Understanding human lung development through *in vitro* model systems

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Abbreviations: BADJ, bronchoalveolar duct junction; **ECM,** extracellular matrix; **hPSC,** human pluripotent stem cell; **MYF,** myofibroblast; **scRNAseq,** single cell RNA sequencing

Abstract

An abundance of information about lung development in animal models exists; however, we know comparatively little about lung development in humans. Recent advances using primary human lung tissue combined with the use of human in vitro model systems, such as human pluripotent stem cell-derived tissue, has led to a growing understanding of the mechanisms governing human lung development. They have illuminated key differences between animal models and humans, underscoring the need for continued advancements in modeling human lung development and utilizing human tissue. This review discusses the use of human tissue and the use of human in vitro model systems that have been leveraged to better understand key regulators of human lung development and that have identified uniquely human features of development. This review also examines the implementation and challenges of human model systems and discusses how they could be applied to address gaps in our knowledge.

1. Introduction

The respiratory system is comprised of the trachea and airways of the lung, the branched network of epithelial tubes forming the bronchi and bronchioles, and the alveoli, where gas exchanges with the vascular system. Each of these structures are made of multiple specialized epithelial cell types that help carry out the lung's unique functions of air intake and gas exchange, epithelial barrier function, protection from microbes and pathogens, and

the maintenance of fluid and electrolyte homeostasis [1]. The diverse repertoire of respiratory epithelial cells that comprise the trachea, airways, and alveoli are derived from a common population of progenitor cells that are specified in the endodermal germ layer early during development [2–4]. In addition to the endoderm-derived epithelium, both the developing and mature respiratory systems contain cells derived from the mesoderm (e.g. smooth muscle) and ectoderm (e.g. neurons) germ layers, and the complex interactions between cells from all three germ layers are absolutely critical for proper respiratory system development and function [5–8].

Development of the respiratory system is broadly divided into five stages, each representing major morphological changes that take place [9] (Figs. 1a, 1b, 1c). The embryonic stage is defined by respiratory specification, the establishment of the nascent tracheal domain, and the emergence of two primary lung buds from the ventral anterior foregut endoderm (Fig. 1a). Following these events, the lung enters the pseudoglandular stage where lung buds undergo repeated rounds of bifurcations during a process called branching morphogenesis, which establishes the arborized network of bronchi and bronchioles [10] (Fig. 1b).The alveoli form across several stages, with alveolar cell-type specification beginning during branching morphogenesis and finalizing their differentiation in the terminal stages of lung development [11], which includes the canalicular stage where alveolar ducts form at terminal bronchioles, the saccular stage where alveolar cells functionally mature and alveolar sacs form, and the alveolar stage where alveoli continue to mature and increase their surface area through septation (Fig. 1c). The lung is one of the few organs that continues to develop in post-natal life as the alveoli continue to grow in size and complexity for seven years after birth in humans and one month after birth in mice.

The structure and function of the adult mouse and adult human respiratory systems have multiple differences, including anatomical differences such as the number of airway branches, the identity and localization of adult stem cells, and the morphology of alveoli [12] (Fig. 1d). These physiological differences likely contribute to the failure of most human clinical trials using lung therapeutics developed in mouse models [13]. Until recently, it has been difficult to assess the mechanistic differences that emerge during respiratory development that lead to differences in the mature lungs of mice and humans. However, contemporary research has addressed this issue by using primary human tissue and by developing *in vitro* model systems that mimic human respiratory development. Coupled with technological advances such as single cell RNA sequencing (scRNAseq) [14–17], these studies have shed light on many of the similarities and differences between mouse and human respiratory development. In this review, we will discuss our understanding of human lung development during each stage of respiratory development, focusing on signaling and transcriptional networks that regulate the developing human respiratory system. We will also discuss the current state of human model systems to accurately model human respiratory development and disease and highlight the challenges that remain.

2. The embryonic stage of respiratory development

2.1. Contribution of cellular signaling pathways to respiratory endoderm specification

The respiratory system is specified at E9.5 in mice and at 4 weeks of gestation in humans as the trachea and primary lung buds separate ventrally from the esophagus in the anterior foregut endoderm [18–20]. The respiratory system is first marked by the transcription factor

NKX2.1 [21,22], which is also necessary for lung specification [23]. Respiratory specification in mouse models has been reviewed extensively [24–28]. These studies have identified many of the signaling pathways that are essential during respiratory specification and have been used as a framework to differentiate human pluripotent stem cells (hPSCs) into respiratory lineages *in vitro*. This strategy, known as 'directed differentiation', is an attempt to recapitulate a series of developmental events in a stepwise manner by modifying the growth factor signaling environment in the tissue culture dish. This approach has allowed us to gain an appreciation of the signaling and transcriptional regulators that are necessary for respiratory specification in a human-specific context. The major developmental milestones for lung specification using directed differentiation include definitive endoderm differentiation [29,30], followed by anterior-posterior patterning into anterior foregut endoderm [31], at which point NKX2.1$^+$ respiratory progenitor cells can be specified [22,32–38].

Studies using directed differentiation from hPSCs as well as studies in animal models have stressed the importance of WNT signaling for initiating the expression of NKX2.1 from anterior foregut endoderm [37,39–41] (Figs. 2a, 2b). However, activation of WNT signaling that induces NKX2.1 expression requires cooperation from multiple other signaling pathways (Figs. 2a, 2b). The complex signaling network that induces the respiratory fate is dependent on retinoic acid (RA) signaling, which is required prior to respiratory specification and renders the ventral foregut endoderm competent to respond to cues that induce the respiratory lineage, although the mechanisms through which RA signaling acts are unknown [33,35,37,42]. It has been shown in mice that Sonic Hedgehog (SHH) ligands emanating from the endoderm induce the expression of WNT ligands in the mesoderm, which signal back to the endoderm to activate NKX2.1 expression [37,43,44]. NKX2.1$^+$ cells have been induced from hPSCs without the addition of SHH signaling components to the media; however, since SHH signaling acts upstream of WNT in mice, it is possible that directed differentiation strategies using hPSCs bypass the need for SHH components through the addition of exogenous WNT ligands. In mice and humans, BMP signaling represses SOX2 in the endoderm, which is required for the endoderm to properly respond to WNT ligands and express NKX2.1 [37,45]. Genetic gain- and loss-of-function studies in mice have also established a role for FGF signaling during respiratory specification [46–49]; however, like SHH, FGF has not played a prominent role in differentiation of hPSC into NKX2.1$^+$ respiratory progenitor cells, and its role in human respiratory specification remains unknown.

