involved in specifying the respiratory progenitors. (A) During gastrulation, signals secreted from the adjacent mesoderm and ectoderm specify the foregut endoderm, which expresses marker genes *Hhex* and *Sox2*. (B) As the gut tube folds, multiple signals interact to induce trachea and lung fate in the ventral endoderm. NOG = Noggin, CHO = Chordin

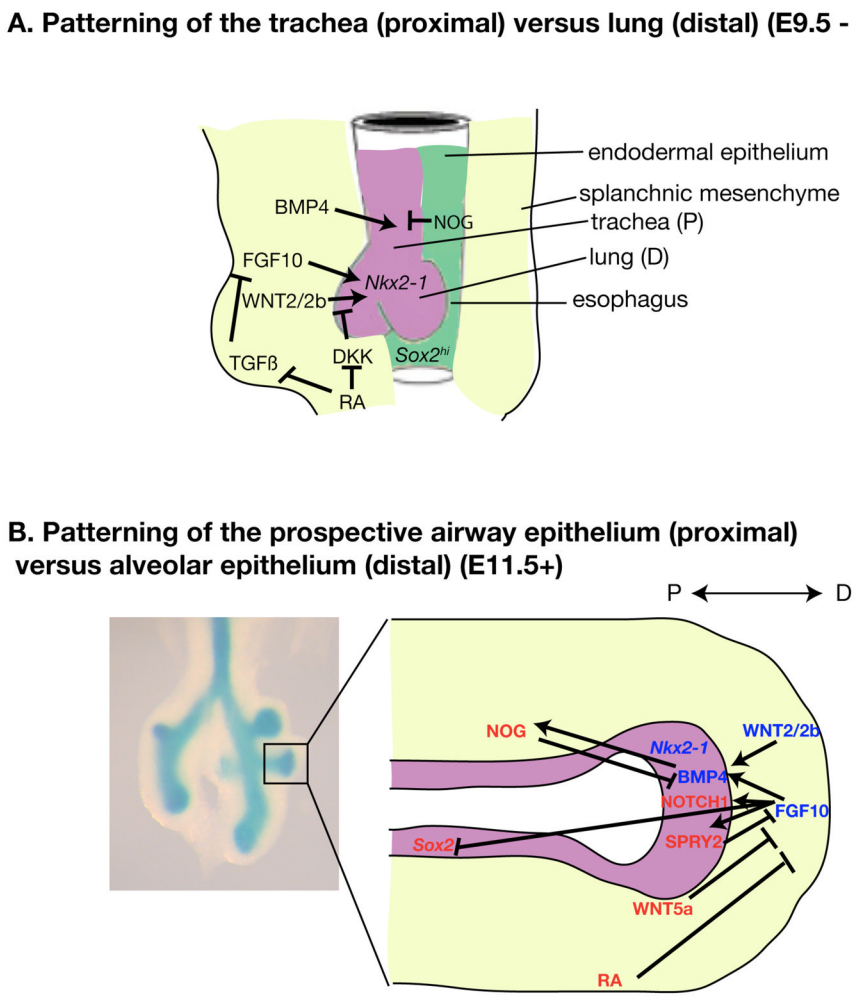

Figure 2. Signaling interactions during P-D patterning of the respiratory epithelium

Schematic depictions of key secreted ligands and transcription factors involved in patterning the developing respiratory system. (A) During lung budding, multiple ligands are secreted from epithelium and the surrounding mesenchyme to distinguish the trachea and nascent lung buds. (Modified from Que et al., 2006). (B) During lung branching morphogenesis, multiple secreted ligands and transcription factors interact to distinguish the airway epithelium versus alveolar epithelium. Left panel is a wholemount image of lung at E11.5. Boxed area is illustrated in the right panel, with the epithelium depicted in blue. Signals that promote distal fate are labeled in blue. Signals that promote proximal fate are labeled in red.

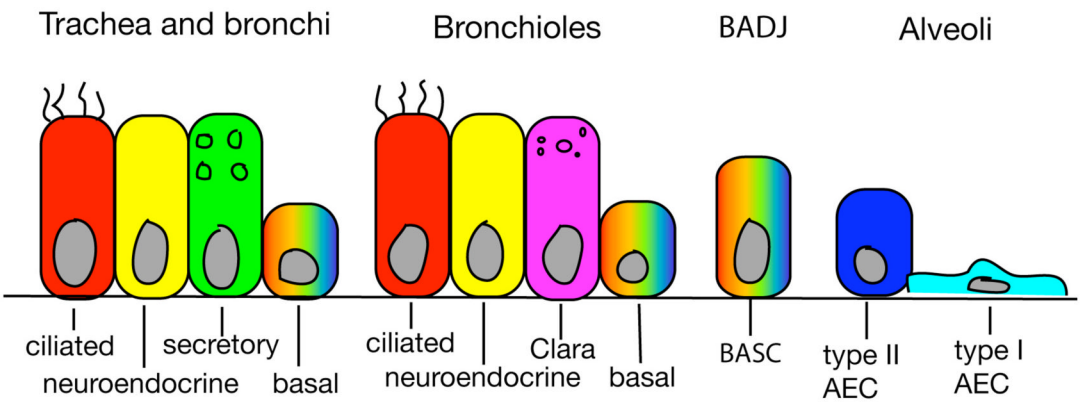

Figure 3. Differentiated cell types within the respiratory epithelium

Schematic depiction of relative locations of the various cell types in the mature lung. Notch signaling regulates the fate decision between secretory and non-secretory cell fates, while TGFβ specifically promotes the differentiation program of Clara cells. The location of putative multipotent stem cells (basal cells and BASCs) are indicated with rainbow shading. BADJ – bronchioalveolar duct junction. BASC – bronchioalveolar stem cell.