2.2. Signaling involved in self-organization of 3D lung models

Many directed differentiation protocols that induce anterior foregut endoderm lineages from hPSCs use 2D cultures; however, it is also possible to generate 3D anterior foregut endoderm structures, called spheroids, using directed differentiation techniques [36,50]. Spheroids are immature multicellular tissue structures that arise during directed differentiation through unknown mechanisms and which mimic a primitive gut tube-like structure. Spheroids provide an opportunity to direct the differentiation of hPSCs into lung cells with the correct cellular organization. The cues that are needed to pattern hPSCs into 3D lung spheroids seem to require a different set of signals compared to cells grown in 2D. For example, Dye *et al.* has shown it is possible to derive 3D ventral anterior foregut structures that can give rise to mature lung lineages by simultaneously inhibiting SMAD, which is required for anterior foregut patterning, and by activating FGF4, WNT, and SHH, which are required for both inducing 3D spheroid formation and robust NKX2.1 expression [36,51,52]. The necessity of FGF4, WNT, and SHH for the formation of 3D structures suggest

that these signaling pathways may be responsible for cell migration and patterning during respiratory fate specification in humans.

2.3. Different signaling pathways contribute to mouse and human respiratory mesoderm specification

In the mouse, respiratory mesoderm is *Nkx2.1*$^{-}$ but is marked by *Tbx4* and *Tbx5*, both of which are necessary for respiratory mesoderm development and specification of the lung and trachea [53]. A recent study from Kishimoto *et al*. showed that WNT signaling originating from the mouse endoderm induces *Tbx4* expression in the primitive lung mesoderm independent of *Nkx2.1* expression (Fig. 2a). Using mouse pluripotent stem cells and hPSCs, they showed that tracheal mesoderm (chondrocytes and proximal smooth muscle cells) could be specified from lateral plate mesoderm by BMP4 and WNT signaling in the mouse and SHH, BMP4, and WNT signaling in the human [54]. This demonstrates that the primary molecular mechanisms responsible for tracheal mesoderm specification are different between the mouse and human as mouse tracheal mesoderm specification does not require SHH. In another study, *Wnt2*$^{+}$/*Gli1*$^{+}$/*Isl1*$^{+}$ mesodermal cells that arise prior to respiratory specification were shown to give rise to some lung and cardiac mesodermal lineages [55]. SHH signaling regulates specification of these “cardiopulmonary progenitors” into smooth muscle lineages in the lung [55]; however, the mechanisms regulating cardiopulmonary progenitor specification into other distal mesenchymal cell types are currently unknown [56].

3. The pseudoglandular stage of respiratory development

The pseudoglandular stage occurs between E10.5 to E16.5 in mice and 5 to 17 weeks of gestation in humans. This stage is defined by branching morphogenesis, where progenitor-rich lung bud tips begin to undergo repeated bifurcations to create the complex arborized network of the airways [2,8,10,12,25,57–59] (Fig. 1b). Humans undergo extended rounds of branching relative to mice (17-21 in humans, 7-17 in mice) [10,60], raising the possibility of regulatory divergence in human branching morphogenesis. Complex reciprocal signaling between the epithelium and mesenchyme during this stage creates a unique hurdle in characterizing the signaling pathways important for branching. Other changes in the lung during pseudoglandular development include the emergence of smooth muscle and vasculature, which both contribute to the environment that influences branching morphogenesis. Here, we discuss the emergence of lung cell types during branching morphogenesis, their role in establishing the lung microenvironment, and how these environments dictate local signaling.

3.1. Cellular differentiation during branching morphogenesis

A significant event during the pseudoglandular stage is the specification of airway cell types in the lung epithelium. As branching tips of the epithelium continue to grow and bifurcate, bud tip progenitors leave progeny behind, which differentiate into airway cell types including basal, ciliated, secretory, and neuroepithelial cells (Fig. 3b). Lineage tracing in mice suggest there is a specific developmental window where bud tip progenitors preferentially give rise to airway cell types [61,62]. Until recently, there was limited knowledge about how these processes differ in humans. Several groups performed scRNAseq on human fetal lung samples [3,4,63], and these studies established important *in vivo* benchmarks of cellular transcriptional states that can be directly compared with *in vitro*-derived cells, providing a roadmap for developing directed differentiation approaches to generate specific airway cell types. For example, methods to direct the differentiation of bud tip progenitors to TP63^{+} basal cells have been developed by manipulating SMAD signaling [63,64] (Fig. 3b). Alternatively, inhibition of Notch signaling directs hPSC-derived lung epithelium to differentiate into ciliated and neuroendocrine cells [65] (Fig. 3b). There remains debate over the role of WNT on bud tip progenitor fate [66,67]. Some groups conclude that high WNT

signaling supports a proximal airway cell fate and other groups conclude it supports alveolar cell types. As organoid models continue to improve, coupled with single cell studies, it is likely that more questions can be answered about cell lineage specification in the human airway.

3.2. Regulation of bud tips during branching morphogenesis

Branching morphogenesis is a complex morphological process that relies on highly proliferative progenitor-rich distal bud tips of the developing lung. RNAseq data on bud tips found differences in both gene and protein expression between human and mouse [3,51,52,61]. In mice, bud tip progenitors express *N-myc*, *Id2*, and *Sox9* but are *Sox2* negative [61,68–70]. This contrasts with humans where bud tip progenitors express *SOX9* in addition to *SOX2* [3,4,71] (Figs. 2c, 3d). Loss of *SOX2* does not occur in human bud tips until the canalicular stage. In cultured human lung explants where RAC1 inhibition causes decreased $SOX9^+$/$SOX2^+$ bud tip progenitors, there is also decreased epithelial proliferation and impaired branching [71]. As *Sox2* has been shown to be essential for airway cell fates in mice [72], longer perdurance of *SOX2* expression in human bud tip progenitors may suggest that human bud tips retain the potential to differentiate into airway cell fates much later into development than in mice.

Studies of branching morphogenesis in the mouse have elucidated important mechanisms that regulate this process, which are reviewed extensively elsewhere [26,73–75]. Here, we focus on comparing the signaling regulation of murine and human bud tips during branching. A thoroughly investigated signaling pathway in branching is FGF10, which is expressed in the mesenchyme near the most distal bud tips and is critical for branching and proximal-distal patterning in mice [76,77] (Fig. 3a). *Fgf10*$^{-/-}$ mice do not undergo branching and conditional knock-outs of *Fgf10* or *Fgfr2* also disrupt lobe growth and have fewer branches [47,48,78]. In the developing human lung, *FGF10* is expressed from 10-21 weeks [76,77,79] diffusely throughout the lung parenchyma [76,80]. Murine lung explants cultured with FGF10 show increased branching, while in contrast, human lung explants cultured with FGF10 exhibit enlarged buds and fewer branches [76,77]. Human lung organoid models suggest that FGF10 is not required for bud tip maintenance [3,4], though long term culture in FGF10-rich media results in differentiation of airway cell types [36]. Another important signaling pathway during branching is WNT (Fig. 3a). In mice, loss of both *Wnt2* and *Wnt2b* results in complete lung agenesis [39], and conditional epithelial knock-out of *β-catenin* results in malformed distal airways with aberrant proximal airways [81]. Similarly, RAC1-mediated WNT inhibition in human lung explants decreases branching and results in loss of bud tip progenitors, although the molecular mechanisms remain to be investigated [70,71]. It was recently discovered that humans with mutations in the WNT activator *R-spondin 2* (*RSPO2*) exhibit lung agenesis [41], which is a surprising contrast to murine lung, where *Rspo2* mutants have mild branching defects [82]. The continued use and advancement of human *in vitro* models are required to fully appreciate the molecular mechanisms of FGF and WNT signaling in human lung branching morphogenesis.

3.3. Molecular and mechanical cues from the mesenchyme during branching morphogenesis

The mesenchyme undergoes significant morphological changes as the branching epithelium continues to bifurcate and alter the landscape of the lung. We are only beginning to understand the diversity of mesenchymal cell types and changes they undergo during human lung development [83]. Therefore, i*n vitro* human models of lung mesenchyme are less developed compared to epithelial models. Engineering approaches using microfluidic chambers with mouse lung explants as well as *in silico* modeling have begun to examine the changes that occur during branching morphogenesis and show promising innovation for human models [84,85]. In mice, it has been shown that both *Fgf10*$^+$ mesenchymal cells and *Pdgfra*$^+$ mesenchymal cells give rise to airway smooth muscle [86], the latter through WNT2 and WNT7b signaling [87]. Blocking smooth muscle differentiation also prevents epithelial buds from bifurcating [88], and it was recently shown that smooth muscle differentiation defines specific domains along the airways that propagate branches in mice [89]. In humans, α-SMA^+ smooth muscle cells support the proximal fate of the human airway and branching

[71], although the signaling mechanisms involved are unknown (Fig. 3a). More *in vitro* models using organoid and explant-like cultures will be required for understanding the signaling changes in the mesenchyme during human lung development. Single cell analysis will continue to help shed light on the complexity of the mesenchyme and identify key signaling factors involved in the morphing landscape of the lung.

4. The canalicular stage of respiratory development

During the canalicular stage of respiratory development, the lung transitions from generating airway (bronchi, bronchioles) to generating the gas-exchange units of the lung, the alveoli. This is characterized by the continued differentiation of bud tip progenitor cells towards alveolar fates [3,4,61,90–94] and by the formation of the bronchoalveolar duct junction (BADJ) in mice [95,96], which demarcates airway-fated epithelial cells from alveolar-fated epithelial cells and can be identified in mice by the terminal border of *Sox2* expression [14,97,98]. The existence of a BADJ in humans has not been demonstrated, but both human and mouse bud tips lack expression of the airway cell fate marker SOX2 prior to generating alveolar cells [3,4,71] (Fig. 4a).

4.1. Alveolar cell specification during the canalicular stage

Classic models of alveolar development proposed that alveolar cell types are specified in a sequential manner, with bud tip progenitors giving rise to alveolar progenitors, which give rise to alveolar type II (ATII) cells, which give rise to alveolar type 1 (ATI) cells [99]. More recent studies of alveolar cell specification at the single cell level have proposed an alternative model that ATI and ATII cells are specified from a bipotent alveolar progenitor [14,92], whose existence in mice was recently proved with lineage tracing strategies [94]. However, this latter study suggested that bipotent progenitors are rare and most likely remnant undifferentiated cells that remain at the end of branching morphogenesis [94]. It was further demonstrated that commitment to alveolar fates occurs much earlier than previously appreciated, taking place concurrently with branching morphogenesis, rather than afterwards. The majority of mature alveolar cells are the progeny of unipotent alveolar progenitor cells fated towards either an ATI or ATII cell early in development, with ATII cells being specified first at the most distal tip of the lung and ATI cells being specified just after ATII cells [94]. Interestingly, in humans, markers of ATI and ATII cell fate aren't detected before 16 weeks of gestation (canalicular stage) and are not robust even at 20 weeks of gestation. It is likely that epithelial bud tip progenitors choose their eventual alveolar cell fate at the molecular level days before becoming morphologically and functionally distinct in mice, but alveolar specification may occur much later in humans. This data also poses a question about whether or not an alveolar progenitor cell state even exists; it is possible that ATI and ATII cells are directly specified from multipotent bud tip progenitor cells, obfuscating the timing of when it is appropriate to term a bud tip progenitor an alveolar progenitor.

In mice and humans, both paracrine signals from the mesenchyme and endocrine signals involving glucocorticoids appear to direct bud tip progenitor cells to give rise to alveolar cells [90,100–104]. Premature human infants are frequently given glucocorticoids in order to speed the maturation of ATII cells such that they begin producing surfactant to have functional lungs [105–109]. Although glucocorticoids are used to mature already specified alveolar cells in the human, studies using mice suggest that endocrine glucocorticoid signaling drives the formation of the BADJ. Interestingly, manipulation of glucocorticoid signal timing or strength alters the size of the future alveolar compartment of the lung without disrupting the appearance of mature alveolar cell types [93,96], suggesting that glucocorticoid signaling acts

to restrict the developmental potential of bud tip progenitor cells away from airway fates without being required for alveolar differentiation. Laresgoiti *et al.* showed that glucocorticoid signaling may interact with inflammatory pathways via STAT3 to initiate the switch from bud tip progenitors giving rise to airway cell types to alveolar cell types in the mouse [93]. Beyond this data, the signaling mechanisms that glucocorticoid signaling works through to propel alveolar formation and maturation is unknown, and given the clinical applications of glucocorticoid signaling in the developing human lung, understanding the precise role of glucocorticoid signaling in alveolar specification could have drastic impacts on preventing chronic respiratory disease in premature infants.

Signals originating locally from the mesenchyme are also involved in regulating the differentiation of bud tip progenitor cells into alveolar cells. Mesenchyme surrounding the bud tips in both humans and mice secrete FGF ligands [46,76,110]. In mice, it has been shown that mesenchyme-derived FGF acts on the epithelium through KRAS to maintain the progenitor state of bud tips [91,96,111]. Secretion of FGF from the mesenchyme is promoted by WNT ligands, which are thought to partially originate from the epithelium, creating a positive feedback loop [39,112–115]. HIPPO signaling terminates branching morphogenesis and promotes alveolar differentiation through degradation of *β*-catenin in the epithelium, disrupting the WNT-FGF feedback loop and directing bud tip progenitors to differentiate [115,116] (Fig. 4a). Active FGF and WNT signaling are known to be important for maintaining bud tip progenitor identity in the human as well [3,4,71,76]; therefore, these pathways may perform analogous roles in maintaining the progenitor state of human canalicular stage bud tips. It is important to note that the specific FGF ligand(s) involved in human bud tip progenitor maintenance are likely different than those in mice [76]. A role for HIPPO signaling in the human lung has not been examined.

4.2. Mesenchyme development during the canalicular stage

While the diversity of mesenchymal cell types in the developing lung is still being uncovered [17], two distinct mesenchymal populations in mice have been defined to undergo significant changes during the canalicular stage: *Fgf10*$^+$ mesenchymal cells and *Pdgfra*$^+$ mesenchymal cells. Importantly, the appearance of lipofibroblasts in the human lung has not been confirmed [117]. However, in mice, *Fgf10*$^+$ mesenchymal cells give rise to lipofibroblasts (LIFs), which are lipid droplet-containing mesenchymal cells that have a regulatory role during alveolar development [79]. Unlike during the pseudglandular stage when *Fgf10*$^+$ mesenchymal cells give rise to myofibroblasts (MYFs), smooth muscle cells, and LIFs in the distal lung, the large majority of *Fgf10*$^+$ cells give rise to LIFs (and other unknown mesenchymal cell types) but not MYFs during the canalicular and later stages of development [118]. Likewise, it was found that the majority of *Pdgfra*$^+$ mesenchymal cells give rise to MYFs, which lay down much of the ECM important for alveolar formation and function [119–121], during the canalicular and later stages of development [121]. TGF*β* signaling negatively regulates FGF10 signaling in the mesenchyme to control the differentiation of mesenchymal progenitor cells to MYFs versus LIFs such that higher FGF10 signaling favors LIF identify and lower FGF10 signaling favors MYF identity [79,122,123].

5. Saccular and alveolar stages of respiratory development

Sacculation and alveologenesis are the terminal stages of lung development, beginning late during development and completing sometime during the first decade of life. The saccular

stage culminates with the formation of primitive alveoli called saccules. Saccules are further divided during alveologenesis through a process called septation that maximizes the area available for gas-exchange. Similar to previous stages of lung development, formation of saccules (sacculation) and their maturation into alveoli is driven by changes occurring in both the epithelium and mesenchyme. In the epithelium, ATI cells transition from a cuboidal to a squamous morphology and then stretch to 10x their original size to form the majority of the surface area within the alveolar epithelium [124] (Fig. 4b). ATII cells become highly proliferative and build specialized organelles dedicated to surfactant production called lamellar bodies (Fig. 4b). Meanwhile, new cell types appear in the alveolar mesenchyme that secrete extracellular matrix (ECM) and further remodel it, thus contributing to development of saccules and setting the stage for further septation during alveologenesis. Importantly, defects in these late stages of lung development in model organisms mimic features of bronchopulmonary dysplasia [125–129], a disease prevalent in premature births that leads to chronic respiratory difficulty throughout life. Thus, the mechanisms that ensure proper sacculation and alveologenesis are of great importance for developing interventions that will rescue lung function in the neonatal ward.

5.1. Sacculation and alveologenesis – signaling active in the epithelium

Sacculation occurs relatively late in human gestation (3rd trimester), making access to human lung tissue at this stage rare. To circumvent this limitation, several groups have developed methods to differentiate hPSCs into progenitors of the lung epithelium [33,34,36,52,63,66], which can then give rise to alveolar cell types, partially recapitulating the development of the lung epithelium during sacculation and alveologenesis. Methods also exist to generate more purified populations of alveolar cells called alveolospheres [22,34,101,102,130], which have already proven useful for modeling congenital disease of the alveoli [131,132] and alveolar injury [133]. These methods, although state-of-the-art, provide an incomplete picture of sacculation and alveolar development in humans because they either lack mesenchyme [63,66,101], require exogenous mesenchyme for alveolar differentiation [34,102], generate immature alveolar cells stochastically [33,36,52,63,66], or give rise to ATII cells only [101,102]. Furthermore, although the methods mentioned above generate alveolar cells, it is unknown whether these cells pass through intermediate states that represent the true signaling, timing, and cell fate trajectories that occur *in vivo*. Never-the-less, establishment of these *in vitro* models has provided insights into cues necessary for alveolar cell specification and maturation in humans.

Methods to generate alveolospheres generally follow directed differentiation paradigms in order to induce lung progenitors from anterior foregut endoderm progenitors, followed by purification of putative alveolar progenitors. Alveolar progenitors are placed into various media types, but common to many protocols is the stimulation of cyclic AMP as well as the WNT, FGF, and glucocorticoid signaling pathways [34,101,102]. These studies suggest that WNT, FGF, glucocorticoid, and cAMP-signaling pathways act to specify and/or mature alveolar cells in humans (Fig. 4c).

A role for WNT in ATII cell specification and/or maturation is consistent with insights from animal models. During late sacculation and early alveologenesis, ATII cells exhibit an increase in WNT signaling activity that correlates with an expansion in ATII cell number [11],

and constitutive WNT increases ATII cell number while loss of *β-catenin* during sacculation reduces ATII cell number and leads to an increase in ATI cell number [11]. Together, this suggests that WNT signaling promotes the proliferation of ATII cells and may regulate the identity of alveolar progenitor progeny. The role of FGF and cAMP-signaling in alveolar cell fate specification in animal models has yet to be elucidated.

Studies from animal models also suggest a key role for the HIPPO-signaling pathway in promoting ATI cell fate. HIPPO-signaling pathway mouse mutants exhibit defects in saccular architecture that phenocopy aspects of human emphysema and bronchopulmonary dysplasia [127,128,134]. Of note, mutations in the HIPPO-signaling pathway leading to overactive TAZ activity generate lung epithelium with precocious and ectopic expression of markers of ATI cell identity [135], suggesting that defects in the saccular architecture reflect a specific role for TAZ in promoting ATI cell fate. Intriguingly, physical association between the lung epithelial transcription factor NKX2.1 and TAZ has been demonstrated [136], and more recently, NKX2.1 was demonstrated to perform a role in ATI cell specification distinct from its earlier role in specification of lung epithelium [137], suggesting that NKX2.1 and TAZ may partner to drive development of ATI cells. Despite the important role of HIPPO signaling in lung epithelial progenitor specification (see canalicular section) and the development of ATI cells in mice, the dynamics of HIPPO signaling in human models of lung development are not yet known.

5.2. Sacculation and alveologenesis – contributions from the mesenchyme

Three major populations of alveolar fibroblasts have been defined that guide the development of alveolar epithelium through sacculation during the formation of mature alveoli. Myofibroblasts (MYFs), expressing α-SMA, localize to developing septal tips where they remodel existing networks of elastin, which is necessary for proper formation of alveoli and provides elasticity for the lung during respiration [119,129,138–140]. Cues for remodeling the lung ECM may be primarily physical, as stretching induces the activity of elastase [88]. MYFs are also thought to play a key role in driving secondary septation during alveologenesis [119,138,141]. Similar to MYFs, matrix fibroblasts are intimately associated with the saccule during its development and are distinguished from MYFs by high levels of PDGF-signaling activity and high levels of WNT5a production [120,142]. Matrix fibroblasts secret collagen and other ECM components [142] that are essential for sacculation and alveolar maturation [143–147]. In contrast to MYFs and matrix fibroblasts, which are thought to play more structural roles in sacculation and alveolar maturation, lipofibroblasts (LIFs) are thought to guide development and maturation of ATII cells through trafficking of lipids to ATII cells to assist in production of surfactant [148]. Interestingly, development of LIFs is dependent on signaling from ATII cells, which secrete PTHRP to antagonize Hedgehog and WNT signaling in LIF progenitors, which in turn leads to PPARγ mediated transcription of Leptin and ADRP [149–151], molecules that induce surfactant production in ATII cells [150,152], thus linking the co-maturation of ATII cells and LIFs.

hPSC-derived models of human lung development highlight the important contribution of mesenchyme to human sacculation and alveologenesis. For instance, hPSC-derived alveolospheres normally contain only ATII cells [101] but will give rise to cells with features of ATI cells when co-cultured with fetal lung fibroblasts (Fig. 4c) [34,102]. Likewise, fetal-derived lung bud tip progenitors cultured *in vitro* readily differentiate into airway cell types but require co-culture with fetal lung mesenchyme for alveolar cell fate specification to occur [3].

Together, these studies suggest that human fetal lung mesenchyme provides cues that induce alveolar cell fates in human lung epithelium. Mesenchyme-derived cues for alveolar cell fate specification are likely partially ECM-derived, as decellularized lung ECM supports the development of multiple alveolar cell types in hPSC-derived lung epithelium [153,154]. Notably, many hPSC-derived alveolospheres are grown in hydrogels that don't necessarily recapitulate the properties of the lung ECM during alveolar development. How ECM instructs alveolar differentiation is not known, but given the mechanosensitivity of the HIPPO signaling pathway, and the evidence for a central role of HIPPO signaling in ATI cell specification and maturation [116,128,134,135,155], it is tempting to speculate that an ECM to HIPPO signaling axis guides the development of ATI cells *in vivo*. A greater understanding of the roles of mesenchyme during sacculation and alveologenesis will be essential to recapitulate cues that instruct hPSC-derived lung epithelium to specify alveolar cells.

6. Conclusions and future directions

We can never fully understand the unique aspects of human respiratory development without the use of *in vitro* model systems. In order to continue answering unknown questions in human respiratory development and properly model disease and genetic defects, several challenges must be overcome. For example, most *in vitro* human lung model systems are still overly simplistic, where the epithelium is cultured alone and relies on the addition of signaling components to media, or where epithelium is co-cultured with poorly characterized mesenchymal cells that organize in an unclear way. Neither of these approaches meticulously recapitulate an *in vivo* environment, and it would be invaluable to develop model systems where the mesenchyme and epithelium are cultured together in the correct organization. It is also important to note that these systems often lack a functional vasculature and a nervous system, although efforts to improve complexity have been reported recently [156,157]. Access to developing human tissues as well as the advancement of technologies such as scRNAseq coupled with *in situ* hybridization and immunofluorescence have begun to provide temporal and spatial gene expression patterns and have laid a strong foundation for the description of cell types, cell type-associated gene expression signatures, transcription factors, and signaling pathway components [14–16,63]. Translation of genetic manipulation techniques such as CRISPR to *in vitro* human model systems is evolving and will be instrumental to the functional understanding of signaling pathways during human lung development [158]. In addition to molecular mechanisms and cellular functions guiding lung development, it is also appreciated that mechanical cues play important roles in lung development and function; thus, establishing complex *in vitro* human model systems that incorporate and/or mimic aspects cellular, signaling, and biomechanical cues important for human lung development remains a critical obstacle. Current challenges include incorporating mechanical forces that occur during lung development, such as local forces involved in branching morphogenesis, peristaltic contractions observed in the developing lung, blood sheer stress, transmural pressure, and surface tension [84,85,88,89]. It is likely that lung-on-chip [159] and microfluidic technologies [85] will serve as useful tools to understand the influence of mechanical forces on human lung development. As all of these technologies continue to be integrated into *in vitro* human model systems of respiratory development, we will better appreciate the mechanisms conserved among species as well as the uniqueness of human biology.

In vitro models of human lung development will likely also play an important role in personalized medicine. With the ability to use cultured primary patient tissue or generate

patient-derived induced pluripotent stem cells to generate human *in vitro* models, we have the capability to model human lung disease and perform large-scale screens for patient-specific reactions to toxins, new drugs, and therapies. This could serve as a powerful tool for diseases such as cystic fibrosis and COPD, where current therapies are often ineffective or can be extremely costly; personalized screens could save months of trial-and-error with various medications to determine the optimal drug regime for a patient [160,161]. As chronic lung disease is a major cause of death worldwide [160], the need for new therapies and better treatments is critical, and *in vitro* model systems of the human lung will provide a high-throughput opportunity to develop personalized treatments for lung diseases.

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Respiratory Specification	Branching Morphogenesis

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The role of **SHH and FGF** signaling in NKX2.1^{+} lung progenitor cell specification	The molecular mechanisms of **FGF and WNT** in branching morphogenesis
The signaling required for respiratory cell **specification vs. organization**	Signaling mechanisms for **mesenchymal cell maintenance** and differentiation
Mechanisms involved in **distal pulmonary mesenchymal cell specification** during the embryonic stage	The role **physical pressures** (i.e. thoracic cavity) have on branching morphogenesis
Alveolar Cell Fate Specification and Maturation	
The **timing** of alveolar cell specification	
Signaling pathways **regulating ATI vs ATII cell fate choice**	
The role of **glucocorticoid signaling** in alveolar cell fate specificiation/maturation	
The role of **mesenchyme-epithelial cross-talk** in alveolar cell fate specification/maturation	

Table 1. Summary of the major unknowns in human lung development.

Human *in vitro* model systems have been instrumental to understand specific features of lung development that are unique to humans. In this review, we explore how these models have been used to interrogate human lung development, unlocking the potential to answer unknown questions in human biology and physiology.

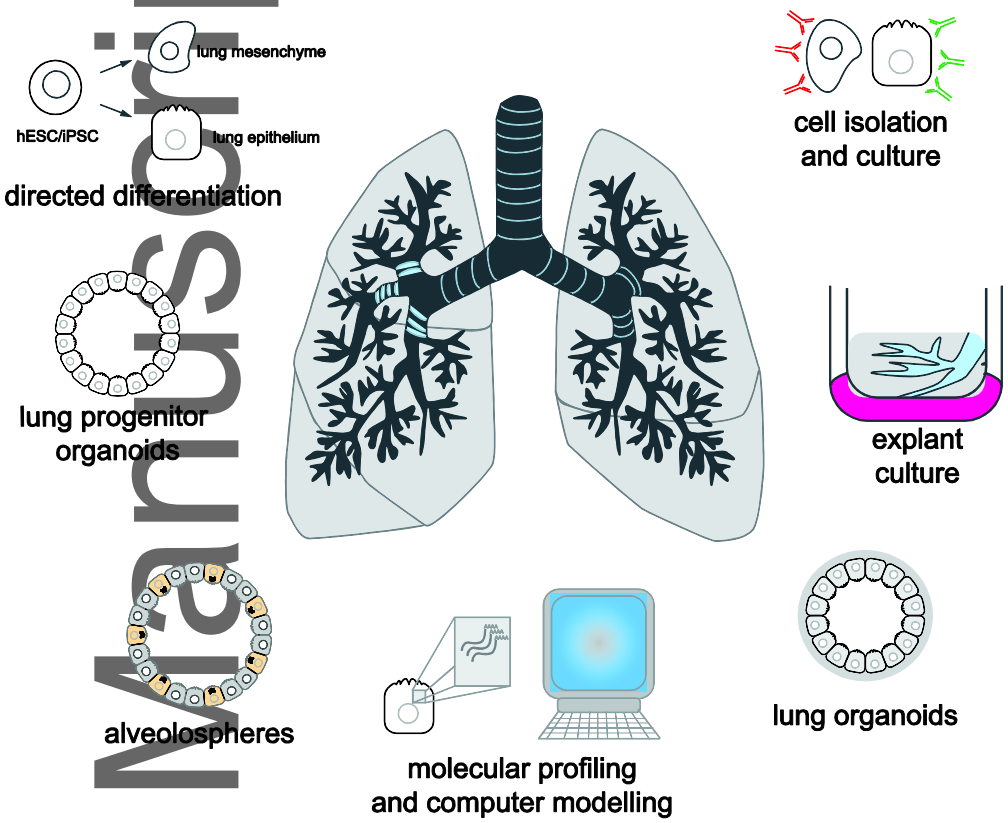
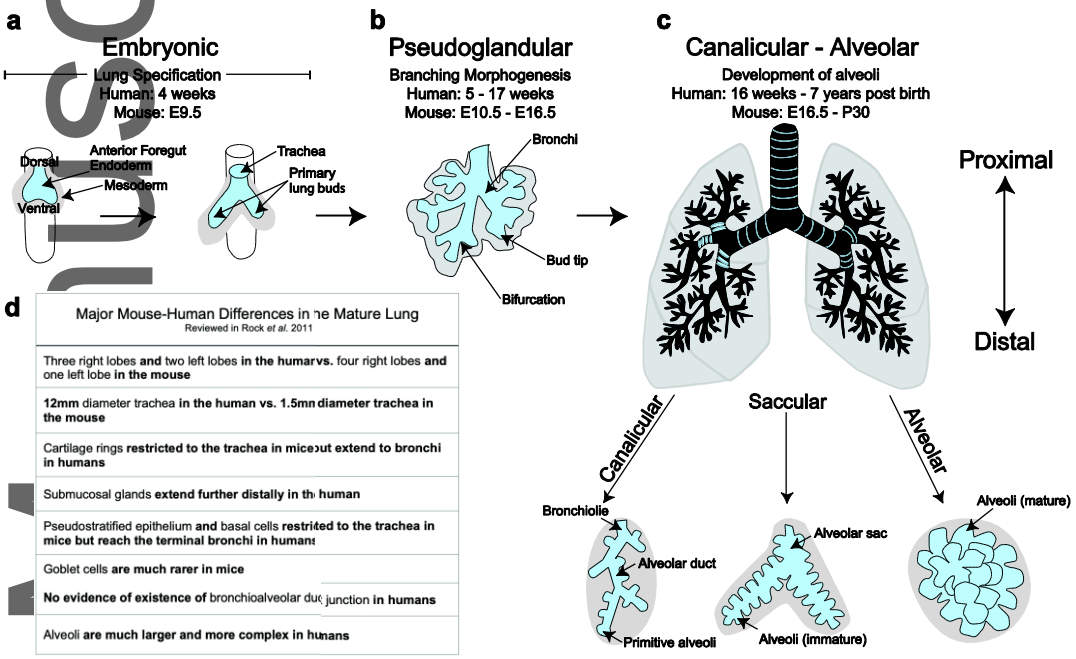


Figure 1. Morphology of the respiratory system during the five stages of respiratory development. a) During the embryonic stage, the lung arises ventrally from the anterior foregut endoderm, giving rise to two primary lung buds that branch off from the trachea into the surrounding mesoderm. b) The pseudoglandular stage is characterized by the processes of branching morphogenesis, whereby distal bud tips undergo repeated rounds of bifurcations to create the arborized network of airways. c) The alveoli, the air sacs that allow for gas exchange, are formed during the canalicular, saccular, and alveolar stages. This occurs as alveolar ducts form at the most distal airways, the bronchioles, which then form terminal sacs that will give rise to functional alveoli. d) The adult mouse and adult human lungs contain many morphological differences.



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Major Mouse-Human Differences in the Mature Lung Reviewed in Rock *et al.* 2011
Three right lobes **and** two left lobes **in the human vs.** four right lobes **and** one left lobe **in the mouse**
12mm diameter trachea **in the human vs. 1.5mm diameter trachea in the mouse**
Cartilage rings **restricted to the trachea in mice but extend to bronchi in humans**
Submucosal glands **extend further distally in the human**
Pseudostratified epithelium **and** basal cells **restricted to the trachea in mice but reach the terminal bronchi in humans**
Goblet cells **are much rarer in mice**
No evidence of existence of bronchioalveolar duct junction **in humans**
Alveoli **are much larger and more complex in humans**

Figure 2. Signaling mechanisms required for respiratory specification in vivo (a) and in vitro (b). TGFβ/SMAD signaling drives definitive endoderm specification, and SMAD inhibition through Noggin drives anteriorization of definitive endoderm. BMP4 from the mesoderm inhibits SOX2 expression in the mesoderm while SHH from the endoderm activates WNT ligands in the mesoderm that turn on NKX2.1 expression. RA is required for this process. WNT, BMP4, and SHH (humans only) from the endoderm specify the tracheal mesoderm, which is marked by TBX4. FGF10 is required for lung bud outgrowth. c) The mouse respiratory epithelium is initially made of SOX9+ bud tip progenitor cells, which become restricted to the budded tips of the lung as the primary lung buds grow out from the trachea. The bud tip progenitors that are left behind proximally become SOX2+. d) The human respiratory epithelium is initially made of SOX2+/SOX9+ bud tip progenitor cells, which become restricted to the budded tips of the lung as the primary lung buds grow out from the trachea. The bud tip progenitors that are left behind proximally lose SOX9 expression but remain SOX2+.

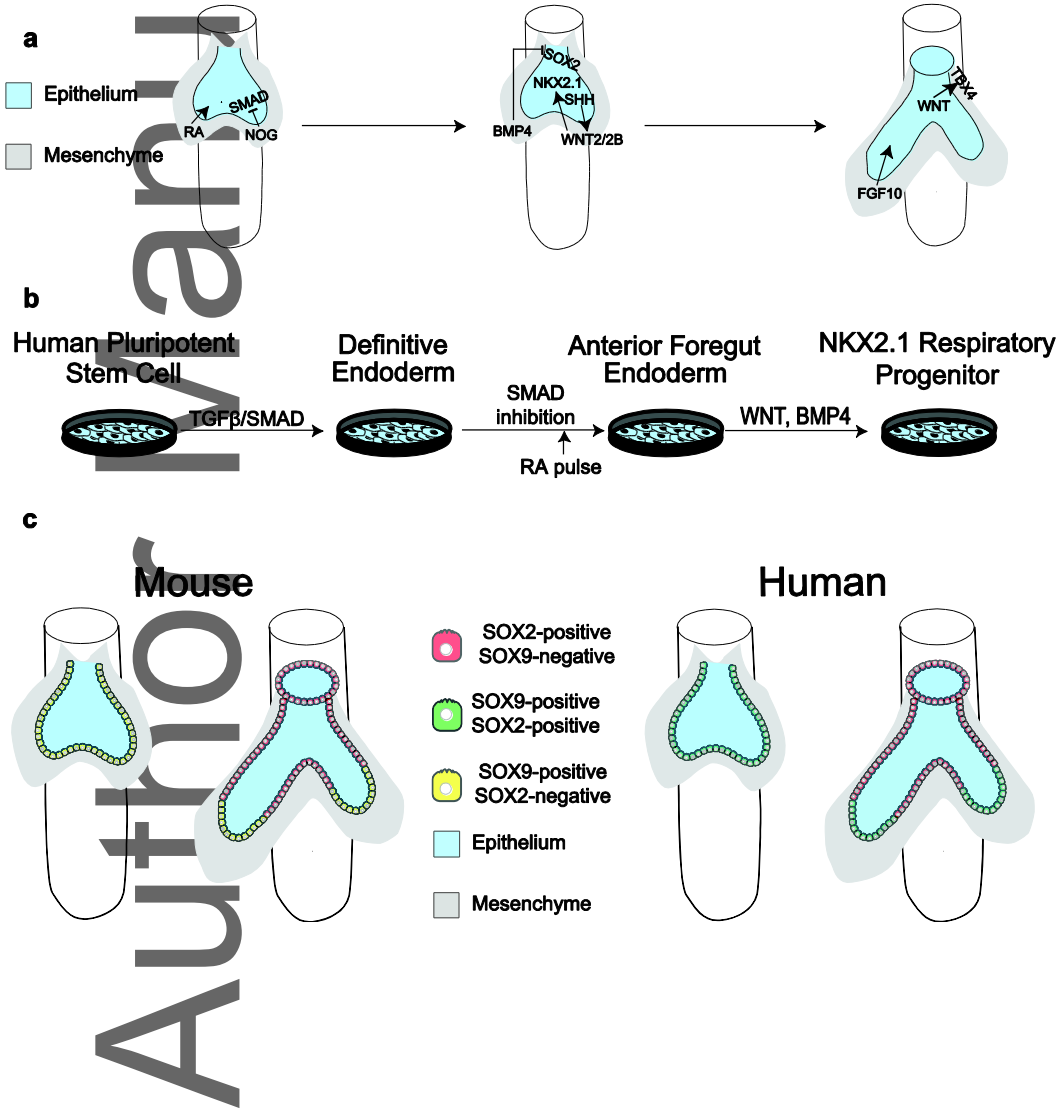

Figure 3. Signaling and cell types of branching morphogenesis. (a) FGF10 signaling from the mesenchyme interacts with FGFR2 receptors on epithelium. WNT signaling from the mesenchyme also supports branching of the epithelium. An important physical cue for branching is the smooth muscle (pink). (b) The signaling pathways important for airway cell differentiation include SMAD signaling from bud tip progenitors to TP63+ basal cells. Terminal differentiation into club/secretory cells is facilitated by active Notch signaling, and inhibition of Notch gives rise to multiciliated cells. Neuroendocrine cells also form from an epithelial progenitor through Notch inhibition, although it is less clear if they are specified directly from bud tip progenitors. (c) In vitro directed differentiation approaches have enabled expansion of bud tip progenitors as well as their differentiation into airway cell types using mechanisms that mimic in vivo signaling. (d) Organization of SOX2+ cells and SOX9+ cells vary between mice (left) and humans (right) where SOX2+ cells are limited to proximal airway cells, but bud tip progenitors are SOX9+ in mice and SOX2+/SOX9+ in humans.

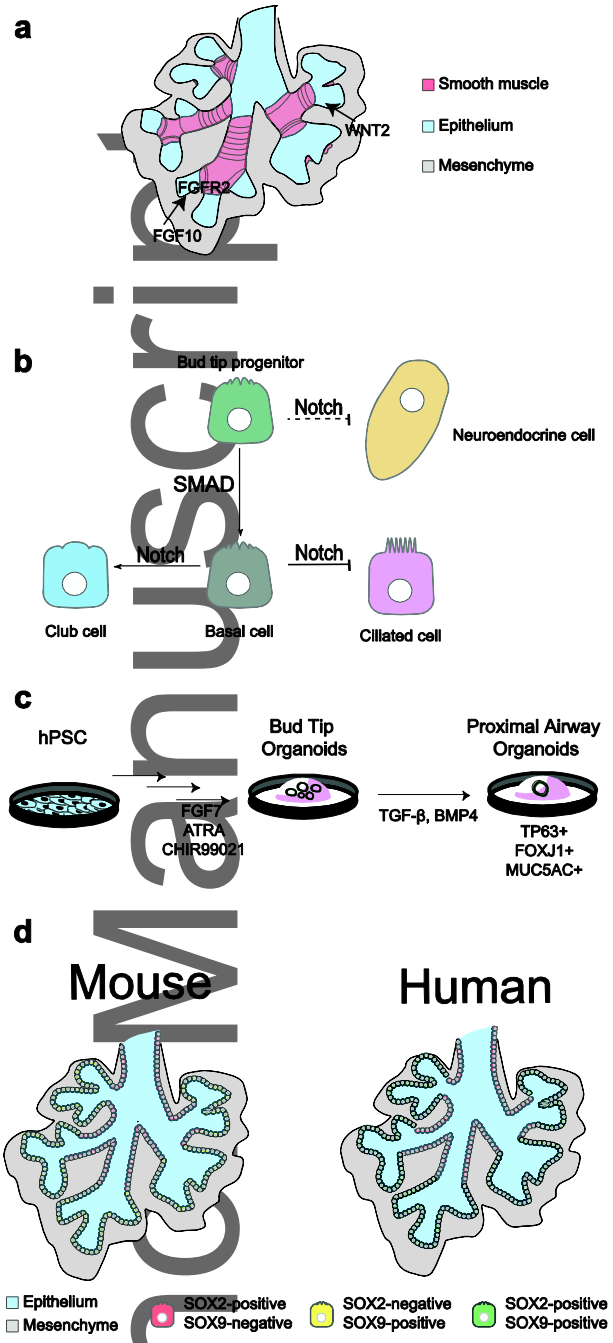

Figure 4. Alveolar cell fate specification. a) In mice, glucocorticoid signaling establishes the bronchoalveolar duct junction (BADJ), which demarcates the airway from the future site of alveoli formation. Bud tip progenitor identity is maintained by high levels of WNT and FGF signaling. High levels of HIPPO signaling in bud tip daughter cells born after BADJ formation leads to differentiation into alveolar progenitors, which coexpress markers of ATI and ATII cells. In humans, whether BADJ formation occurs is unknown. Human bud tip progenitors downregulate SOX2 by week 16 of development, suggesting a change in the developmental potential of human bud tips occurs at 16 weeks. b) Morphology of alveoli. ATI cells are thin to facilitate gasexchange. ATII cells contain lamellar bodies, a surfactant producing organelle. c) In vitro models of alveolar cell fate specification. Putative alveolar progenitors are purified from hPSC-derived NXK2.1+ lung epithelium. Conditions of high WNT, glucocorticoid, cAMP, and FGF signaling lead to the formation of ATIIIlike cells containing lamellar bodies. Incorporation of human fetal lung mesenchyme leads to the generation of ATI-like cells.